CHEMISTRY OF MARINE NATURAL PRODUCTS

A THESIS SUBMITTED TO THE

GOA UNIVERISTY
FOR THE DEGREE OF
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BY

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1993

DEDICATED TO

MY FATHER

LUIS CARACIOLO FERNANDES

AND

MY MOTHER

ANTONIETA AUGUSTA PEREIRA E FERNANDES

STATEMENT REQUIRED TO BE SUBMITTED UNDER ORDINANCE 19.8 0F 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

MD'Souza

(Maria Lisette de Lumen D'Souza)
Candidate

CONTENTS

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Above all, I thank profoundly my husband, **Oswald** for encouraging me and sparing me from a multitude of chores during the lengthy process of this thesis and my daughters, **Valerie and Evelyn** for using the time that was rightfully theirs.

MD'Souza
Maria Lisette de Lumen D'Souza
Feb 1993

GENERAL REMARKS

1. All the figure numbers, table numbers, scheme 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 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 (20x20cms.) coated with 0.25mm layer of TLC grade silica gel with 13% $CaSO_4$ as binder. The plates were activated at 110°C for 1 hour before use.
6. Spectral data reported for the different compounds were mainly obtained through the courtesy of various institutions. The details regarding the instruments are therefore not given. These are duly acknowledged.
7. The chemical shifts parameters in the ^1H NMR and ^{13}C NMR spectra are expressed in 'δ', ppm with TMS as internal standard.
8. All known compounds were identified by direct comparison of the spectral data and physical constants reported in the literature. Molecular formulas of the compounds were assigned on the basis of molecular weight by mass spectroscopy.

INTRODUCTION

CHAPTER I

SECTION I

MARINE PROSTANOIDS

Since ancient times, the coastal waters have been of great significance to man economically and scientifically. At present due to human population explosion and dwindling resources on land, man is increasingly focussing his attention towards ocean, as a major source of food, industrial chemicals, pharmaceuticals and energy; and in this process the marine organisms are becoming of immense importance.

The oceans and seas of the world cover approx 71% of the earth's surface which is very rich in marine life but the wealth of the medicines that have been isolated from terrestrial organisms is in extreme contrast to the poverty of drugs that have been obtained from marine organisms. These organisms have shown to contain unique compounds that are capable of destroying tumors, viruses and infectious bacteria or in other words compounds which will provide treatment for various human maladies. Other marine organisms contain poisons, which when diluted are novel agents that stimulate failing heart, while still others provide models that in their simplicity enhance our understanding of human disorders such as night blindness, artereosclerosis, coronary spasm, arthritis, shock, etc. Marine organisms are not only capable of providing drugs for the treatment of disease but more importantly they are a rich source of new and novel structures having useful biological activity, some of which could serve as models for new drugs, antifouling compounds, pheromones, neurotransmitters, antifertility compounds and starting materials from which new drugs could be synthesized. In other words, it could provide leads for natural product chemist to embark on a synthetic or semi-synthetic programme.

It is worth mentioning that the lack of application of substances from marine source to medicine is more due to lack of study of highly potent agents available and search for others than to a paucity of pharmacologicaly interesting substances from the sea. The contributing factors are that the required biological testing for isolation of bioactive substances makes a)

purification of new compounds a slow process, b) obtaining them in sufficient quantities which may occur as p.p.m. among thousands of other substances is a laborious process and c) the necessary research requires co-ordination among biologists, chemists, pharmacologists, pharmacognosists, physiologists and microbilogists. Added to this are the difficulties encountered in collection of the organisms, processing and the lack of adequate financial support.

In spite of all these limitations a number of important marine natural products are already in commercial use and several interesting bioactive and novel substances have been characterized and are undergoing further study but only few of these are in widespread use in therapy today. Noteworthy among them are the novel nucleosides 1-B-D-arabinofuranosyl derivatives spongothymidine, spongouridine and spongosine which served as models for D-arabinosyl cytosine or cytarabine or Ara-C [1], an important synthetic agent used clinically in the treatment of tumors. A similar compound Ara-A, isolated from a gorgonian *Eunicella cavolini*, is being used clinically against encephalitis.

Cephalosporin C [2], a metabolite of the fungus *cephalosporium acremonium*, is an antibiotic active against penicillin resistant microbes widely used in medicine and is being marketed as *Keflin* by Lily Pharmaceuticals. The antihelmintic, Kainic acid from *Digenea simplex* marketed as *Digesan*, a combination of santonin and kainic acid, by Takeda Pharmaceuticals. Among the toxins so far studied mention may be made of fish toxins, tetrodotoxin and saxitoxin[3], the former being used clinically as a muscle relaxant and pain-killer in neurogenic leprosy and terminal cancer. Nereistoxin, a metabolite of annelid, *Lumbriconereis heteropoda*, is the only insecticide of marine origin which served as a model for the manufacture of "Cartap", an insecticide being used in Japan against rice stem borer.

One of the most noteworthy marine chemical discoveries was the finding of two new

prostaglandins in the gorgonian, *Plexaura homomalla*, which are of considerable interest in medicine, as they are extremely potent compounds exhibiting a variety of biological activities. Their medical applications are at the stage of extensive clinical trial in the area of reproductive biology, fertility control, labour induction and also in certain renal pathology and treatment of intestinal ulcers.

Perhaps the most widely used products of seaweeds in medicine and pharmaceuticals are the gel forming polysaccharides, agar, carrageenan and alginates and at present there do not appear to be any artificially prepared alternatives.

All these discoveries have generated interest in India on developing drugs from the Indian Ocean. As far as India is concerned this is one of the untouched fields, though some work on the chemical and biochemical aspects of Indian seaweeds has been in progress since 1950's. In order to tap these marine resources, a project has been initiated at National Institute of Oceanography in collaboration with Central Drug Research Institute.,The project on "Drugs from the sea" has directed efforts towards detecting various pharmacological activities in the extracts of marine organisms and towards isolating and characterizing those active substances which show promise as potential therapeutic agents.

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2. Abraham, E. P. In: **Biologically Active Substances — Exploration and Exploitation,** D. A. Hems, Ed., Chapter 1, John Wiley & Sons, New York, 2, (1977).

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Prostaglandins represent a large and still expanding group of biologically potent lipid acids first discovered in seminal fluids and extracts of Icelandic sheep[1]. They have also been known to occur in various terrestrial animal organs and in recent years shown to be distributed in marine life such as fish[2].

A literature survey has indicated that there are reports of isolation of prostaglandins from *Gracilaria lichenoides*, an alga belonging to the family Rhodophyceae. Screening results of algae showed that a few number of them exhibit antifertility activity. Since prostaglandins are known to be good antifertility agents and already reported in the red alga, *G. lichenoides*, it was presumed that the activity observed could be due to the presence of prostaglandins. Hence an extensive review on marine prostanoids was undertaken and it is being presented under this section of the thesis.

Several reviews which dealt with marine prostanoids include those of Nomura Tadashi[3] which has stressed upon the distribution, structure, biosynthesis and their function in marine animals. Crabbe Pierre[4] in 1979 discussed the synthesis of modified prostaglandins from the PGA2 of corals, prostaglandins of fish alongwith the hormones of hypothalamus, pituitary glands, thyroid glands and sex glands have been reviewed by Lin Haoran[5] and finally in 1985 Nagasaka[6] presented the chemistry and synthesis of recently discovered anti-tumor marine prostanoids clavulones, chlorovulones and punaglandins. From the biosynthetic point of view, Corey has discussed this subject on several occasions.

In the present review, an attempt has been made to review all known marine prostanoids that have appeared in the literature through December '92. Emphasis will be placed on the compounds actually isolated, their chemistry and their probable biogenesis. No synthetic chemistry will be considered.

In 1969 Weinheimer and Spraggins[7] made a remarkable discovery of presence in relatively large amounts of two non-mammalian type of prostaglandin derivatives, 15 epi PGA_2 (1) and its diester (2) in air dried cortex of the Caribbean seawhip *Plexaura homomalla*. They differed from the mammalian prostaglandins (3) in having the unnatural R configuration at C_{15} and unlike the terrestrial ones, they did not exhibit blood pressure lowering effect. They do not occur in easily detectable amount in any related corals or other marine animals investigated so far.

This interesting discovery brought considerable attention to *P. homomalla* particularly because of their high concentrations and as potential synthetic precursors to biologically active prostaglandins A_2, E_2 or F_2 and stimulated worldwide survey of PG's in marine life. This resulted in isolation by Light and Samuelsson[8] of minute amounts of 15S isomers besides the predominant 15R compounds consisting of complex mixture including 11α(15R)-dihydroxy-9-oxo-prost-5,13-dienoic acid (5), its methyl ester (6), (15R)-PGA_2 ester (4) from *P. homomalla* of Florida. It is interesting that some species of *P. homomalla* from Caribbean exclusively produce 15S prostaglandins and some 15R isomers and rarely same species contained both the isomers[9].

The presence of natural mammalian type (15S)-PGA_2 acetate methyl ester (7) and (15S)-PGE_2 (8) was detected in some variety of *P. homomalla*[9]. It was observed that the two epimers differ only slightly in the silica gel TLC polarity using AIX[10] system and both the forms may be present in a single species. The presence of (15S)-PGA_2 acetate methyl ester (7) was confirmed by its conversion to (15S)-PGE_2 and (15S)-$PGF_{2\alpha}$ and base catalysed rearrangement to (15S)-PGB_2

A re-examination of the extracts of *P. homomalla* var. (S) by Bundy et al[11] allowed a more detailed analysis showing that more related products were present to which the

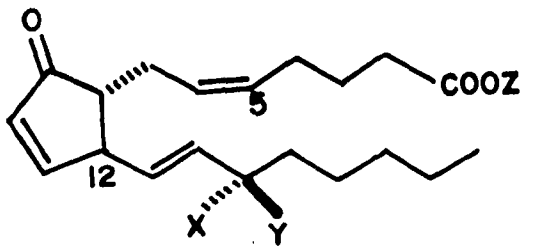
structure (15S)-15 hydroxy-9-oxo-(5 trans PGA_2) (9) was assigned.

Monoacetate of $PGF_{2\alpha}$ methyl ester (10), $PGF_{2\alpha}$ methyl ester (11), $PGF_{2\alpha}$ (12) and small amounts of PGE_2 (8) have been reported[12] in addition to (15S)-PGA_2 acetate methyl ester (7) *P. homomalla* from Cayman Island. This gorgonian was also found to contain yet another prostaglandin characterized as 5,6 trans PGA_2 (9) by its subsequent conversion to 5,6 trans PGE_2 (13). 13-14 cis PGA_2-15 acetate (14); 13-14 dihydro PGA_2 acetate methyl ester (15) and 13-14 dihydro PGA_2 internal Michael adduct (16) have been reported from the sample from Cayman Island by Schneider[13] in 1977. Compound (14) was identified by its conversion to methyl ester, whose configuration at C_{15} was established as 15S by its ozonization oxidation to -acetoxyheptanoic acid whose ORD curve was identical to that of (S)-

-acetoxy heptanoic acid obtained by ozonization of 15(S)-PGA_2 acetate methyl ester. UV absorption of its methyl ester at 217nm was shifted to 325nm due to elimination of CH_3COOH on addition of a base. The ^1H NMR of this elimination product (17) was found to be identical to the one found from PGA_2 acetate methyl ester[14]. IR of the methyl ester was indicative of absence of trans double bond. 13-14 cis nature of the double bond was established by alkaline epoxidation of methyl ester to a mixture of 10, 11 epoxides which on reduction yielded acetate methyl ester PGE_2 (18) identical to the synthetic product reported by Kluge et al[15]. The extract of the same gorgonian sp. also contained the adduct (16) and 15 α -acetate methyl ester (15) of (13) and (14).

Later on by monitoring the activity towards isolated guinea pig ileum, PGF_2 (19) was detected in the Japanese coastal gorgonian Euplexaura erecta[16].

The occurrence of prostanoids in marine life other than gorgonians has also been reported. By monitoring the antihypertensive activity by bioassay towards the pentabarbitone anaesthetized hypertensive rats, Gregson et al.[17] isolated PGE_2 (20) and $PGF_{2\alpha}$ from the



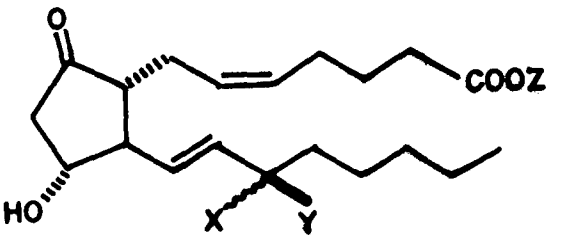
1 : X = H ; Y = OH ; Z = H

2 : X = H ; Y = OAc ; Z = Me

3 : X = OH ; Y = H ; Z = H

4 : X = H ; Y = OH ; Z = Me

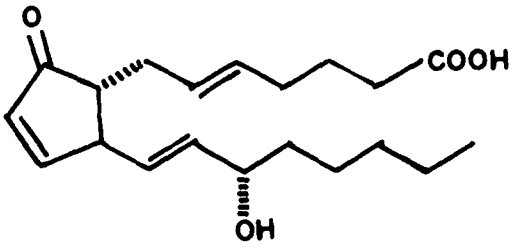
7 : X = OAc ; Y = H ; Z = Me



5 : X = H , Y = OH , Z = H

6 : X = H , Y = OH , Z = CH_3

8 : X = OH , Y = H , Z = H



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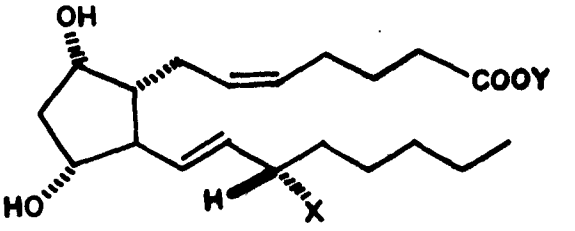
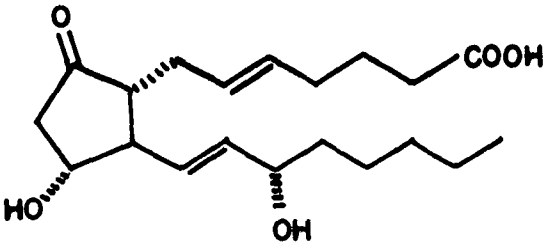


10 : X = OAc , Y = Me

11 : X = OH , Y = Me

12 : X = OH , Y = H



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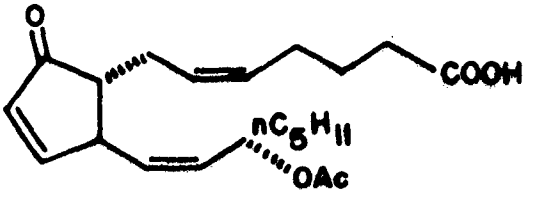

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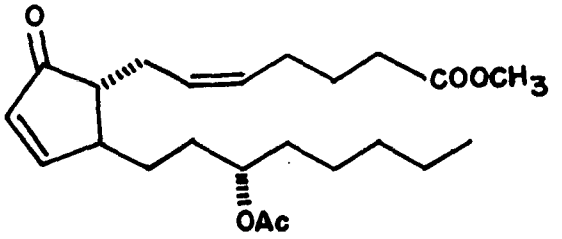

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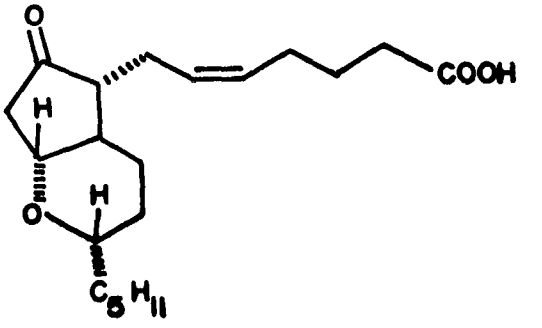


16

marine red alga *Gracilaria lichenoides*. This is the only report of the occurrence of prostaglandins in plants.

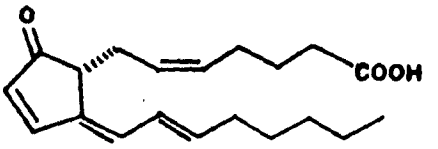
The Red Sea soft coral *Lobophytum depressum* was shown to contain four PGF derivatives (15S)-$PGF_{2\alpha}$ -11-acetate methyl ester (21), its 18-acetoxy derivative (22) as well as two corresponding free carboxylic acids (23) and (24).

More recently new prostanoids possessing anti-tumor activities and having oxygenated function at C-4, C-12 positions and olefeins at C-7, C-14 positions designated as clavulones have been identified in Japanese soft coral *Clavularia viridis*[19]. The structures of these clavulones I, II and III (25, 26 and 27) were assigned on the basis of spectral data and chemical evidence. The presence of a cross conjugated system was indicated by the UV data of allylic alcohol (28) obtained by $NaBH_4$ reduction of the clavulone (25) and the ketone (29), a pyridinium chlorochromate oxidation product of the alcohol (28).

The UV data of the ketone (30) and the mass spectrum of its dihydroderivative (31) obtained by hydrogenation of (25) with Pd/C established the nature and positions of the side chain in a α—ß unsaturated cyclopentenone system.

The stereochemistry of the chiral centres of these new prostanoids, clavulones, at the C-4 position was established by isomerisation of (25) with oxalic acid in methanol to (26) and (27) and by converting the aldehyde (32) obtained by ozonolysis of (25) to the lactone (33) with known stereochemistry[20].

The examination of CD spectra of the p-bromobenzoate derivatives (34), (35) prepared from the clavulone established the stereochemistry at C-12. The antileukemic effect of these compounds on human promyelocytic leukemia (HL-60) cells has also been studied[21].



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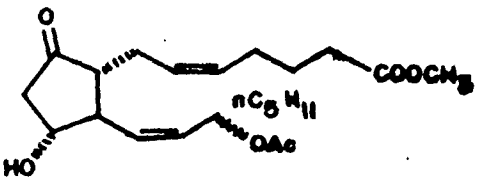

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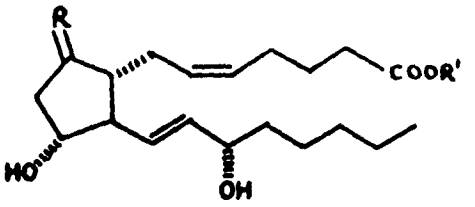
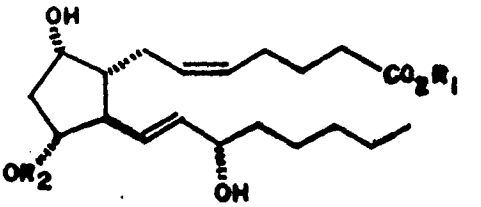


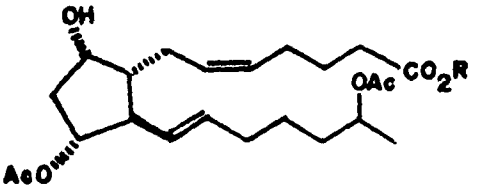
19 : R = α-OH ; β-H, R' = H

20 : R = O ; R' = H



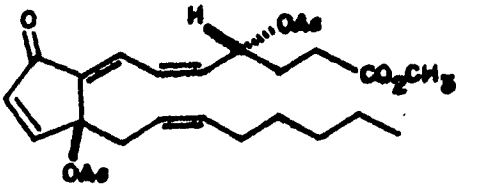
21 : R_1 = Me , R_2 = Ac

23 : R_1 = H , R_2 = Ac



22 : R = H

24 : R = Me



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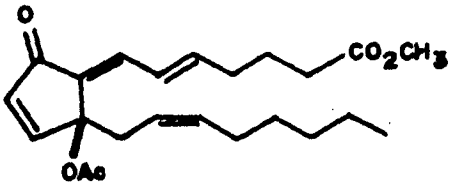

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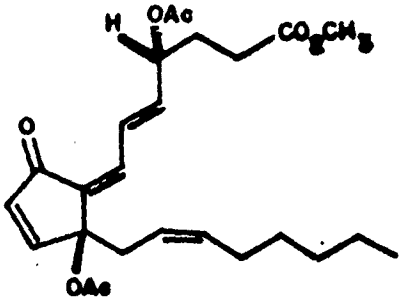

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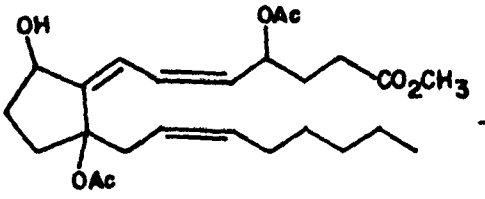

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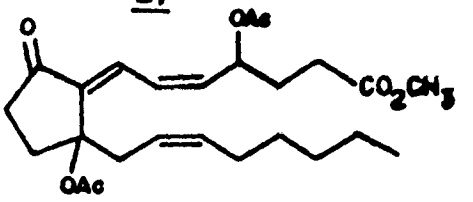


29

Further investigations[22] on the same soft coral led to the isolation of oxygenated prostanoids (36), (37) and (38) having an acetoxy group at C-20 position. These were the first examples of C-20 oxygenated prostanoids from marine source, 20-hydroxy prostaglandins are recognised in metabolites of mammalian prostanoids[23].

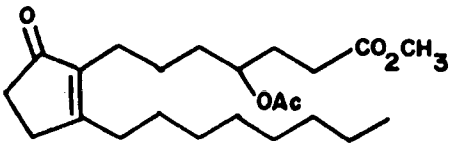
The structure and stereochemistry was obtained by CD spectra and comparison of their ^{1}H and ^{13}C NMR with those of clavulones (25), (26) and (27).

From the same source, C. *viridis*, Kitagawa et al.[24] published the isolation of claviridenone a, b, c and d (39 to 42) and 20-acetoxy claviridenone-b (43) and 20-acetoxy claviridenone-c (44), the stereostructure being determined on the basis of physical and physico-chemical evidence including the application of the CD excitation chirality method[25]. While preparing this review, physico-chemical data of claviridenones c, b and d was compared with that of clavulone I, II and III (25-27) and established that they are identical. The comparison of ^{1}NMR and ^{13}C NMR spectral data of (37) and (38) with the spectral data of (43) and (44) isolated independently by two different groups of Japanese workers indicated that they are also identical.

Further, investigation of clavulone congeners from C. *viridis* resulted in the isolation of a series of new halogenated prostanoids, chlorovulones I, II, III & IV with strong anti-tumor activity. Their stereostructures were established as (45-48) on the basis of spectral data and CD measurement of chlorovulone derivatives[26] and enantioselective synthesis of chlorovulone II, (46).

Chlorovulone I (45), showed 13 times stronger anti-proliferative and cyto-toxic activities in human promyelocytic leukemia (HL-60) cells in vitro than clavulone I.

Recently, punaglandins[27] characterized by C-12 oxygen and unprecedented C_{10}



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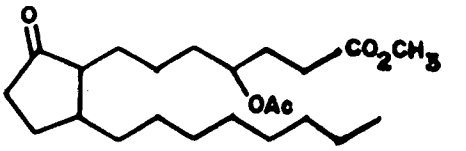

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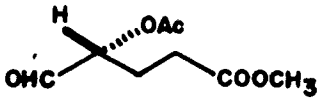

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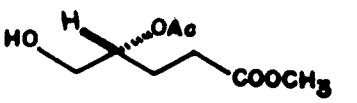

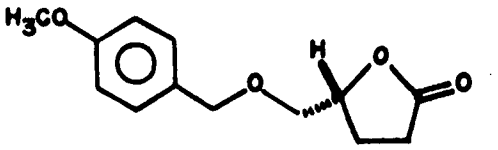

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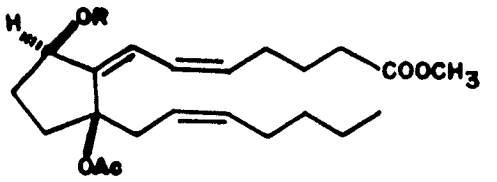
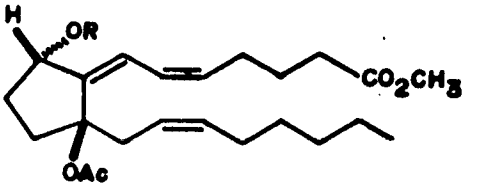


34 – R = – C(=O) – C_6H_4 – Br



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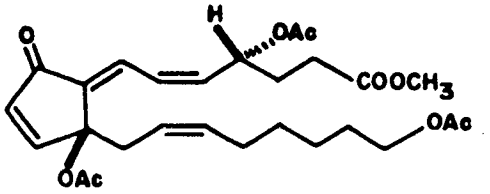

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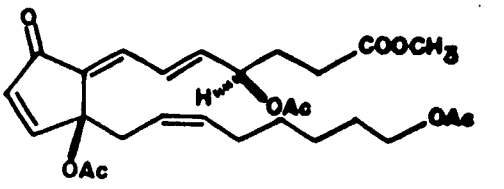

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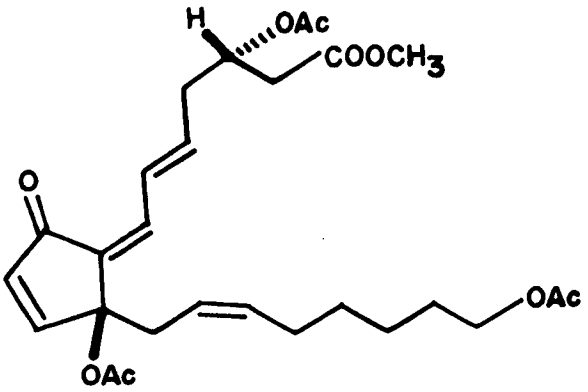

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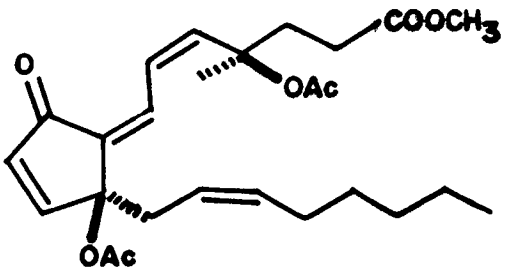

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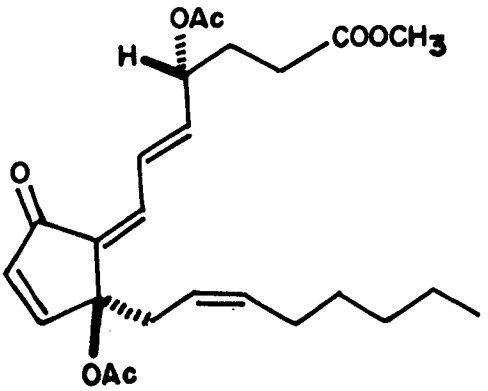

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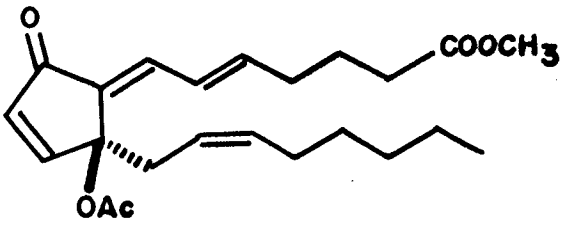

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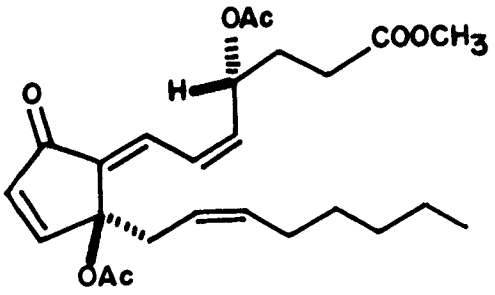

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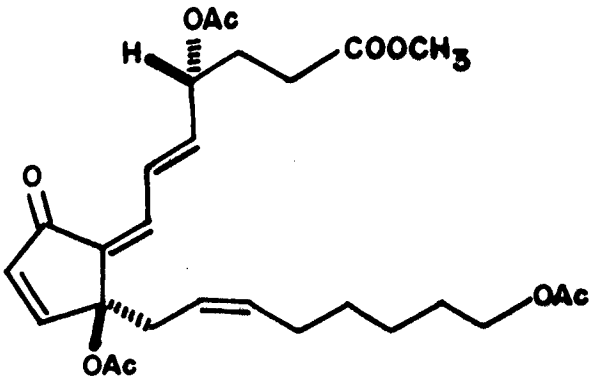

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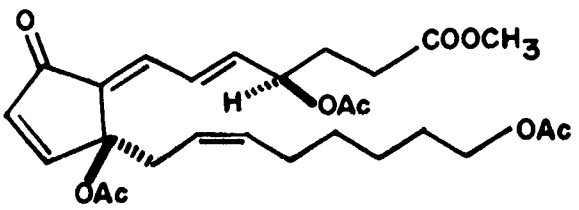

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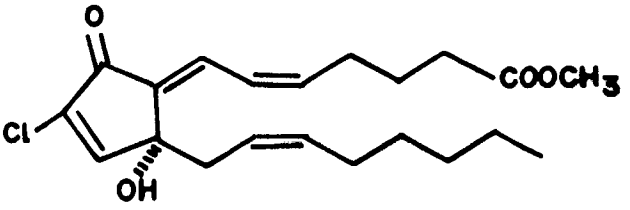
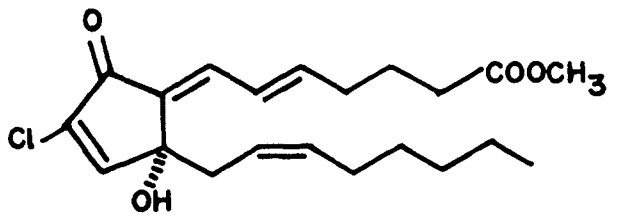


45

chlorine functions, with the structure related to those of clavulones isolated from *Clavularia* viridis were detected in *Telesto riisie*, an octocoral that lacks symbiotic algae. The following structures were proposed originally for PUG 1-6, i.e. (49-54) based on the spectroscopic data and biosynthetic pathway of related marine prostanoids clavulones or claviridenones but later, the structures of PUG 3 and 4 i.e. (51) and (52) were first revised[28] to (55) and (56), followed by second revision[29] to (57) and (58).

The revision of structure was based on the synthesis of possible diastereoisomers with respect to C-5, C-6 and C-12.

In the family of punaglandins, PUG-3, (51) and PUG-4, (52), have received particular attention because of the potent inhibitory effect on L_{1210} leukemia cell proliferation[30].



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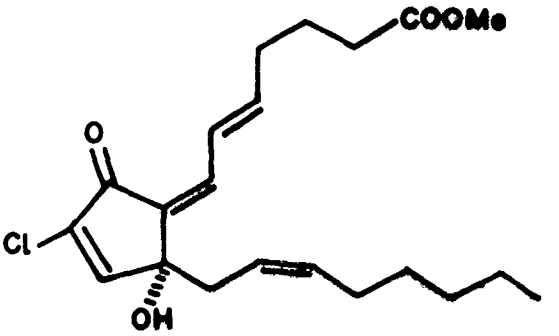

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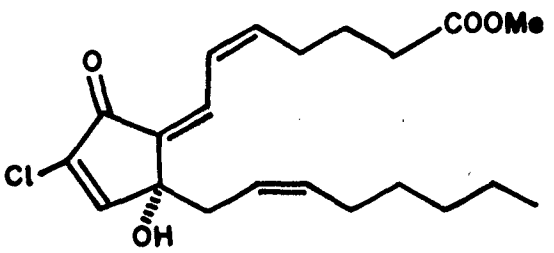

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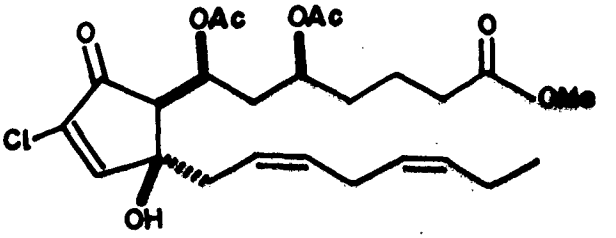
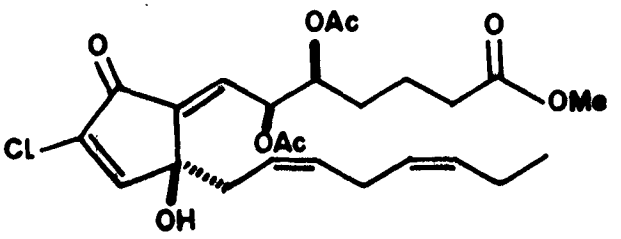


49

50 : 17, 18 dihydro - 49



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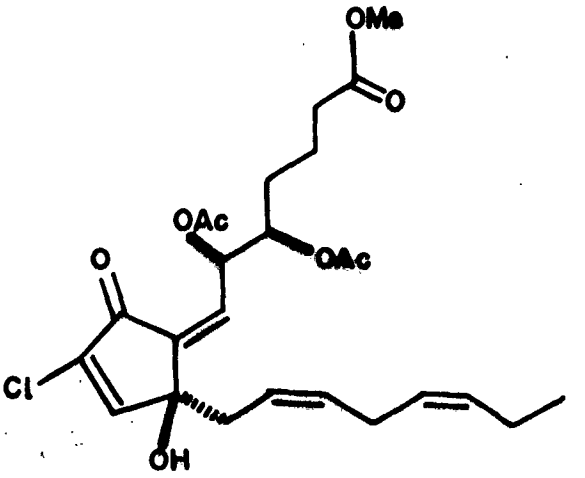

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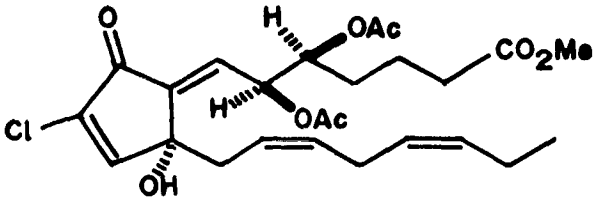

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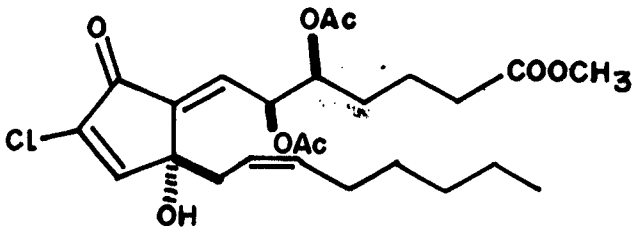
56 : 17, 18 dihydro - 55



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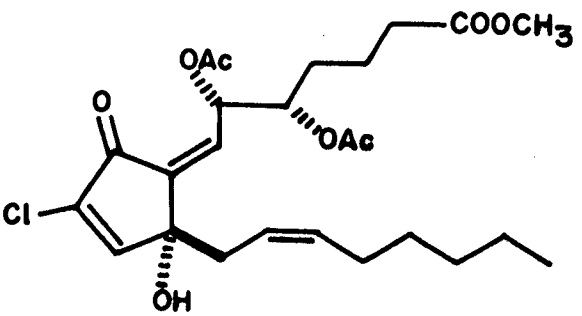


58

Biosynthesis of marine prostanoids

Biosynthetic pathways for marine prostaglandins have been proposed and studied by Corey *et al.*[34]. It is surprising that the biosynthesis of these substances in corals proceed by a pathway which differs from that of formation of prostaglandins in mammals (endoperoxide pathway)[33]. Because of several practical difficulties including extreme instability of enzyme preparations from *P. homomalla* and self degradation of this coral, progress in defining this biosynthesis has been slow.

In 1973 Corey *et al.*[34] published biosynthesis data based on ^{3}H-labelled arachidonic acid. Incubation of this radiotracer with a freshly prepared homogenate of *P. homomalla* yielded PGA_2 (59) provided an optimum pH range (7.5-8.0) and appropriate salt concentration was used. Further, it was of interest to note that mammalian and coral synthetase respond very differently to co-factors and inhibitors. Thus indomethacin, an anti-inflammatory agent, is a potent inhibitor of mammalian enzyme but without any effect on sea whip synthetase.[35,36]

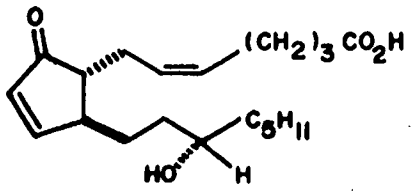
According to a recent report[37] by Fenical, some soft corals of genus *Pseudoptergorgia* produce diterpenoids with anti-inflammatory activity. It seems quite possible that somehow these diterpenoids regulate arachidonic metabolism in marine animals and it is therefore expected that indomethacin being also an anti-inflammatory agent does not affect the biosynthesis. In addition, it was also observed that the pathway of PGA_2 biosynthesis in coral does not involve PGE_2. The endoperoxide pathway proposed for mammalian prostaglandin does not apply to marine prostanoids was validated experimentally[33] through the use of several different preparations of PGA_2 synthetase of *P. homomalla* and mammalian enzyme preparation with either [1-^{14}C] PGH_2[38] or PGG_2[39] for conversion to PGE_2 and $PGF_{2\alpha}$. It was observed that this conversion is possible only with mammalian synthetase preparation and

not *Plexaura homomalla* enzyme indicating that endoperoxides PGH_2 (60) and PGG_2 (61) are not intermediates in PGA_2 synthesis by *P. homomalla*. Another possibility via endoperoxide (62) in *P. homomalla* was ruled out by the same authors[33] when synthesis of 10-[3H]-11-epi PGE_2 (63) from PGA_2 failed (by the incubation with active synthetase from *P. homomalla*).

In 1986 Bundy *et al.*[40] discovered the presence of arachidonic lipoxygenase in the gorgonian coral *Pseudoplexaura porosa*. This enzyme is capable of converting exogenous arachidonic acid (64) into (8S)-8 hydroperoxy-5,9,11,14 eicosatetraenoic acid (65) (8-HPETE) and catalyses the introduction of oxygen at C-15 forming (15S)-15 hydroperoxy-5,8,11,13 eicosatetraenoic acid (66) (15-HPETE) but surprisingly, even though the coral contains arachidonic acid as one of the most abundant of the common fatty acids, its transformation products like (8R)HPETE and the hydroxy compound 8HETE has not yet been identified in the coral i.e. the role of lipoxygenase in *Pseudoplexaura porosa* is still unknown.

As mentioned earlier due to extreme instability of enzyme preparations from *P. homomalla* and self degradation of coral even at -78°C, the progress in the biosynthesis of marine prostanoids has been slow: Recently, however, another family of prostanoids, the clavulones, has been identified from the Okinawan soft coral *Clavularia viridis*[41] which has proved to be much more amenable to biosynthetic studies.

Corey *et al.*[42,43] proposed that preclavulone A (PC-A) (67) a probable precursor of clavulones (and punaglandins), obtained by enzymatic transformation of tritiated arachidonic acid via 8(R)HPETE is converted to clavulones exemplified by (68,69) by a series of three hydroxylations at C_4, C_7 and C_{12} followed by esterification and elimination of C_7 oxygen. By analogy with the biosynthesis of plant regulator cis-jasmonic acid,[44,45] it was proposed that cis-4,5 disubstituted 2 cyclopentenone (67) is formed from hydroperoxide 8(R)HPETE (70) possibly by pericyclic closure of 2-hydroxypentadienyl cation (71).



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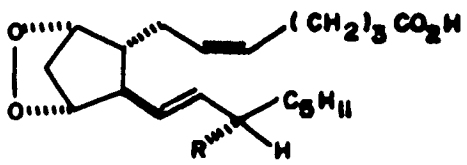
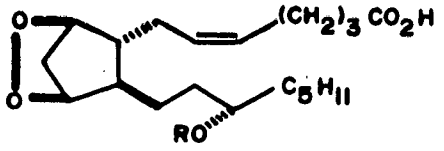
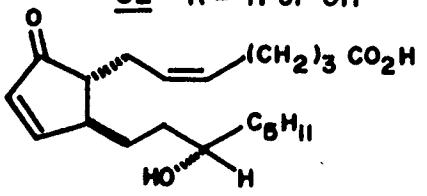


60 ; R = H

61 ; R = OH



62 R = H or OH



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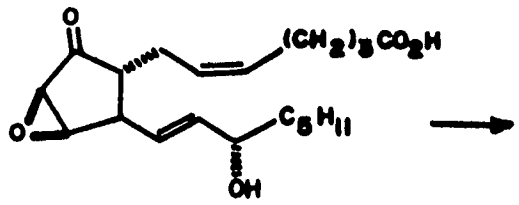
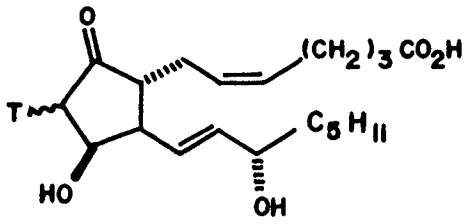
H_2O_2, Na OH, Me OH

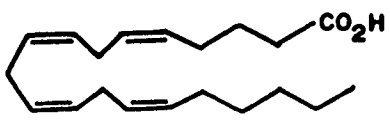


+ ∝. epoxide

Al (Hg), THF-T_2O



63 + PGE_2



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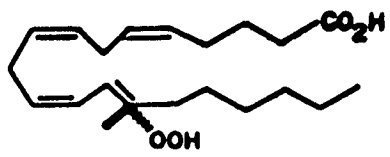

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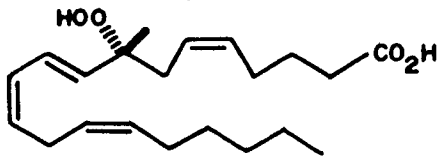

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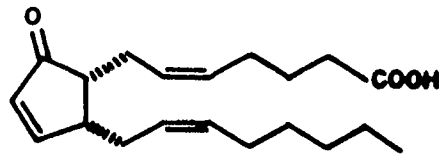


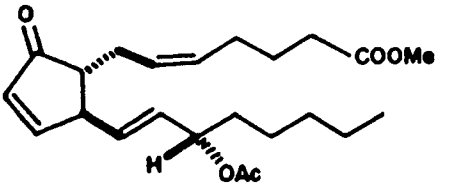
67

Strong evidence for this pathway was obtained when radiotracer studies[46] on ^{3}H-8(R)HPETE with coral homogenates indicated formation of (67) (PC-A) which on methylation followed by catalytic hydrogenation of the methylated product and epimerization provided 9-oxoprostanoic acid (72) identical with the catalytic hydrogenation product of PGA_2 methyl ester acetate (73).

Subsequently it was proposed that during biosynthesis of PC-A (67) oxygen must migrate from C_8 to C_9 as in cis jasmonic acid[44,45] and 8(R)HPETE (70) is converted via allene oxide (74) and oxidopentadienyl cation (71) intermediates with antarafacial pericyclic closure of cation leading directly to PC-A. The mechanistic feasibility of formation of PC-A has been demonstrated recently by biomimetic chemical synthesis.[47] Additional proof for the 8-lipoxygenation-oxidopentadienyl cation cyclisation involved enzymatic conversion of 8R HPETE and arachidonic acid to PC-A by homogenate preparation of *Pseudoplexaura porosa*.

If preclavulone-A (PC-A) is possibly the precursor of not only clavulones but also of other marine prostanoids[46], it is difficult to explain why no preclavulone or clavulones have yet been detected in coral *Pseudoplexaura porosa* even though 8(R)HPETE has been found to be present in it[40].

It is fascinating that marine organisms and mammals have evolved totally different biosynthetic paths to prostanoids. It is quite possible that the environment is playing a role in the biosynthesis of these metabolites as it has been observed that enzymatic biosynthesis of PGA_2 using tritiated arachidonic acid with homogenates of *P. homomalla* takes place only if the buffered homogenates were prepared using seawater[34] i.e. it required alkaline conditions (pH - 7.5-8.0) and appropriate concentrations of NaCl, conditions prevailing in the marine environment.



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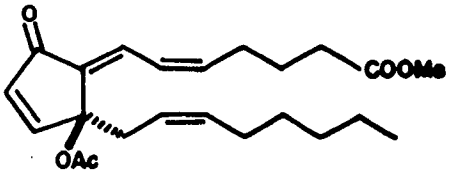

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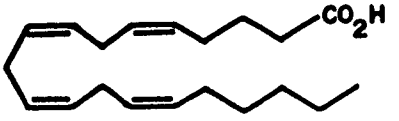

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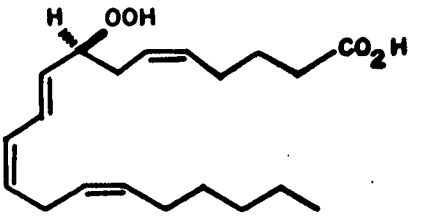

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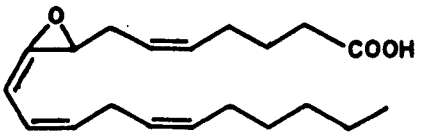

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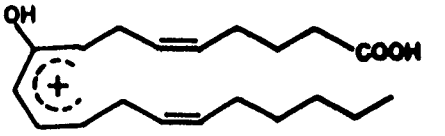

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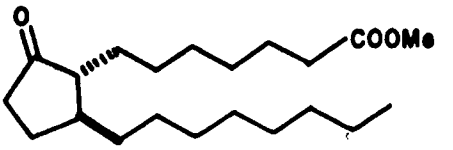

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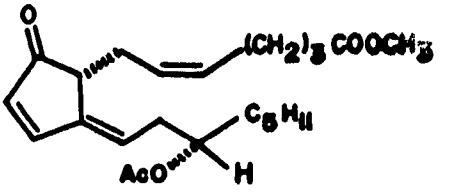

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SECTION II

SCREENING OF MARINE ORGANISMS FOR BIOLOGICAL ACTIVITY

It is now well established that sea is a rich source of biologically active materials which can be exploited for the benefit of mankind. It is not only capable of providing drugs for the treatment of disease but more importantly it is a rich source of new and novel structures which could be used as model for the synthesis of biodynamic compounds. So far only some isolated groups of scientists and laboratories have been working on this problem due to difficulties faced in collection, identification, repeat collection and biological evaluation. Even the laboratories engaged in this work are either content in reporting the activities in crude extracts without trying to correlate the activity with structure or in isolating some new structures in amounts insufficient for biological evaluation. In spite of these limitations, considerable amount of work has been reported in the literature.[1-3]

In India, a serious effort for exploration of marine flora and fauna was carried out only in the last 5-7 years with the collaborative effort of National Institute of Oceanography, Goa, Central Drug Research Institute, Lucknow and Bose Institute, Calcutta. This joint effort has resulted in broad based biological screening of nearly 500 marine organisms alongwith their identification and in some cases identification of biologically active constituents.

The results of the screening of extracts from marine plants, mainly seaweeds belonging to the families Rhodophyceae, Chlorophyceae and Phaeophyceae are presented in this section.

Marine plants used for the study were collected from the West coast of India during pre- and post-monsoon periods depending upon their seasonal availability. Precaution was taken during collection to reduce the possible contamination due to epiphytes and other marine organisms. Fresh material was washed free from extraneous matter, air dried, ground to a fine powder and extracted thrice at room temperature with 50% aqueous ethanol as described by Bhakuni *et al.*[4],except in the case of red alga, *Amphiroa fragilissima* which, owing to its

calcareous nature, was extracted with absolute ethanol. The solvent from the combined alcoholic percolate was removed at reduced pressure below 50°C. Almost all the extracts were tested for antibacterial, antifungal, antiviral, antifertility and a wide range of other pharmacological activities. These biological tests have been described briefly under the experimental section.

The results of biological screening alongwith the place and time of collection, activity observed and their intraperitoneal $LD_{50's}$ in mice are summarized in Table-1.

Table I — Results of biological screening of extracts of seaweeds

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
CHLOROPHYCEAE (green)				
Acetabularia crenulata (L) Kuntze	Andaman Feb.	E1-3; J1-4; K1; L1-4; N.	—	> 1000
Caulerpa peltata Lamour	Malvan, M April	B1-5; E1-3; J1-4	Hypotensive	> 1000
Caulerpa scalpelliformis (R.Br.) Web V.Bosse	Okha, Guj. Jan.	A2,4,9-11; B1-3,6,7; E1,2; G2; L1-4	Anti-implantation 40%	681
Caulerpa sertularioides (Gmel.) Howe	Malvan M Nov.	A2,4,9-11; B1-3,6,7; L1-4	CNS stimulant	—
Caulerpa taxifolia (vahl.) Ag.	Okha, Guj. April	A2,4,9-11; B1-3,6,7; H; J1-4; K1; L1-4; N	Hypotensive, diuretic	825

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Caulerpa veravalensis (Thivy and Chauhan)	Okha, Guj. Dec.	A2,4,9-11; B1-3,6,7; E1,2; J1-3; L1-4.	—	825
Chaetomorpha linum (O.F. Muller) Kuetz	Kilakarai island, T March	A2,4,9-11; B1-3,6,7; J1-3; K1; L1-4; N	—	> 1000
Chaetomorpha media Kuetz	Vengurla, M April	A2-5; B1-5; J1-4; H	—	> 1000
Chamaedoris auriculata (Boergesen)	Veraval, Guj. Dec.	A2,4,9-11; B1-3,6,7; G2; J1-4; K1; L1-4; N	—	825
Cladophora pinnulata Kuetz	Malvan, M Nov.	A2-5; B1-5; J1-4; N	toxic	825
Codium Dwarken Boergesen	Dwarka, Guj. Feb.	A2,4,9-11; B1-3,6,7; E1-3; G2; J1-2; L1-4	—	> 1000
Codium elongatum (Turner) Ag.	Cabo de Rama, G Feb.	E1-3	E1 (50%) E3 (45%)	681
Enteromorpha flexuosa (Wulf) J.Ag.	Cabo de Rama, G Dec.	A2-5; B1-5; E1-3; N	Diuretic	—
Enteromorpha intestinalis (L.) Nees	Chapora, G Feb.	E1-3	E1	> 1000
Ernodesmis verticillata (Kutzing) Borgesen	Karwar, Ka Dec.	A2,4,9-11; B1-3,5,7; E1,2; H; J1-3; K1; L1-4; N	—	> 1000

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Halimeda gracilis Harv.ex.J.Ag.	Mandapam, T Feb.	A2,4,9-11; E1-3; H; J1-2; K1; L1-4	—	> 1000
Halimeda macroloba (Decaisne)	Andaman, Feb.	G2; J1-2; K1; L1-4; N	Diuretic, anti-implantation	> 1000
Halimeda opuntia Lamour	Andaman, March	A2,4,9-11; B1-3,6,7; G2; J1-3; K1; L1-4; N	Diuretic, Hypotensive	> 1000
Halimeda simulans Howe	Andaman March	A2,4,9-11 B1-3,5,7; G2; J1-3; K1	—	—
Monostroma sp. Wittr	Sirgaon, G Nov.	A2,4,9-11; B1-3,6,7; G2; J1,2; K1; L1-4; N	60% anti-implantation	> 1000
Pseudobryopsis sp. Berthold	Okha, Guj. Dec.	A2,4,9-11; B1-3,6,7; E1-3; H; J1-3; L1-4; N	Hypoglycaemic	681
Udotea indica A.&E.S. Gepp	Porbander, Guj. Dec.	A2,4,9-11; B1-3,6,7; J1-3; K1; L1-4; N	Cytokinin	1000
Ulva fasciata Delile	Baga, G Dec.	E1-3, L1-4	E1 (75%)	46.4
Ulva lactuca Linnaeus	Okha, Guj. March	A2,4,9-11; B1-3,6,7; E1-3; G2; H; J1-3; L1-4; N	—	1000

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Ulva reticulata Forskal	Andaman Feb.	A4,9-11; B1-3,5,6,7; J1-3; K1; L1-4; N	—	681
Valoniopsis sp. Borgesen	Krusadai, T March	A2,4,9-11; B1-3,6,7; J1-3; K1; L1-4; N	—	1000
Valoniopsis pachynema (Martens) Boergs	Adatra, Guj. Dec.	A2,4,9-11; B1-3,6,7; J1-3; K1; L1-4; N	—	825
PHAEOPHYCEAE (Brown)				
Chnoospora implexa (Her.)J.Ag.	Mandapam, T May	A2-4; B1-5; E1-3; J1-4	CNS stimulant	681
Cystoseira indica (Thivy et Doshi) Mairh	Okha, Guj. March	A2,4,9-11; B1-3,6,7; E1; H; J1-4; K1; L1-4; N	Hypoglycaemic	> 1000
Dictyopteris australis (Sonder) Askenasy	Cabo de Rama, G Dec.	A2,4,9-11; B1-3,5,7; E1-3; J1-3; K1; L1-4; N	Diuretic, Hypotensive	681
Dictyota bartayresiana Lamour	Anjuna, G. April	E1-3	E3	> 1000
Dictyota dumosa Boergesen	Anjuna, G. Dec.	A2,4,9-11; B1-3,6,7; E1-3; J1-3; K1; L1-4; N	—	> 1000
Hydroclathrus clathratus (Bory) Howe	Dwarka, Guj. Dec.	A2,4,9-11; B1-3,6,7; E1-3; H; J1-3; L1-4; N	—	> 1000

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Iyengaria stellata (Boergs) Boergs	Adatra, Guj. Dec.	A2,4,9-11 B1-3,6,7; J1,2; K1; L1-4; N	Adrenergic blocking agent	825
Padina gymnospora (Kuetz) Vickers	Adatra, Guj. March	A2,4,9-11; B1-3,6,7; E1-3; H; J1-4; L1-4; N	Hypoglycaemic	1000
Padina tetrastomatica Hauck	Baga, G. Dec.	A2,4,9-11 B1-3,6,7; E1-3; H; J1-4; L1-4; N	CVS, spasmogenic hypotensive, 100% antifertility, antiamoebic	464
Pocockiella varjiegata (L) papenfuss	Krusadai, T March	A2,4,9-11; B1-3,6,7; G2; J1,2; K1; L1-4; N	CNS stimulant	1000
Sargassum cinereum J.Ag.	Malvan, M March	A2,4,9-11; B1-3,6,7; G2; J1,2; K1; L1-4; N	CNS stimulant	1000
Sargassum myriocystum J.Ag.	Gulf of Mannar,T March	A2,4,9-11; B1-3,6,7; E3; J1-4; K1; L1-4; N	E3 (75%)	—
Sargassum tennerrimum J.Ag.	Baga, G Dec.	E1-3	—	178
Sargassum wightii Greville	Gulf of Mannar, T March	A2,4,9-11; B1-3,6,7; E3; G2; H; J1-3; K1; L1-4; N	E3 (50%)	> 1000
Spatoglossum asperum J.Ag.	Anjuna, G March	A2,4,9-11; B1-3,6,7; G2; H; J1-3; K1; L1-4; N	69% antiimplantation and hypotensive	1000

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Turbinaria conoides Kuetz	Kilakarai island T, Feb.	A2,4,9-11; B1-3,6,7; J1-3; K1; L1-3; N	—	> 1000
Turbinaria decurens Bory	Andamans Feb.	J1-3; K1; L1-4; N	—	1000
RHODOPHYCEAE (Red)				
Acantophora spicifera (Vahl) Boergesen	Anjuna, G Feb.	A2-5; B1-5; E1-3; G2; J1-4; K; L1-4; N	Antifertility (100% 200 mg/kg.) E1	> 1000
Amphiroa anastromosans W.V. Bosse	Tuticorin, T April	A2,4,9-11; B1-3,6,7; E1-2; J1-3; L1-4; N	—	> 1000
Amphiroa fragilissima (L) Lamour	Anjuna, G Jan.	A2-5; B1-5; E1-3; G2; J1-4; K1,2; L1-4; N	Antiviral, Oxytoxic, spasmogenic	—
Botryocladia leptopoda (J.Ag.) Kylin	Adatra, Guj. Dec.	A2,4,9-11; B1-3,6,7; G2; J1-4; K1; L1-4; N	Anti-implantation and CNS stimulant	825
Centroceras clavulatum (Ag.) Mont.	Mandapam, T Jan	E1-3	—	681
Cheilosporum spectabile Harvey	Anjuna, Goa Feb.	E1-4; G2; J1-3; K1; L1-4	—	> 1000
Chondria armata (Kuetz) Okamura	Baga, G.	E1-3	E1 (75%)	17.8

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Chondrococus hornemanii (Lyngbye) Silva	Anjuna, G Feb.	A2,4,9-11; B1-3,6,7; E3; J1-4; K1; L1-4; N	E3 (30%)	> 1000
Galaxaura elongata	Andaman March	A4,9-11; B1-3,5,7; J1-4; K1; L1-4; N	—	> 1000
Galaxaura oblongata Lamour	Andaman Feb.	A2,4,9-11; B1-3,6,7; E1-3; J1-4; L1-4; N	—	1000
Galaxaura rugosa Lamour	Andaman Feb.	A2,4,9-11; B1-3,6,7; E3; J1-4; K1; L1-4; N	E3 (20%)	1000
Gastroclonium iyengarii Srinivasan	Adatra, Guj. Dec.	A2,4,9-11; B1-3,7; J1-3; K1; L1-4; N	—	> 1000
Gelidiella acerosa (Forsk.) Feldman et Hamel	Eravali, T Dec.	E1-3	E3	681
Gelidium micropterum Kuetz	Andaman March	J1,2; K1; L1-4; N	Diuretic	> 1000
Gracilaria corticata J.Ag.	Baga, G Nov.	E1-3	E1 (20%)	1000
Gracilaria crassa Harvey	Tuticorin, T Dec.	A2,4,9-11; L1-3	—	681
Gracilaria folifera (Forsk) Boergesen	Andaman Feb.	A2,4,9-11; J1,2; K1; L1-4; N	Diuretic, Hypotensive	> 1000

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Halymenia venusta Boergs	Okha, Guj. Dec.	A2,4,9-11; B1-3,6,7; G2; J1-4; K1; L1-4; N	—	825
Hypnea musciformis (Wulf) Lamour	Baga, G Dec.	E1-3	E1 (100%)	> 1000
Hypnea cervicornis J. Ag.	Baga, g Dec.	E1-3	E3 (40-50%)	1000
Jania adhaerens Lamour	Mandapam, T Dec.	A2,3,4; B1-5	—	—
Laurencia papillosa (Forskal) Greville	Anjuna, G Feb.	A2,4,9-11; B1-3,5,7; E1,2; G2; J1-3; K1; L1-4; N	Diuretic	> 1000
Platisiphonia miniata (Ag.) Boergesen	Verawal, Guj Dec.	A2,4,9-11; B1-36,7; E1,2; J1-3; L1-4; N	—	> 1000
Scinaia hatei Boergs	Okha, Guj. Feb.	A2,4,9-11; B1-3,6,7; E1-3; H; J1-3; L1-4	Hypoglycaemic	> 1000
Solieria sp. Kylin	Anjuna, G Jan.	G2; J1-3; K1; L1-4; N	Diuretic, antifertility	316
Spyridia fusiformis Boergs	Andaman Feb.	A2,4,9-11; B1-3,6,7; E3; G2; J1-3; K1; L1-4; N	Diuretic, Hypoglycaemic E3 (20%)	68.1
Spyridia insignis J. Ag.	Trivandrum, K Dec.	A1-4; B1-4; E3; J1-4; K2; L1,3,4,; N	E3 (50%)	> 1000

Seaweed	Place and month of collection	Biological tests	Activity observed	LD 50 mg./kg.
Wrangelia argus Boergesen	Okha, Guj. Feb.	A2,4,9-11; B1-3,6,7; E1,2;G2; J1-3; K1; L1-4	—	1000

a) G = Goa; Guj. = Gujarat; K = Kerala; M = Maharashtra; T = Tamilnadu, Ka = Karnataka

b) Codes of biological tests given in the text.

As evident from Table 1, of the 71 alcoholic extracts of marine plants tested, significant biological activity was observed in 43 extracts. The diuretic activity was observed in the extracts of 10 algae namely *Caulerpa taxifolia, Enteromorpha flexuosa, Halimeda macroloba, Halimeda opuntia, Dictyopteris australis, Gracilaria folifera, Laurentia papillosa, Gelidium micropterum, Solieria sp.* and *Spyridia fusiformis* belonging mainly to Rhodophyta and Chlorophyta. This may be attributed to the presence of sodium chloride, as the marine red algae as a whole are considered to be sensitive to mesohaline conditions (5-18% salinity), as relatively few representatives are recorded from salinities below 15%.[5,6] Paradoxically the most extreme halotolerance range reported from macroalgae (0-142%) is shared by five Rhodophyceae species of mangrove algae.[7] Since red algae have high halotolerance one would expect higher content of sodium chloride and hence the diuretic activity observed. The extracts of algae belonging to genus *Halimeda* like *H. opuntia, H. gracilis* are reported to exhibit diuretic activity.[8] The genus Halimeda has been found to contain highly active but unstable sesquiterpenes and diterpenes.[9] Twelve species of Halimeda have been studied and 4 diterpenes were found common to all the species. Some of the diterpenes are reported to exhibit cytotoxic and antimicrobial activities.

Hypotensive activity has been exhibited by the green algae *Caulerpa taxifolia and C. peltata*, the former also being diuretic. LD_{50} of *C. taxifolia* collected during post-monsoon period was found to be 825mg/Kg.

Acute poisoning from the seaweed has not been reported in the literature but mention has been made that, one of the most popular edible alga, *Caulerpa lamourouxii* in the Philippines is known to be toxic during rainy season.[10] It has furnished caulerpicin, caulerpin, cholesterol, taraxerol, ß-sitosterol and palmitic acid.[11] Caulerpin has also been isolated from *C. paspaloides, C. cupressoides, C. prolifera, C. sertularoides,*[12] *C. peltata, C. racemosa,*[13] etc. whereas caulerpicin has been obtained from *C. ashmeadii, C. paspaloides, C. sertularoides, C. racemosa var. uvifera,*[12] *C. scalpelliformis,*[14] etc. Caulerpin produces mild anaesthetic action, difficulty in breathing, sedation and loss of balance.[15] The toxic syndrome has been reported to be somewhat similar to that produced by Ciguatera fish poisoning.[16] Caulerpin is reported to have been isolated in over 80% species of *Caulerpa*. The toxicity of *Caulerpa* sp. of Philippines had been attributed earlier to the presence of caulerpicin which had been found to exhibit neurotropic effects.[17] Recently Vidal *et al.*[14] reported that, taking into account the caulerpin content of the edible algae, it alone cannot explain the acute poisoning that may occur in man, although long term toxicity may be possible.

The hypotensive and diuretic activities observed in *C. taxifolia* do not seem to be either due to caulerpin or caulerpicin but to other common constituent of the genus *Caulerpa* as the same activities have also been reported in *C. racemosa*.

Hypoglycaemic activity has been observed in *Pseudobryopsis sp.* (Chlorophyceae), *Padina gymnospora, Cystoseira indica* (Phaeophyceae) and *Scinaia hatei* (Rhodophyceae). Saito and Nakamura[18] reported isolation of a phenolic substance sargaline from *Sargassum confusum* and Guven and Guven[19] reported a hypoglycaemic substance having polypeptide structure from *S. vulgaris*. It is noteworthy that *S. wightii* tested by us did not show this activity, it being reported to be due to the presence of peptides in three species of Cystoseira: *C. corniculata, C. barbata and C. crinita*.[20,21] Guven *et al.*[22] have

also observed that *Pterocladia capillacea* had a protein fraction responsible for hypoglycaemic activity.

It is hypothesized that a protein type substance is responsible for this activity in the seaweed extracts and *Sargassum wightii* seems to be devoid of it presumably due to the absence of sargaline or protein-like substance or it could also be attributed to the varying solubility behaviour of the proteins responsible for it or to the well known variation in phyto constituents depending upon the ecological factors prevailing at the time of collection and upon the growth stage of the plant.

The cytokinin activity observed in *Udotea indica* is not unprecedented as the use of seaweed extracts of *Ascophylum nodosum* in agriculture and horticulture has been known for many years. The presence of hormones in algae essentially similar to those found in higher plants and classifiable as auxins, gibberillins and cytokinins has been reviewied by Augier.[23,24] The presence of gibberillins in commercially available seaweed extracts has also been reported by William *et al.*[27] with the activity falling rapidly on storage at room temperature, being negligible after three months. Even in fresh extracts the levels of activity reported are too low to produce any noticeable effect when applied at the recommended rates of application to a crop. William *et al.*[27] and Mowat[28] did not obtain any significant auxin activity in any of the seaweed extract tested. All these reports indicate that the activity observed is due to the presence of cytokinin in that algae and this is well in agreement with the reports of high cytokinin content in the commercial aqueous seaweed extracts.[29,30] Booth[30] has also suggested that the quantities of cytokinin present in the seaweed extracts were sufficient to produce biological effects when applied to plants, even at the low rates of application used in the field. Further. it has also been demonstrated that there is a close correlation between the results from the use of kinetin (6 furfuryl aminopurine) and commercial extract of equivalent cytokinin

activities suggesting that the effect of several seaweed extract were due to their cytokinin contents.[31,32]

It has been demonstrated by several research workers that seaweed extracts exhibited inhibitory effects upon a number of gram +ve and gram -ve bacteria but none of the extracts tested by us were found to be effective against the bacteria and fungi tested. The absence of activity depends upon several factors. It has been reported[33] that the extracts prepared from fresh seaweed samples showed negligible activity as compared with the crude extracts from dried seaweeds and this has been attributed to the dilution (low concentration) of the active principle. In our observation this factor is ruled out as the extracts were prepared from air dried seaweeds. Similar results were obtained by Sreenivasa Rao *et al.*[34] using fresh and dried *Sargassum johnstonii.*

Henriquez *et al.*[35] and Pesando and Caram[36] screened marine algae from Chile and French Mediterranean coast respectively and described Chlorophyceae to be least active but Campos *et al.*[33] reported *Caulerpa sertularoides* from Brazil to be active against gram +ve bacteria, fungi and yeast but inactive against gram -ve. Hodgson[37] reported a chloroform soluble fraction of *Caulerpa prolifera* to be active against Staphylococcus aureus and Bacillus subtilis whereas *Caulerpa taxifolia* has been reported to be active against S. aureus, Escherichia coli and slightly against Myobacterium smegmatis.[37]

Literature also indicates that the solvent used for extraction also plays a role on the antibacterial activity exhibited by different seaweeds. Rao and Parekh[39] selected ethanol as the best solvent for the extraction of metabolites with antimicrobial activity in algae from India. Padma Kumar and Ayyakkannu[40] suggested toluene-methanol as a better solvent for the extraction of active metabolites from a number of seaweeds reported earlier to be inactive.[41,42]

Marine algae are highly susceptible to microbial degradation once harvested and it has been reported that when the seaweed sample is air dried it gets contaminated with primarily bacteria (Micrococcus and Bacillus sp.) and by less than 1% of fungi.[33] The greater the number of days taken for the algae to dry the higher is the number of contaminant bacteria. It is less in summer than in winter and the rainy season.

The differential antibacterial activity reported seems also to depend upon place, time and season of collection and storage; best activity being reported during fertility period.[36] Hornsey and Hide[41] distinguished four main types of seaweed groups based on the seasonal variation in their antibacterial activity. Further, these authors confirmed the independent periodicity in antibacterial activity of the algae observed by Chester and Stott.[43] Sreenivasa Rao and Parekh[39] observed high antibacterial activity during the winter months.

As evident from the above discussion there are so many factors responsible for the exhibition of antibacterial activity that it is difficult to conclude which factor would be responsible for the inactivity observed in all the seaweed extracts tested.

Among the algae examined for antiviral activity, seventeen were found to be active, significant inhibition being exhibited by the green algae *Ulva fasciata* (75% E_1) and *Codium elongatum*(50%E_1 and 45% E_3) and the red *algae, Chondria armata* (75% E_1), *Hypnea cervicornis* (100% in vitro, E_3). An antiviral effect was also observed for two brown algae, *Sargassum myriocystum* (75% E_3) and *S. wightii* (50% E_3). None of these extracts showed any activity against Ranikhet Disease virus.

In following up the leads provided by the screening results, the activity shown by the crude extract of *Codium elongatum* and the two species of *Hypnea* was located in the water soluble fraction and was found to be due to the sulphated polysaccharide, polygalactan from

C. elongatum and the carrageenan from *Hypnea sp.* Carrageenan extraction from Hypnea sp. was done by the method of Matheson and Tvetter.[44]

Carrageenan from *Chondrus crispus* Stackh. is known to be antiviral,[45] the activity has been reported to be due to the sulphated galactose unit of the phycocolloid. Algal polysaccharides such as laminaran, fucoidan and algin, which lack sulphated galactose units exhibited no antiviral activity.[46] Moreover, phycocolloids from the two species of *Hypnea* have been reported to resemble carrageenan of *Chondrus crispus* with k and fractions.[47]

The antiviral principle from the n-hexane and chloroform soluble fractions of *Ulva fasciata*, which gave 80% protection against SFV has been identified as 2-N-palmitoyl-4,5-dihydro 1,3,4,5 tetrahydroxy sphingosine.[48]

Partitioning of the ethanolic extract of *Chondria armata* located the active principle in the chloroform soluble fraction. This fact suggests that the active agent in the lipid extract could be the unusual lipophylic metabolite. As an example, from a *Laurentia sp.*, a halogenated acetylene, chondriol, has been isolated which is mentioned as antiviral principle.[49] Kappa-, lambda- and iota- carrageenans have been reported to be almost inactive against vaccinia virus[45] which is contrary to observations made on *Hypnea* polysaccharides. The absence of activity in this case may be attributed either to the varying solubility behaviour of the sulphated polysaccharide responsible for the activity in aqueous ethanolic solution[50] or the degree of sulfation[51] or to the well known variation in phytoconstituents depending upon the ecological factors prevailing at the time of collection and upon the growth stage of the plant.

It is interesting to note that in some cases like *Hypnea*, two different species belonging to the same genus showed antiviral activity while in other cases not all species belonging to the genera *Sargassum* and *Galaxaura* exhibited activity. Thus the presence

or absence of antiviral activity may or may not be a character of the genus studied.

The most interesting finding is the anti-implantation activity exhibited by the alcoholic extracts of eight seaweeds viz., *Caulerpa scalpelliformis, Halimeda macroloba, Monostroma sp., Spatoglossum asperum, Padina tetrastomatica, Acantophora spicifera, Botryocladia leptopoda* and *Solieria sp.*

Based on the screening results presented here, *Acantophora spicifera, Stoechospermum marginatum, Amphiroa fragilissima,and mangrove plant Acanthus illicifolius* were taken up for detailed chemical investigations with a view to identify the active principle. Some of the results obtained are presented in next two chapters.

EXPERIMENTAL

A. Antibacterial activity

The activity was tested against three Gram positive Bacillus subtilis (A_1), Staphylococcus aureus (A_2), Streptococcus faecalis (A_9) and four Gram negative Salmonella typhi (A_3), Escherichia coli (A_4), Agrobacterium tumefaciens (A_5), Proteus vulgaris (A_{10}) and Pseudomonas aeruginosa (A_{11}) — strains of bacteria in peptone broth at 37±1°C by the two fold serial dilution technique. The observation period was 24 hours and extracts completely inhibiting the growth in a concentration of 25 ug/ml were considered active.

B. Antifungal activity

The following strains maintained on Sabouraud's agar slants[52] were used Candida albicans (B_1), Cryptococcus neoformans (B_2), Trichophyton mentagrophytes (B_3), Microsporum canis (B_4), Aspergillus niger (B_5), Aspergillus fumigatus (B_6) and Sporotrichum schenckii (B_7). The basic schedule of testing was similar to that of antibacterial testing but Sabouraud's broth at 28±° C was used as the medium. The period of incubation of the inoculated broth was dependant on the fungal strain used. Evaluation of antifungal activity was then done as in the antibacterial tests.

E. Antiviral activity

This was determined against Semiliki Forest virus (SFV) in vivo (E_1), Ranikhet disease (E_2) and Vaccinia virus (E_3) grown on chick chorio-allantoic membrane (CAM) culture.

Mice were used in groups of 10 and given 0.5 or 1mg of the extract intraperitoneally and challenged with 100 LD_{50} dose of Smithfurn and Haddow strain of SFV subcutaneously 24 hours later. The number of animals dying following hindlimb and forelimb paralysis during the next two weeks in control and treated animals were used to calculate % protection.

CAM culture was prepared by the method of Babbar[53] and the extract (0.05mg/ml)

and virus (0.064 haemagglutination unit/ml E_2 and 50 pox forming unit/ml E_3) were added to the culture and incubated at 37°C for 48 (E_2) or 72 (E_3) hours and viral titre measured in 6 replicates. Activity was considered significant only after 75 (E_2) or 100 (E_3) % reduction in viral progeny.

Toxicity

For the estimation of approximate LD_{50}, the extract suspended in 0.1% agar or in 1% gum acacia in distilled water was administered by the oral or the intra-peritoneal route. Concentrations were so adjusted that a 20g mouse received a volume of 0.2ml. The initial dose was at a level of 400 or 500mg/Kg going up or down by a faction of 2. Occasionally an interval of 1.5 was used for close approximation. Control animals were observed for 5 to 6 hours after dosage for toxic symptoms. If death occurred during this time the cause of death was recorded. The highest dose used was 1000mg/Kg.

G. Antifertility activity (G_2)

Adult female and male albino rats maintained in air-conditioned quarters under standard husbandary conditions were used. The animals were mated by the mass mating technique and vaginal smears of the female rats were taken every morning. The days on which spermatozoa were seen in the vaginal smears were designated as day 1 of pregnancy. The algal extracts were macerated with equal quantities of gum acacia and suspended in distilled water to achieve the desired dose. The suspension was introduced into the lower part of the oesophagus by a feeding needle. The control rats received a mixture of gum acacia and distilled water in a similar manner. The extract was administered daily for 7 days from day 1 to 7 post-coitum. The rats were laparotomized on day 10 of pregnancy and the number of corpora lutea and implantations were recorded. The presence of spermatozoa in the vaginal smear was considered evidence of coitus. Complete absence of implantation was taken to indicate antifertility activity.

H. Hypoglycaemic activity

Albino rats (about 100g body weight) were fasted for 18 hours but water was allowed ad libitum. Blood was collected at the beginning of the experiment (0 hour sample) and a suspension of the extract was administered orally to a group of three animals. A single dose of 250mg/Kg was used. Blood was collected 2 and 4 hrs. later for estimation of sugar by the method of Nelson and Somogyi[54]. The maximum percent lowering of blood sugar was calculated in each case. The results were compared with blood sugar levels of a group of untreated rats and a third group of sulphonyl urea (250mg/Kg) treated rats. Lowering of the blood sugar by 30% or more was taken as significant.

J. Effect on respiration, cardiovascular system and nictitating membrane

Adult mongrel dogs or cats of either sex anaesthetized with pentobarbitone sodium (30mg/Kg i.v.) were employed and the extracts administered intravenously.

The effect was assessed on respiration (J_1) i.e. on the rate and amplitude; blood pressure level (J_2), nictitating membrane contraction in response to electrical stimulation of preganglionic sympathetic nerve (J_3) and heart rate(J_4).

The trachea was cannulated and connected to a Marey's tambour, one of the common carotid arteries was cannulated and connected to a mercury manometer. Records were made on a slow moving Kymograph. The drugs were administered through a cannulated femoral vein.

Each extract was initially administered in a dose of 25mg/Kg i.v. If an effect was observed, smaller doses were tested and the minimum effective dose determined. If 25mg/Kg produced little or no response, the effect of 50mg/Kg was studied. An extract inactive at this level was not investigated further.

K. Effect on isolated tissues

Guinea-pig ileum (K_1): Three to five cms. long terminal portions of ileum were taken from a freshly killed guinea-pig weighing 300-500g and suspended in aerated Tyrode solution

maintained at 34-35°C in an isolated organ bath of 20ml capacity. After 30min. rest, 2-4 doses of spasmogen were added to the bath to obtain uniform amplitude of contraction which was recorded on a smoked Kymograph by a frontal writing lever with 5-6 fold magnification and 3-4g tension on the tissue.

The spasmogens, acetylcholine chloride ($5x10^{-8}$g/ml) and histamine acid phosphate ($3x10^{-8}$g/ml) were allowed to act for 20 sec. In some experiments nicotine sulphate ($6x10^{-7}$g/ml) and 5-hydroxytryptamine ($6x10^{-7}$g/ml) were used to produce approximately the same height of contraction. The aqueous solutions of the extracts (50, 250ug/ml) were added to the bath one minute before the addition of the spasmogen. Effect of graded doses of the extract on the spasmogen induced contraction was seen and per cent reduction of contraction measured. The tissue was washed twice at one minute interval and three minute rest was given to it. If the test compound itself caused contraction, effects of atropine sulphate ($1x10^{-7}$g/ml) and mepyramine maleate ($5x10^{-8}$g/ml) on this contraction were studied to determine the nature of spasmogenic action.

L. Gross effects and effects on CNS:

These were studied in mice of either sex weighing between 15 and 20g. Generally 5 animals were employed at each dose level. Initial testing has usually been done at 1/4-1/2 LD_{50}. If activity was detected, smaller doses were subsequently tested.

The observations of Gross effects (L_1) extended for 2 hrs. after administration of the extract for any evidence of CNS stimulation or depression and autonomic effects. Hypothermia (L_2): The skin or rectal temperature was measured with a thermometer before and 1/2, 1 and 2 hrs. after drug administration. The fall in temperature in all the 5 animals of a group at any particular time was totalled and a value below 5°F ignored.

Analgesia(L_3) was tested by Haffner's tail clip method[55]. If a positive result was obtained, further confirmation was done by the hot plate method of Eddy and Leimbach[56].

Anticonvulsant (L_4) was judged by the ability of the extract to abolish the tonic

extensor component of supramaximal electroshock seizure induced by 48ma current for 0.2 sec. delivered via ear electrodes.

N. Diuretic activity

This was tested in male rats hydrated with 5ml/100g normal saline via a stomach tube. The extract (1/4 x LD_{50}) was given orally and extracts producing at least 75% of diuresis compared to 100mg/Kg chlorthiazide p.o. in a 4 hrs. period were considered inactive.

O. Anti-inflammatory activity

Oedema was induced by injecting 0.025ml of 1% carrageenan solution into subplanter region of left paw one hour after oral administration of the extract (1/5 x LD_{50}). The animals were killed 4 hrs. later and the difference between weight of the two paws between treated and control animals was the measure of anti-inflammatory activity. A reduction of 20% was considered significant.

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CHAPTER II

CHEMICAL CONSTITUENTS OF THE RED ALGA *ACANTOPHORA SPICIFERA*

Substances from marine organisms have been used in folk medicine all over the world since ancient times. Thus liver oil of some fish has been utilized as a source of vitamin A and D, insulin has been extracted from whales and tuna fish, in addition,the powdered oyster, the gall bladder of cod and many others are included in Chinese medicines. However it appears to be uneconomical to extract and purify a drug from an organism which must be procured before hand; many of these drugs e.g. vit.A and D have thus been replaced by synthesis. Therefore, only a few organisms are currently used as a source of useful drugs. Nevertheless, the oceans are again scrutinized as a potential source of drugs because of vast and diverse range of marine life. Marine organisms seem to be far more attractive as a source of drugs than terrestrial plants and animals with which we have long been familiar.

Pharmacological agents of marine origin developed into drugs, pharmaceuticals already in commercial use have been dealt with briefly in the introduction.

A great deal of chemical work has been done on Indian seaweeds during the last twenty five years. However, this work was confined mostly to the mineral constituents and carbohydrates with special reference to phyco-colloids, alginic acid, agar and carrageenan.[1] Investigations on biomedical potential of Indian Ocean is still at its infancy.

In India, serious effort for exploration of marine fauna and flora was made only in the last five to seven years with the collaborative efforts of NIO, Goa, Central Drug Research Institute, Lucknow and Bose Institute, Calcutta. As mentioned in chapter I, section II, this joint effort has resulted in broad based biological screening of crude methanolic extracts of almost 500 marine organisms with several of them possessing promising biological activities.

The red alga, *Acantophora spicifera* belongs to the order Ceramiales and family

Rhodomelaceae. Acantophora spicifera (Vahl) Boergeesen is generally found in tropical and sub-tropical oceans. This red alga has been reported to possess in vitro antimicrobial activity against *S. aureus, C. albicans* and *myobacterium smegmates*[2]. It has also been reported to be antiviral, having ability to inhibit the maturation of mouse meningo pneumonitis virus in tissue culture cell line[3], New castle disease virus (50-74%) , encephalomyocarditis virus (75-100%) and hepatitis B virus (25-45%)[4]. Extracts of *Acantophora spicifera* are also known to enhance the growth of dinoflagellate *Gambierdiscus toxicus*[5].

During the course of broad biological screening of the extracts of marine organisms from the Indian sea coast, the crude methanolic extract of the red alga *Acantophora spicifera* (Vahl) exhibited 100% anti-implantation activity at 200mg/Kg. in female rats, LD_{50} of the active extract was above 1000mg/Kg. In the follow up studies the activity was concentrated in petroleum ether and chloroform soluble fractions.

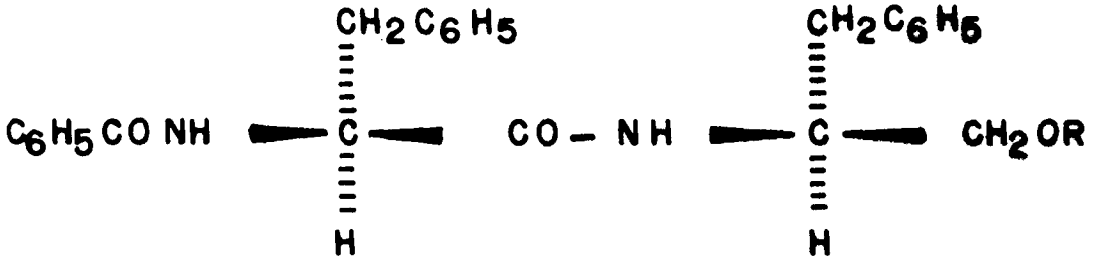
Acantophora spicifera has been extensively investigated for its mineral content[6-8], but the literature on the isolation of specific organic constituents is scanty[9]; there being some studies on its evaluation for total polysaccarides, proteins, amino acids,[10-12] fatty acids and lipids[12-14] content. Tocopherol content has been reported by Jayasree et al.[15] and its ascorbic acid and dihydroascorbic acid by Qasim and Barkati.[16]

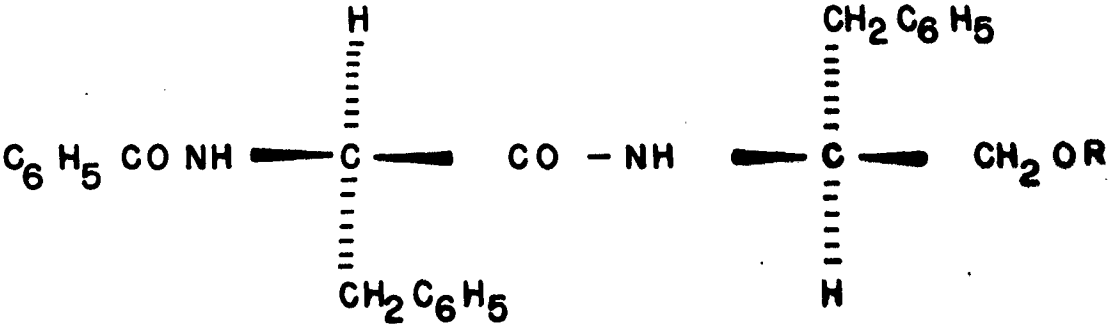
This chapter deals with the isolation and identification of some of the constituents including a novel dipeptide, a novel steroid and a diol from the active chloroform soluble fraction. Some of the well known constituents namely methyl palmitate; steroid cholesterol and fatty acids stearic, palmitic, behenic and arachidonic acids are also being reported here. The steroid, 5 α -cholestane-3,6-dione has been described as the major steroid in Acantophora spicifera.[17]

Rare dipeptide, aurantiamide acetate (1) has been reported earlier from a fungus *Aspergillus glaucus*,[18] and from the plants of Piperaceae,[19] Leguminosae,[20] Sterculiaceae,[21] Rutaceae[22] and Euphorbiaceae.[23] Diastereoisomer of aurantiamide acetate, dia-aurantiamide acetate to which structure (2) is assigned, has also been isolated from A. spicifera. It is a first report of isolation of these dipeptides from marine source and of natural occurence of dia-aurantiamide acetate(2).

Extensive column chromatography of the active chloroform soluble fraction, details of which are described in the experimental, yielded compound (1) m.p. 186°C showed UV (Fig.1) absorption at 202nm indicative of C_6H_5CO- group with sh at 225nm. Its mass spectrum displayed a molecular ion peak at m/e 444 analysing for $C_{27}H_{28}O_4N_2$. Its IR (Fig.2) exhibited bands characteristic of -NH-CO- (3330 and 1660cm^{-1}), acetate (730 and 1255cm^{-1}) and mono-substituted phenyl (735 and 690 cm^{-1}) functionalities. Its ^1H NMR spectrum (Table 1) (200MHz in $CDCl_3$) (Fig.3) confirmed the presence of these groups displaying signals for two CO-NH- at 6.85 (1H,d,J=7.5Hz) and 6.1 (1H,d,J=7.5Hz); both these signals disappeared on deuteration; 15 aromatic protons between 7.12-7.78; 2.03(3H,s) due to acetoxy methyl, a pair of benzylic methylenes 3.07(1H,dd,J=14.5,8Hz), 3.22(1H,dd,J=15.6Hz) and 2.74(2H,d,J=7Hz). In addition signals due to a methylene adjacent to acetoxy was observed at 3.84(1H,dd,J=11.5Hz) and 3.95(1H,dd,J=11.5Hz). The signals at 4.35(1H,m) and 4.78(1H,dd) were assigned to methine protons.

^{13}CNMR spectrum (Table 1, Fig.4) of compound (1) showed signals which on the basis of INEPT experiments (Fig.5) were indicative of the presence of a methyl carbon (20.764); three methylene carbons (64.56, 37.42 and 38.41); two aliphatic methine carbons (49.457 and 54.97), nine aromatic methines (126.723, 127.053, 127.114, 128.557, 128.606, 128.225, 129.109, 129.283 and 131.888) and six quarternary carbons (170.75, 170.31, 167.131,





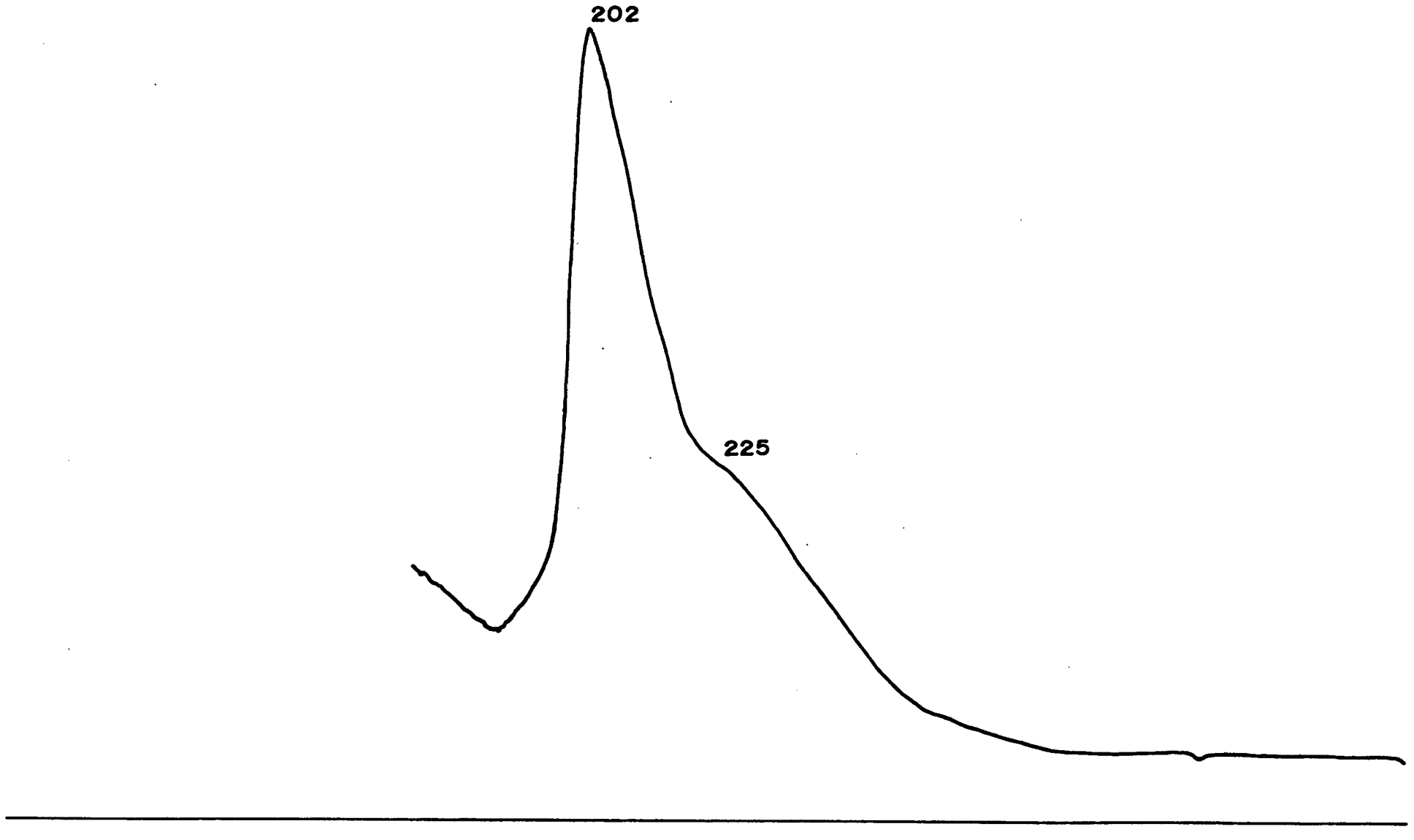


Fig.-1 U V spectrum of N-(N'- Benzoyl S-phenyl alaninyl) S-phenyl alaninol

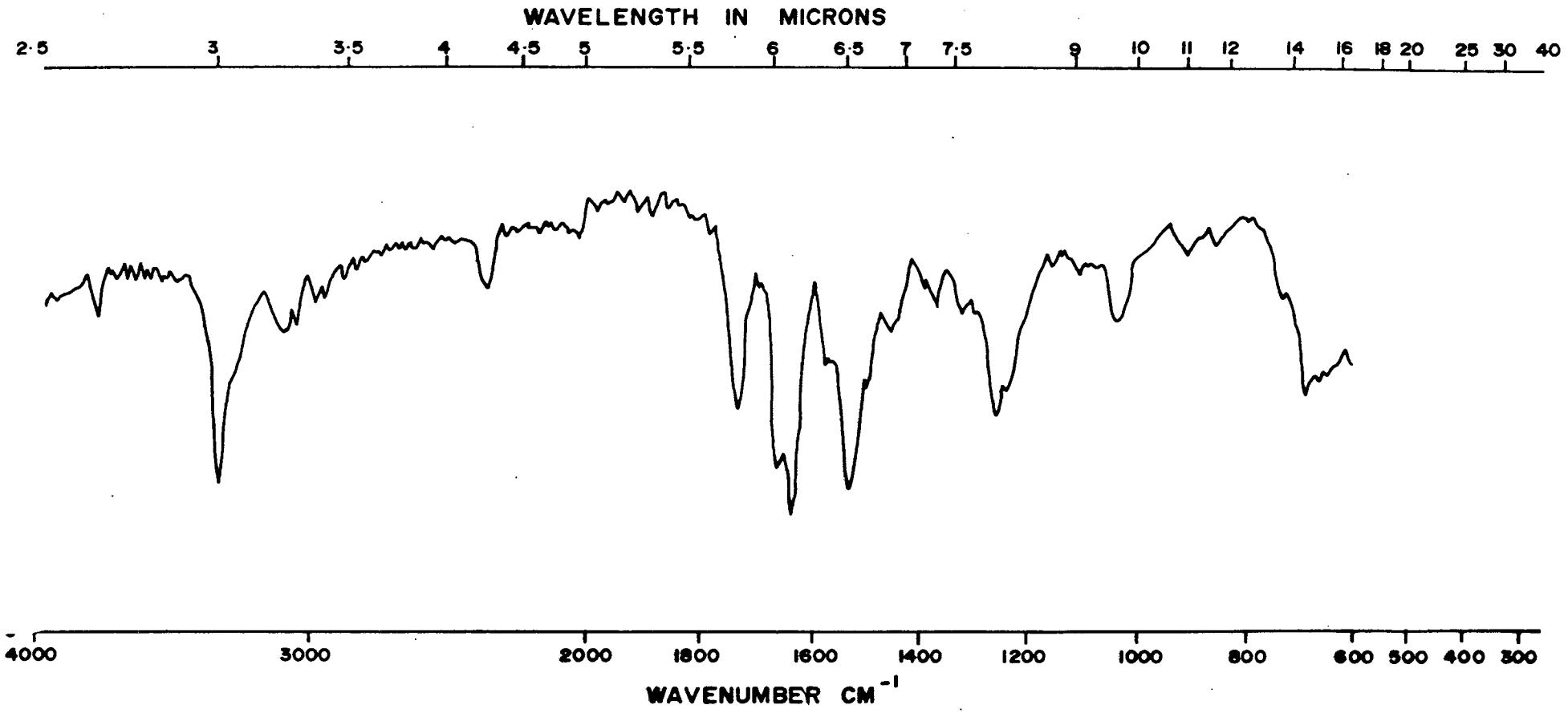


Fig. 2 N-(N'-Benzoyl S-phenyl alaninyl) S-phenyl alaninol acetate (aurantiamide acetate)

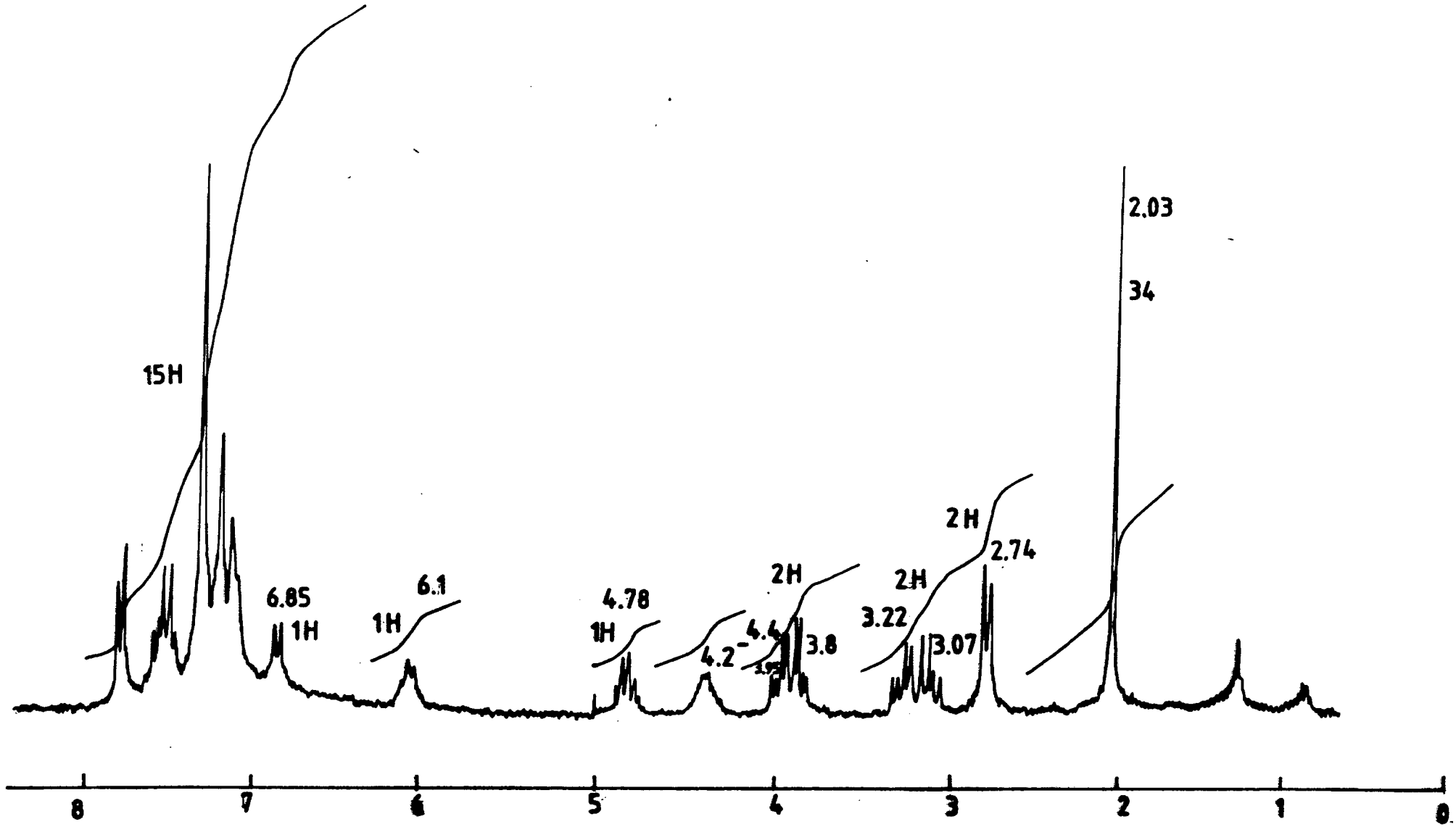


FIG.-3 'H NMR SPECTRUM OF AURATIAMIDE ACETATE

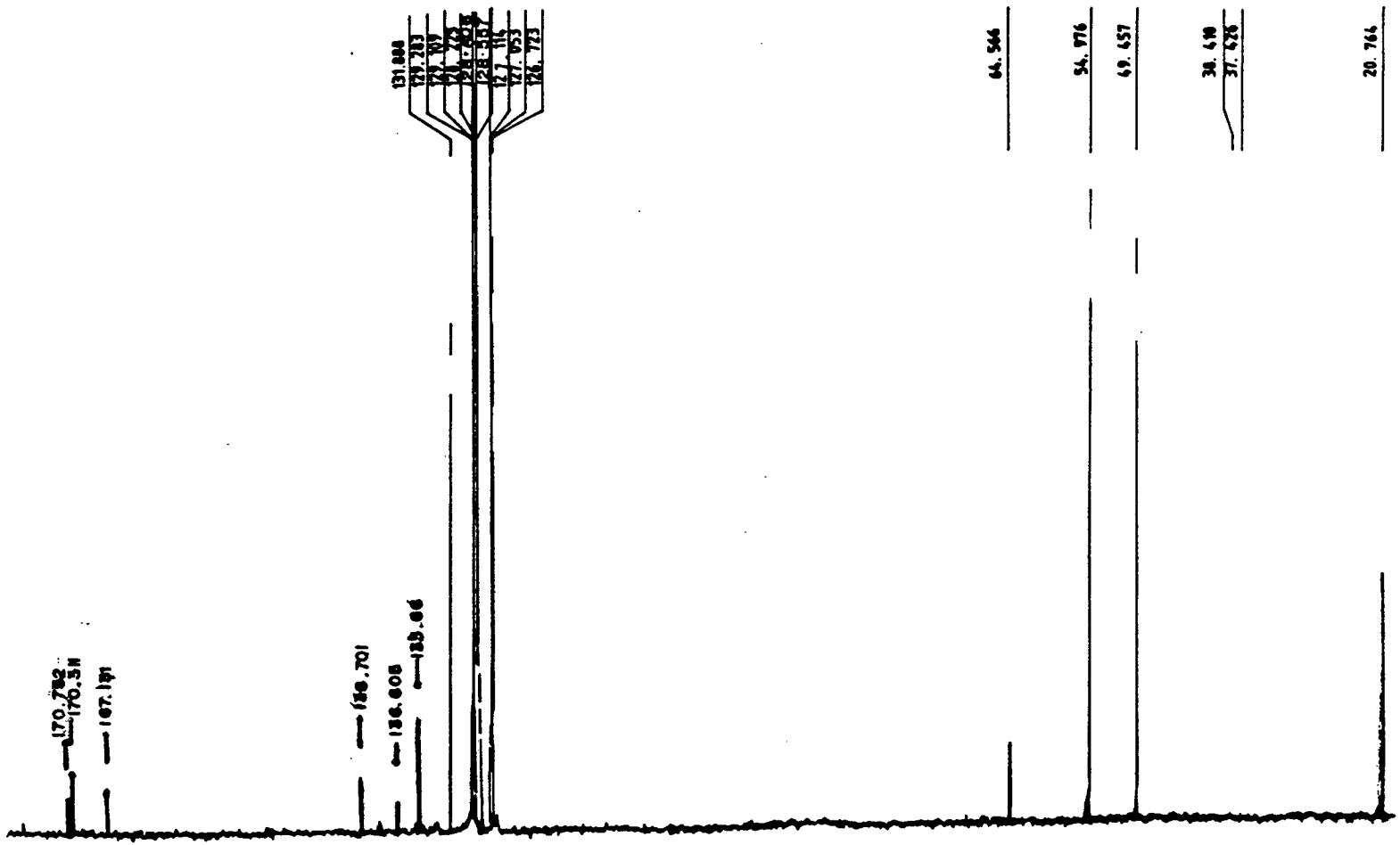
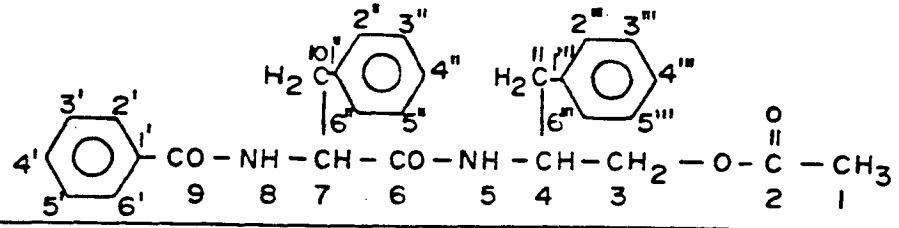


FIG. 4 ^{13}C NMR SPECTRUM OF AURANTIAMIDE ACETATE

Table 1: NMR Chemical shifts of 1 and 2 in CDCl3



	δ-H-atom 1	2	*C-atom	1
H1	2.03 S	2.0 S	C1	20.764
H3	3.84dd (11,5)*	3.90dd (11, 6.5)	C2	170.752
	3.95dd (11,5)	3.96dd (11, 5.5)	C3	64.566
			C4	49.457
H4	4.2–4.47m	4.28–4.31m	C6	170.314
			C7	54.976
H5	6.1 d (7.5)	6.31d (8)	C9	167.131
			**C10	37.426
H7	4.78m	4.81m	**C11	38.410
			C1'	133.66
H8	6.85d (7.5)	6.9d (7.5)	C1"	136.605
			C1'''	136.701
H10	3.07dd (14.5, 8)	2.99 dd (13.6, 9)	C2',6'	128.557
	3.22dd (15, 6)	3.12 dd (14.1, 6.5)	C3',5'	128.606
			C4'	131.888
H11	2.74d (7)	2.79dd (14,8)	C2",6"	127.053
		2.85dd (13.8, 5.6)	C3",5"	127.114
Aromatic			C4"	129.283
15H	7.12 – 7.78	7.1 – 7.72	**C2''',6'''	128.225
			**C3''',5'''	126.723
			C4'''	129.109

* assignement based on cosy experiments.

** Interchangeable.

Coupling constants are given in Hertz in parentheses.

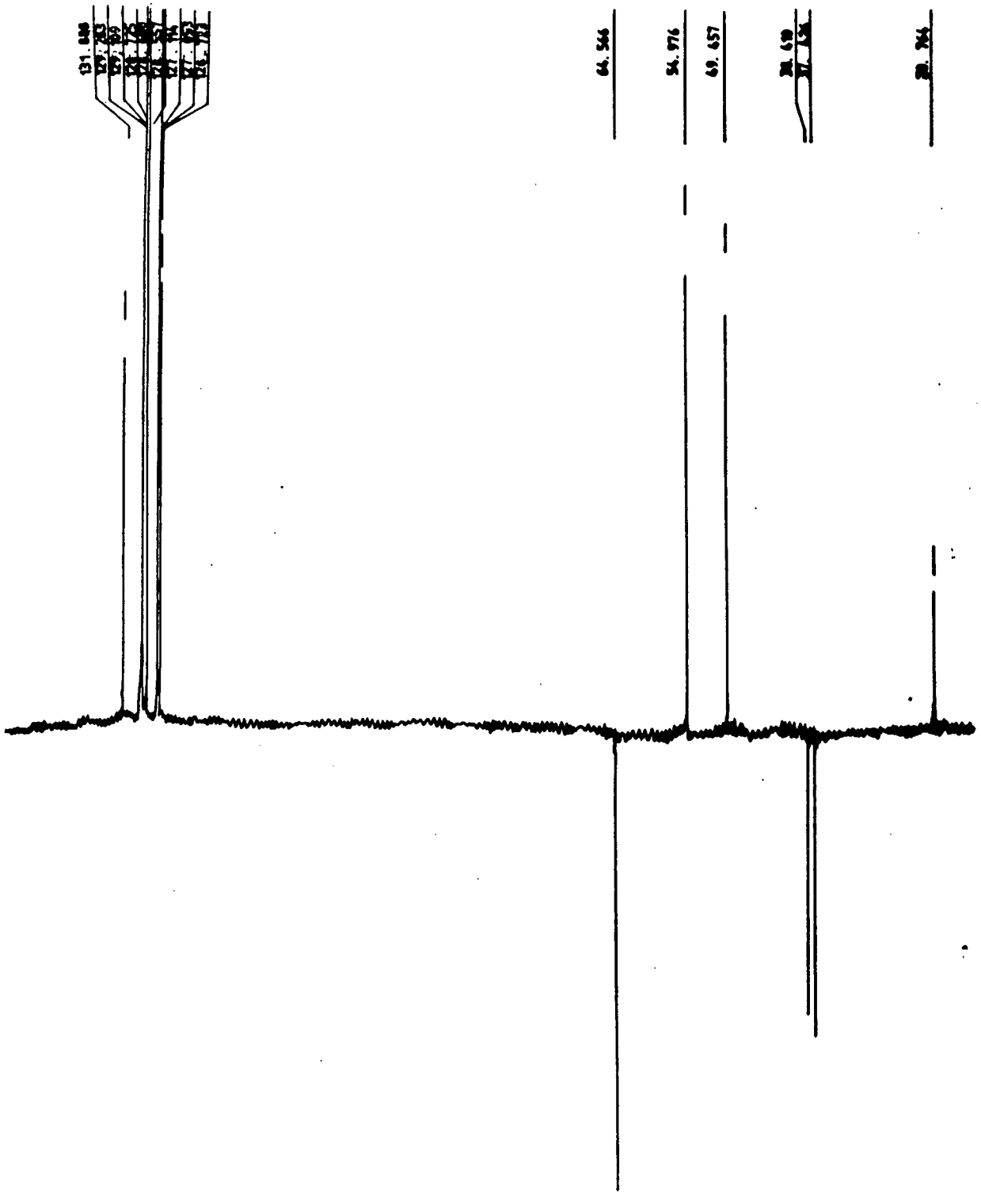


FIG-5. INEPT SPECTRUM OF AURANTIAMIDE ACETATE

133.66, 136.605 and 136.70) of which three were attributed to three carbonyl functionalities and remaining three were aromatic in nature.

Its mass spectrum (Fig.6) also displayed peaks at m/e 43, 51, 77, 91, 131, 134, 172, 176, 224, 252, 269, 293, 311, 323, 353 and 384 besides base peak at m/e 105 and the molecular ion at m/e 444.

Homonuclear proton-proton chemical shift correlated 2DNMR spectroscopy was applied to unambiguously assign the protons. Fig.7 shows a contour plot of COSY experiment. This indicated that 6.1(1H,d,J=7.5Hz,D_2o exchangeable -CONH-) is coupled with the proton resonance at 4.34 (1H,m) which is interacting with a set of methylene protons resonating at 3.84 (1H,dd,J=11Hz and 5Hz) and 3.95 (1H,dd,J=11Hz and 5Hz). HOMCOR experiment also established that 6.85 signal (1H,J=7.5Hz,D_2O exchangeable) is coupled with 4.78 (1H,m) resonance which is in turn interacting with a set of non-equivalent methylene protons resonating at 3.22 (1H,dd,J=15Hz and 6Hz) and 3.07 (1H,dd,J=14.5Hz and 6Hz).

Usual heteronuclear (^{13}C-^{1}H) chemical shift correlation (Fig.8) indicated that the methine carbon signal at 49.4 is associated with the proton resonance at 4.34(m) and the methylene carbon resonance at 37.4 with the proton resonance at 2.7. The proton resonances at 3.95(dd) and 3.84(dd) are related with the resonance at 64.5 for a methylene carbon which should thus be the site of the acetate linkage. The acetate methyl proton resonance (2.03,s) is correlated with the carbon resonance at 20.7. The methine carbon signal at 54.9 is associated with the proton resonance at 4.78(m) and the methylene carbon resonance at 3.22(dd) and 3.07(dd).

The HOMCOR experiment (Fig.7A) further established mutual coupling between the proton resonance at 7.72, 7.43 and 7.52. These signals were related with carbon resonances

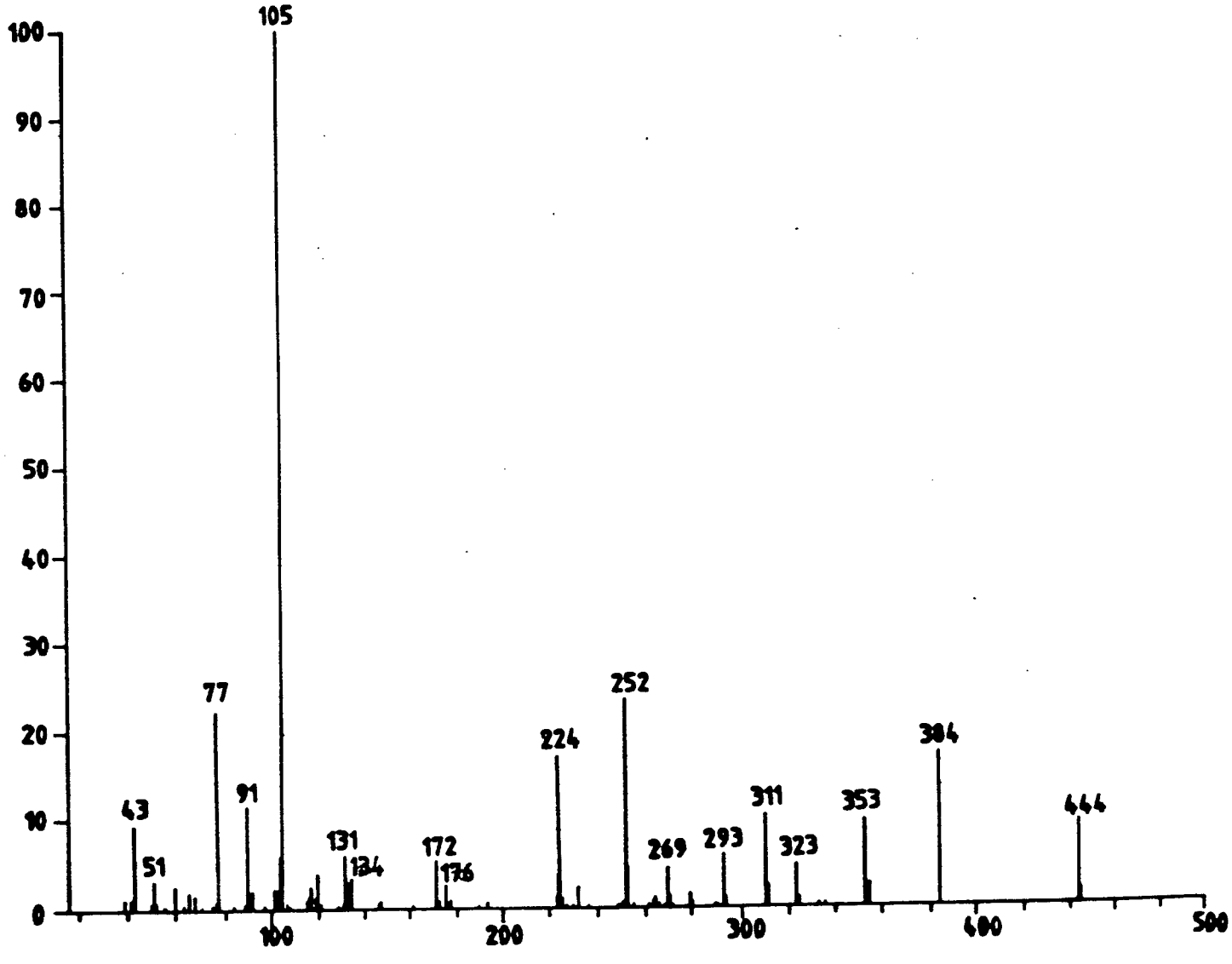


FIG.-6 MASS SPECTRUM OF AURANTIAMIDE ACETATE

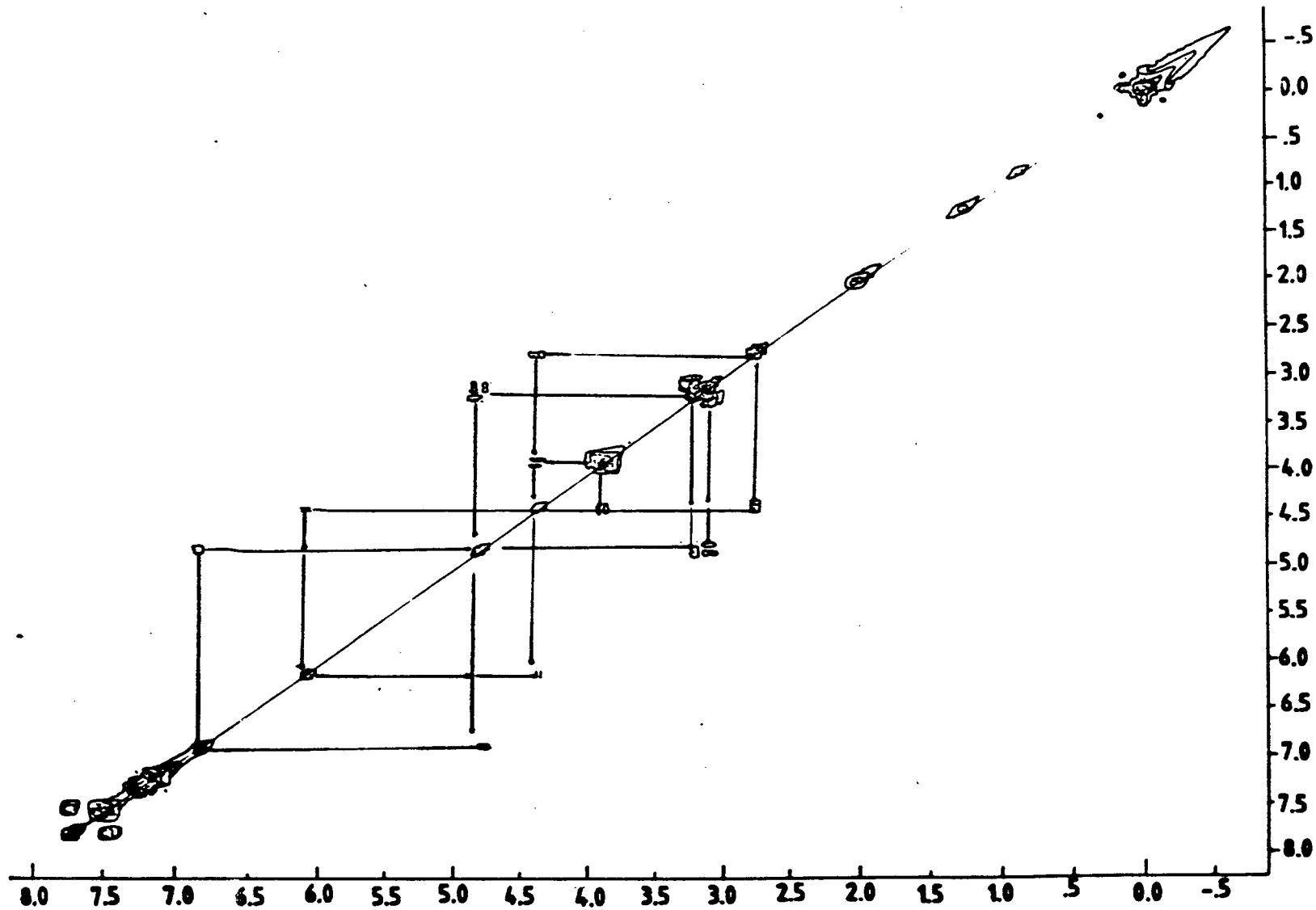


FIG.-7 $^1H - H^1$ COSY SPECTRUM OF AURANTIAMIDE ACETATE

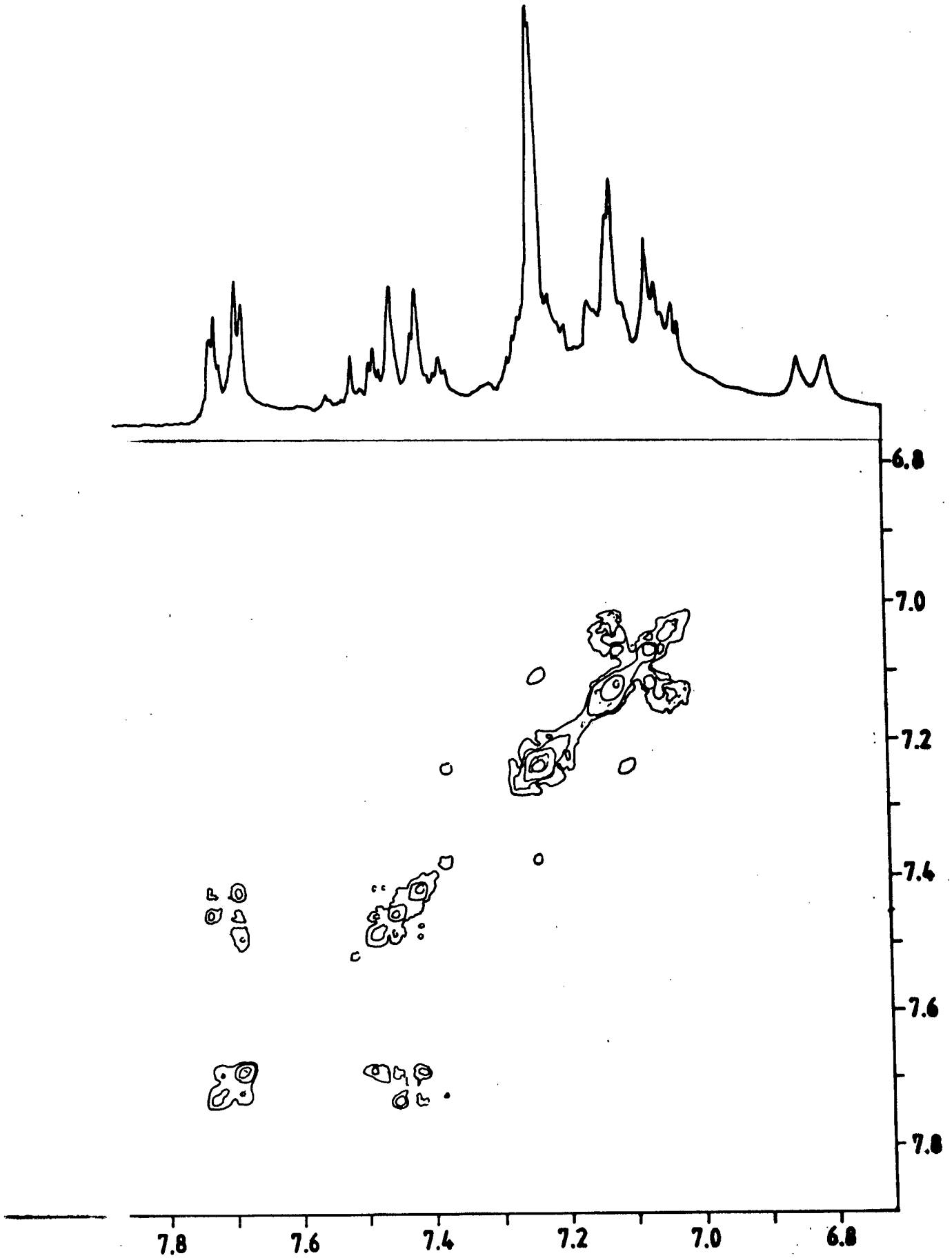


FIG.-7A 'H-H' COSY SPECTRUM OF AURANTIAMIDE ACETATE

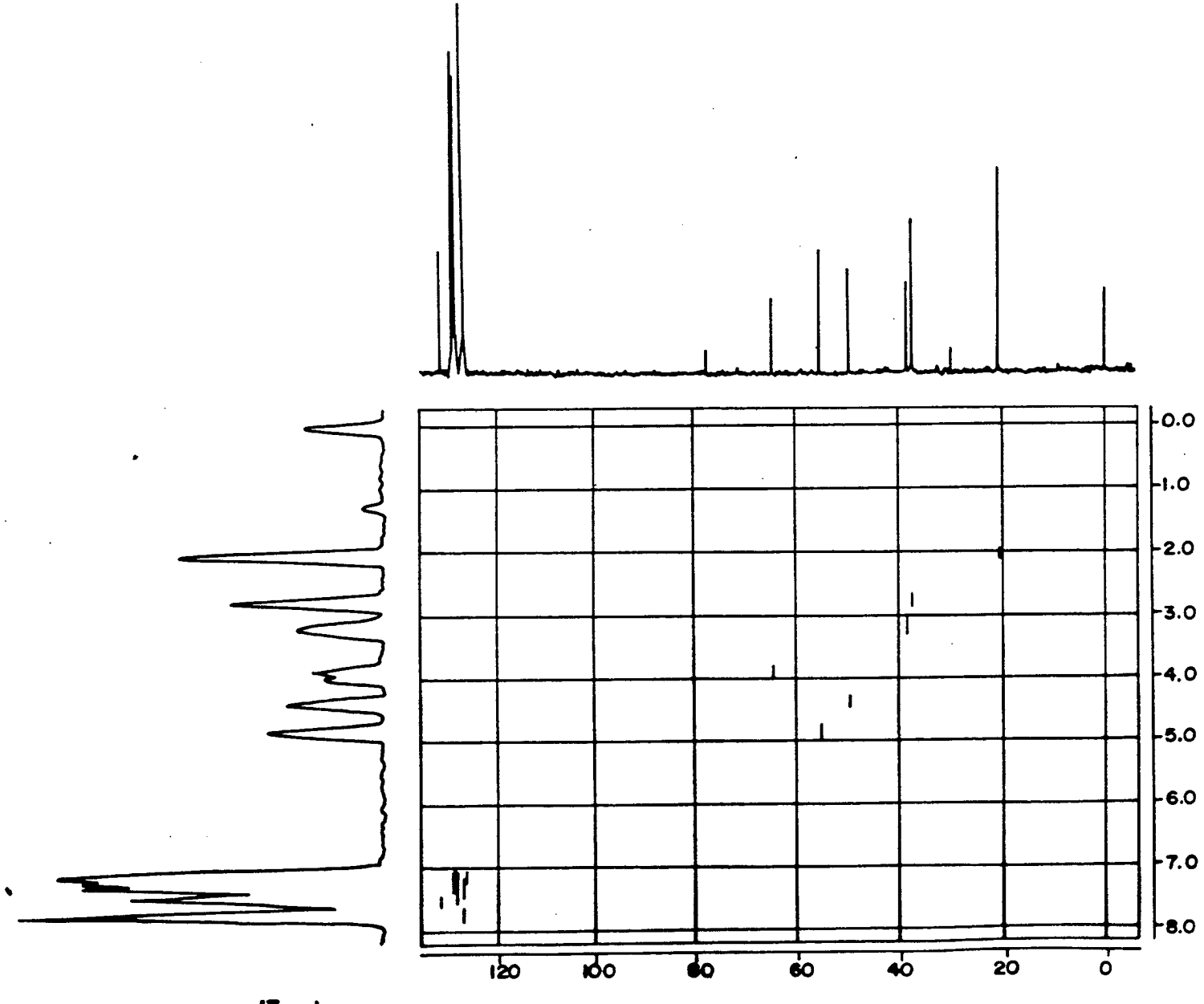


Fig . 8 $^{13}C - ^{1}H$ Cosy spectrum of Aurantiamide acetate

at 128.5, 128.6 and 131.8 resp. of the unsubstituted benzoyl moiety. Similarly mutual coupling was observed between the protons at 7.08(2H) and 7.22(3H) of one of the unsubstituted benzyl group with protons at 7.33(5H) of another unsubstituted benzyl group.

The foregoing evidence together with the mass spectral fragmentation pattern established (1) as the structure of dipeptide without any stereochemical implications. The COSY experiments (HOMCOR and HETCOR) were consistent with this structure which was found to be identical with the aurantiamide acetate. Confirmation of the structure (1) was esablished by comparison of the spectral data with the literature values.[19]

Compound (2), m.p.167°C obtained in traces (2mg) had a RF value lower than that of (1) but MS (Fig.9) virtually identical with that of aurantiamide acetate and ^{1}H NMR(Table 1, Fig.10) differing significantly only in the multiplicity and coupling constant of the signal for the benzylic methylene protons (H_{11}) attached to C-4 indicating that the stereochemistry differed at C-4. This revealed that compound (2) was a diastereoisomer of (1) to which structure (2) was tentatively assigned. Lack of material precluded further spectral investigation of this isomer.

Four stereoisomeric structures depending on the configuration of the two chiral centers of aurantiamide acetate are possible. To ascertain the stereochemistry of the compounds (1) and (2) all the four stereoisomers were synthesised by a known procedure[19] (Scheme 1). The isolated dipeptides (1) and (2) have been identified with the acetylated synthetic compounds obtained from S-N-benzoyl phenylalanine and S and R - phenyl alaninol. Thus compound (1) is O-acetate of N-(N'-benzoyl-S-phenylalaninyl)-S-phenylalaninol and compound (2) is O-acetate of N-(N'-benzoyl-S-phenylalaninyl)-R-phenylalaninol.

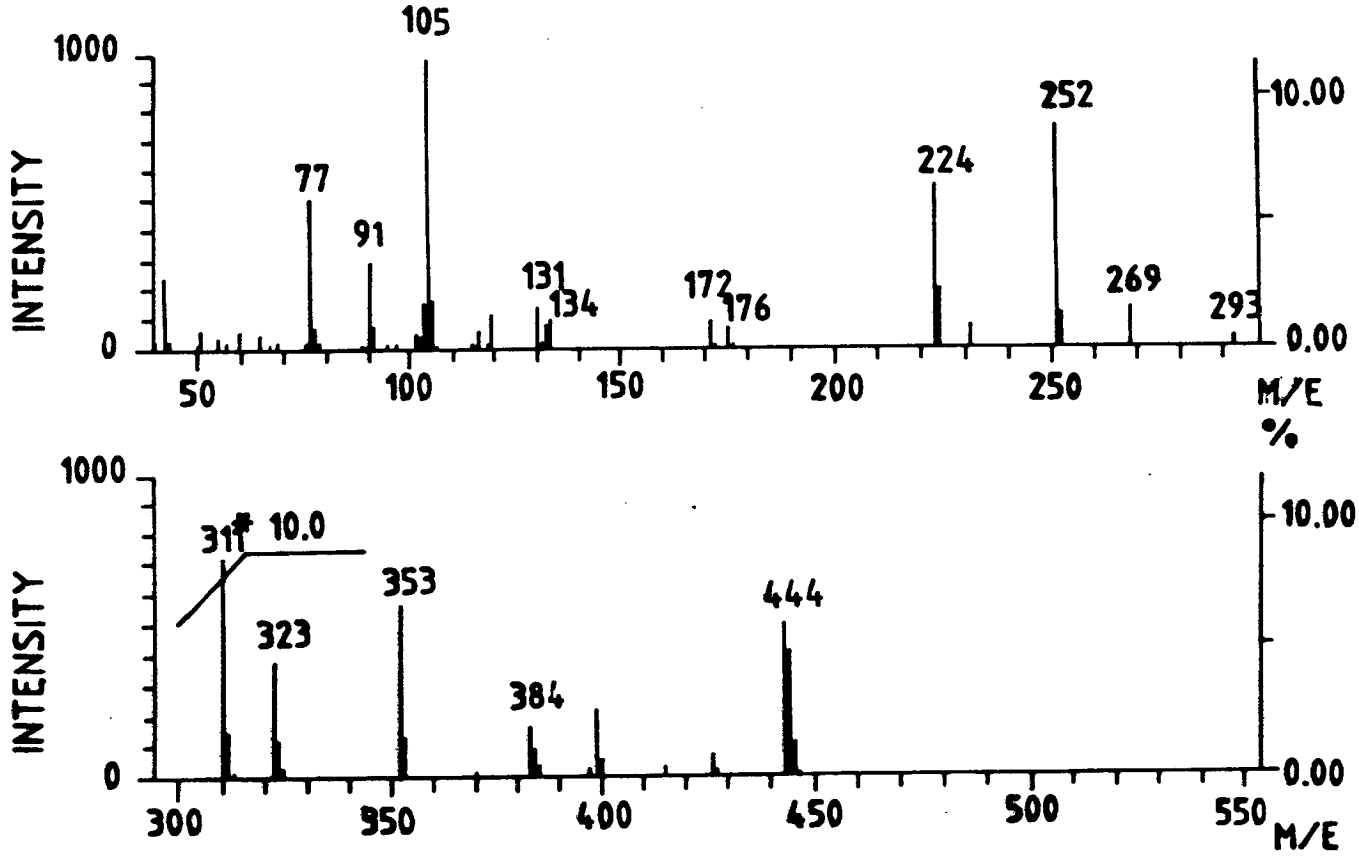


FIG.-9 MASS SPECTRUM OF DIAAURANTIAMIDE ACETATE

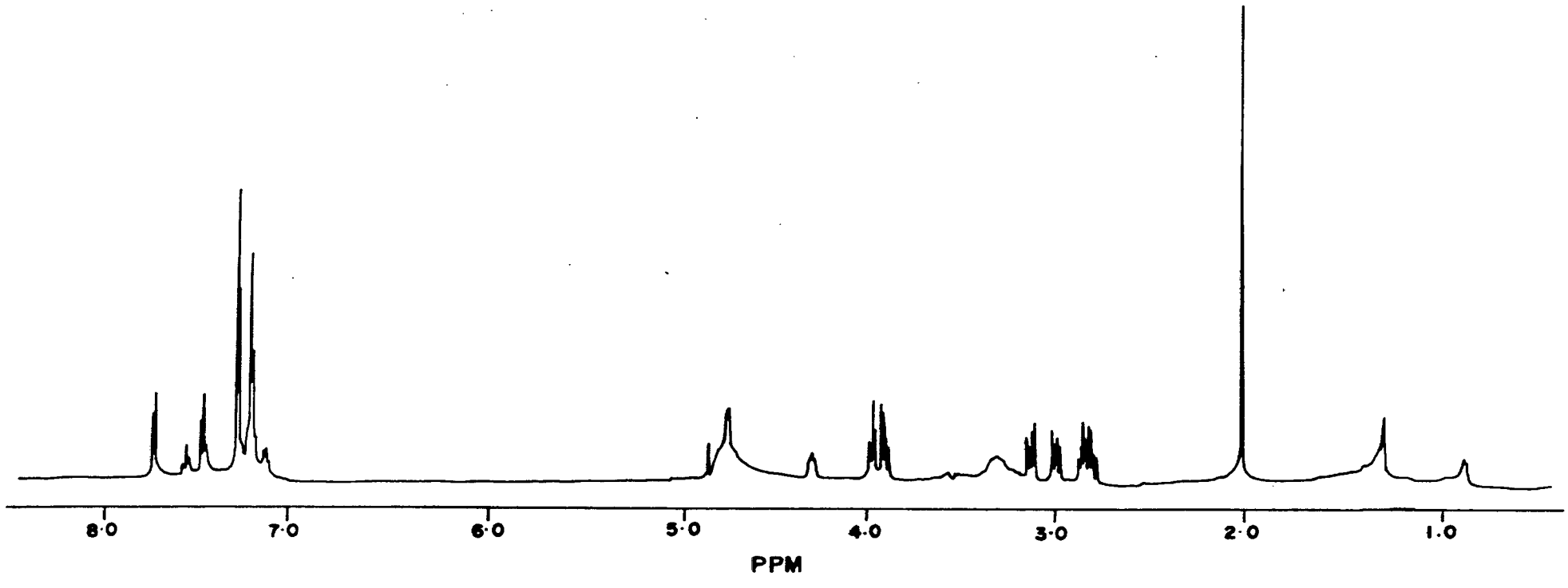
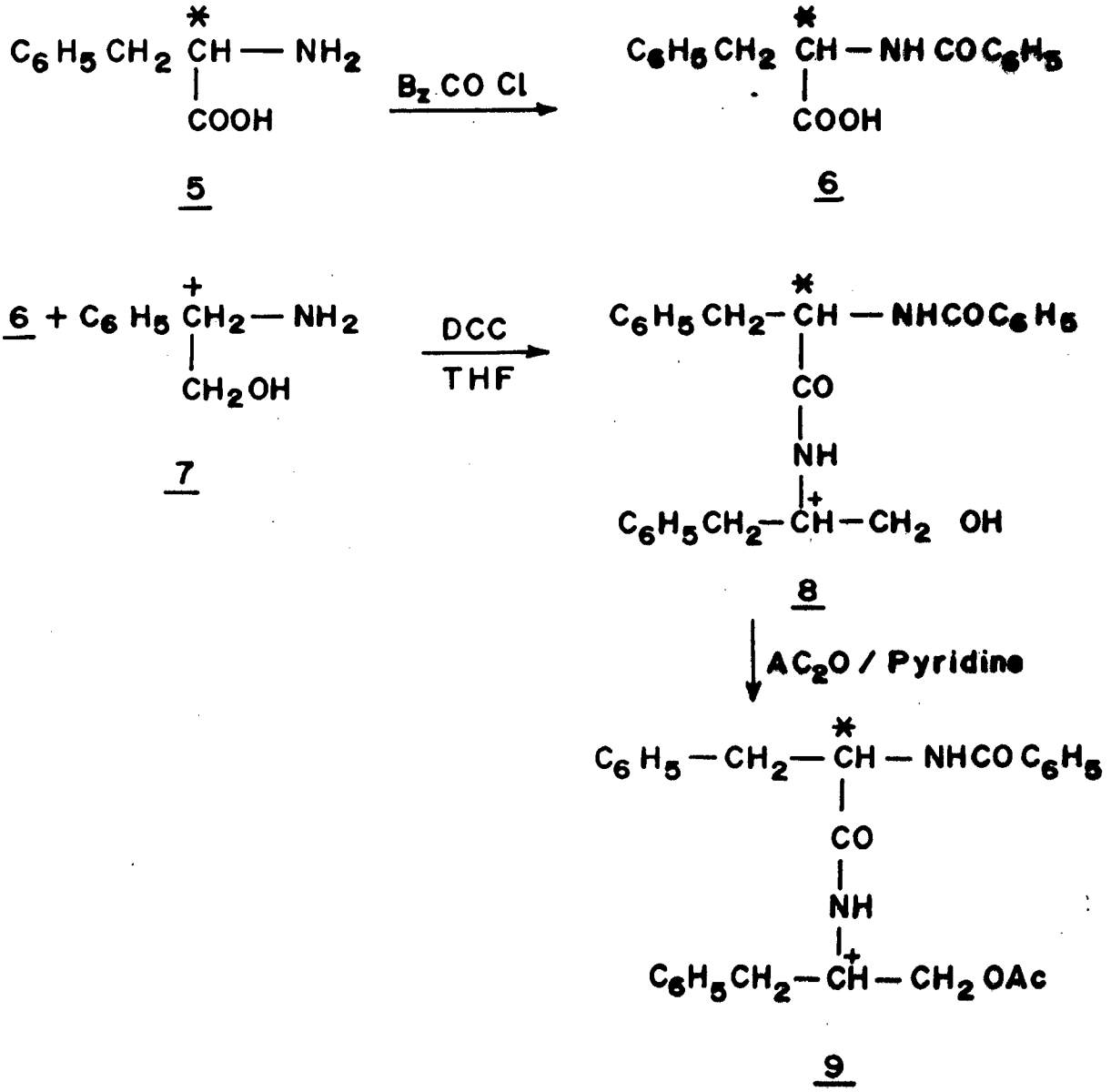


FIG.-10 ^{1}H NMR SPECTRUM OF DIA - AURANTIAMIDE ACETATE



5 6 7 8 9

9 a	* = S ; + = S
9 b	* = R ; + = R
9 c	* = R ; + = S
9 d	* = S ; + = R

Scheme 1

Synthesis of diastereoisomers of N (N'-benzoyl phenyl alaninyl) phenyl alaninol acetate

The mass spectra of both the acetates (1) and (2) also displayed peaks at m/e 311 and 134 corresponding to the elimination of $C_6H_5CH_2$ — fragment and formation of $[C_6H_5CH_2CH{=}CHOH]^+$ ion respectively from the molecular ion M^+ of the corresponding alcohols, aurantiamides (3) and (4). The aurantiamides could have either originated from the acetate by elimination of ketene during fragmentation process or present in it as impurities. To ascertain this the column chromatographed aurantiamide fraction of *Acantophora spicifera* was subjected to HPLC analysis (Table 2) and their presence confirmed through comparison of their retention times and coinjection with synthetic dipeptides. The contamination of the acetate with the alcohol is quite possible since both have nearly the same RF values.

Table 2. Relative R_t S and % composition (uncorrected) of the aurantiamides from the seaweed *Acantophora spicifera*.

Constituent	Rt (Synthetic)	Rt (Natural)	%
SS - Acetate	8.93	9.44	18.549
SR - Acetate	9.00	8.55	0.644
RS - Acetate	9.00	—	—
RR - Acetate	8.49	—	—
SS - Amide	8.37	11.82	1.62
SR - Amide	8.71	10.67	51.748
RS - Amide	8.69	—	—
RR - Amide	8.77	—	—

It may be mentioned here that the column chromatographed fraction used for HPLC analysis was obtained from fresh specimens of *A. spicifera* and not from the same extract from which the acetates were isolated and identified. The different percentage composition shown by HPLC is due to ecological factors and different stages of developmental cycle prevailing at the time of collection.

Literature indicated this to be the first report of natural occurence of dia-stereoisomer of aurantiamide(4) and its acetate(2). Since the two aurantiamides and their acetate co-occur in A. spicifera, one could expect the dia-aurantiamide also to be present in terrestrial plants.

11 — ∝ -hydroxy cholestane-3,6-dione (10)

There has been continuing interest in the sterols and steroids of marine organisms ever since the earliest studies of Henze[24] and Doree[25] showing the potential for new sterols other than cholesterol. The discovery of many new marine sterols confirm the predictions made long ago by Bergmann[26] regarding their diversity. In the sterol composition of the red algae, C_{27} compounds are found to be major constituents in which cholesterol predominates, although in a few species desmosterol has been reported to be present in substantial amount and may even be the major steroid.[27-29] In the red alga investigated, 5 ∝ -cholestane-3,6-dione was found to be the major steroid.[17]

Red algae also contain, though in minor amounts C_{26}, C_{28} and C_{29} sterols and in some species the presence of Δ^4-3-keto steroid has been reported.[30,31]

Extensive silica gel chromatography of the petroleum ether: ethyl acetate(80:20) eluate led to the isolation of yet another crystalline compound, a sterol, (10), m.p.145°C. It gave on TLC plate, solvent system, petroleum ether : ether : acetic acid (70:30:6), a golden yellow spot turning into orange with 2,4-dinitrophenyl hydrazine as the detecting agent. Its ketonic nature was evident from its IR absorption at 1700cm^{-1} and 1680cm^{-1} (Fig.20), (no IR and ^{1}H NMR evidence for aldehydic group). Its IR spectrum also indicated the presence of -OH group at 3460cm^{-1} and -$\overset{O}{\overset{\|}{C}}$-$CH_2$ functionality at 1405cm^{-1}.

The ^{1}H NMR spectrum (Fig. 21) of the compound revealed that it was a steroid but it gave no reaction with the Liebermann-Burchard test thus indicating the absence of 3ß-OH

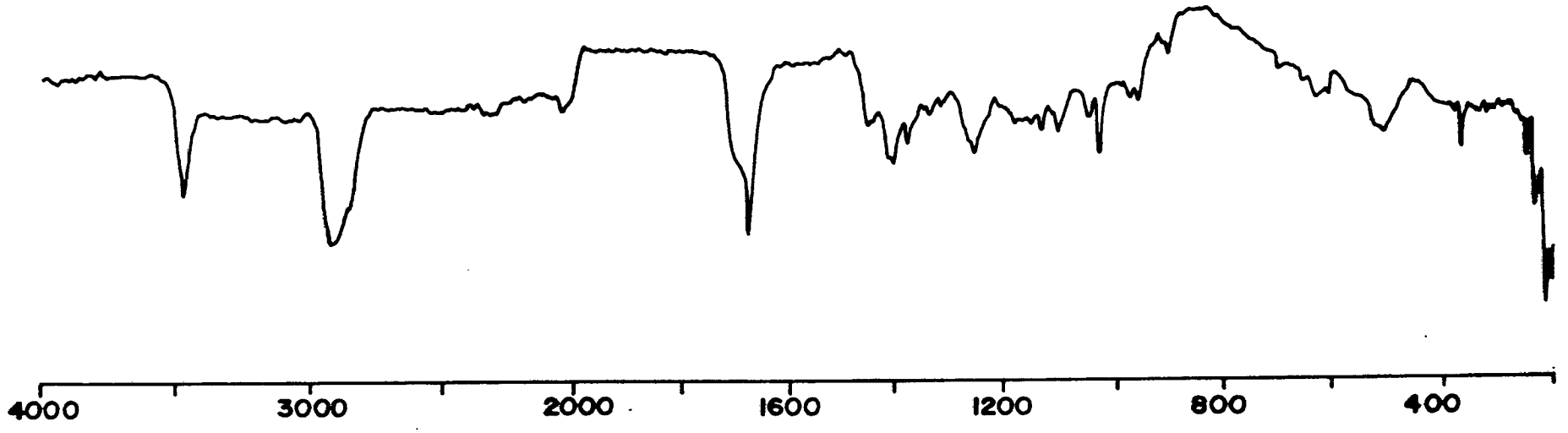


Fig.-20 IR spectrum of 11 ∝ - hydroxy cholestan 3,6 dione

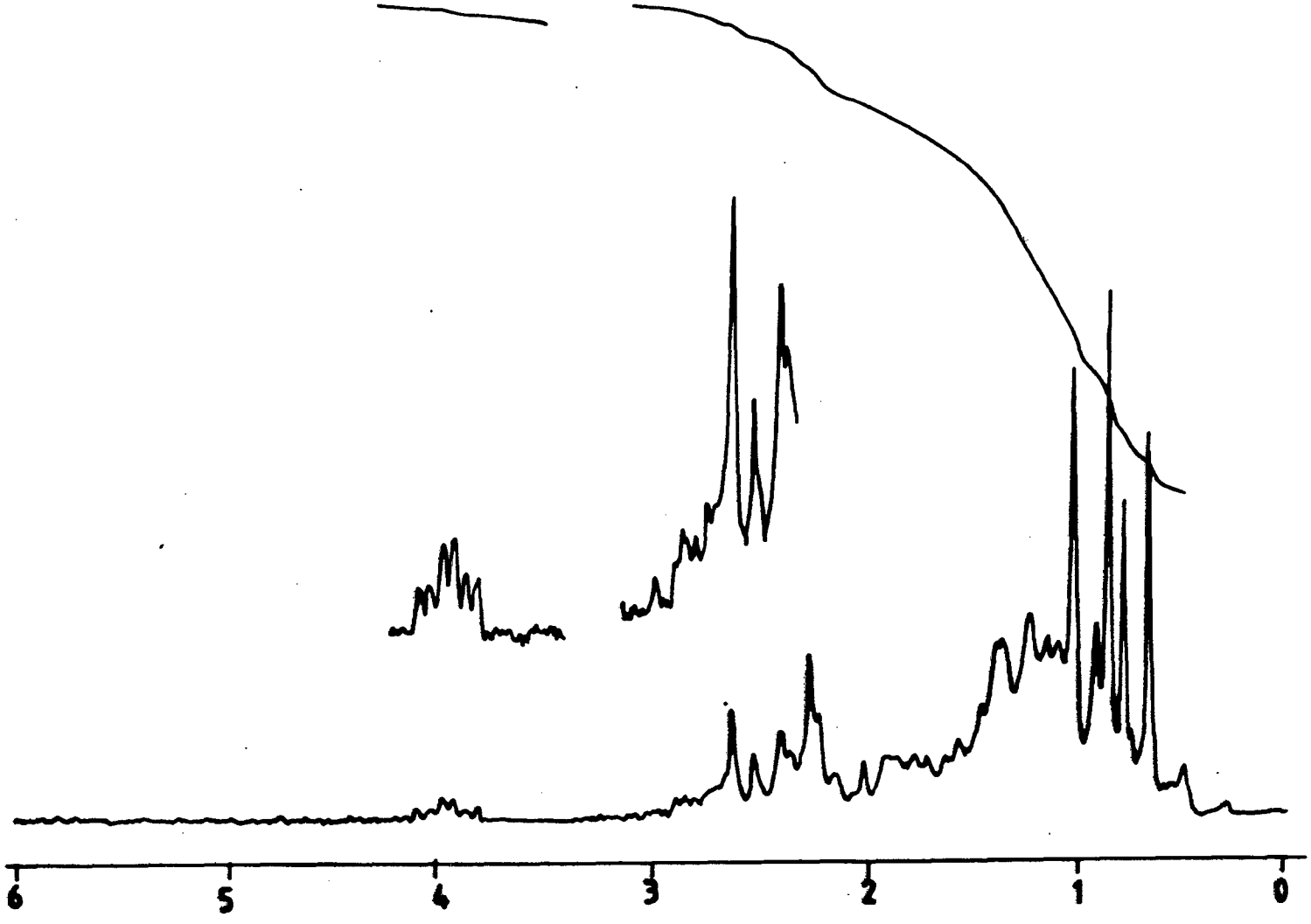


FIG.-21 ^1H NMR SPECTRUM OF 11α HYDROXY CHOLESTAN, 3,6- DIONE

group. It displayed signals at 0.71(3H,s) and 1.08(3H,s) for the two tertiary methyls, a signal at 0.94 was attributed to the secondary methyl at C_{21}(J=6Hz) and a doublet for six protons centered at 0.86(J=6Hz) was assigned to the methyl group at the end of side chain. Moreover the spectrum showed a septet at 4.05 due to methine -CH- proton; seven protons resonating between 2.0-2.85 probably αC to the >C=O group. Signals due to olefinic protons were absent in the ^{1}H NMR spectrum. The presence of two carbonyl groups in the molecule was evident from the ^{13}C NMR signals at 208.5ppm and 211.1ppm respectively. All this spectral data revealed that the compound is a steroid belonging to cholestane series with two carbonyl and a hydroxyl group.

The mass spectrum (Fig. 22) of the compound exhibited a molecular ion peak at M$^+$416, corresponding to molecular formula $C_{27}H_{44}O_3$, a base peak at m/e 43($C_3H_7^+$); and prominent peaks at 398(M$^+$-H_2O); 387(M$^+$-29); 383(M$^+$-H_2O + CH_3); 285(M$^+$-sidechain + H_2O); 370; 355; 301; 300; 259; 243; 105; 81; 69 and 55. The peak at m/e 387(M$^+$-29) indicated that the stereochemistry at C_5 is α[32].

In order to fix the positions of the functional groups in the compound(**10**), ^{1}H—^{1}H COSY, COSYLR and 2D XHDEPT NMR experiments were carried out. The ^{1}H and ^{13}C chemical shifts are given in Table 3.

Table 3. 3^{13}C and 1H NMR chemical shifts of (10) in $CDCl_3$

Atom	δC	δH	$J_{H,H}$(Hz)
1α	37.7	2.37	m^e
1ß		2.44	m^e
2α		1.82	m^e
	39.9		
2ß		2.81	J_{gem} = 14.1; $J_{2,1}$ = 6.5; $J_{2,1}$ = 2.3
3	208.5		

4 ∝		2.32	m[e]
	37.3		
4ß		2.60	m[e]
5	57.9	2.68	$J_{5,4} = 2.7$; $J_{5,4} = 12.9$
6	211.1	—	m[e]
7 ∝		2.09	$J_{7,8} = 12.9$; $J_{gem} = 14.1$
	46.1		
7ß		2.36	m[e]
8	36.5	1.83	m[e]
9	35.7	1.41	m[e]
10	42.9[a]	—	—
11	68.7	4.01	$J_{11,12} = 11.0$; $J_{11,12} = 4.7$; $J_{11,9} = 10.6$
12 ∝		1.32	m[e]
	51.7		
12ß		2.36	m[e]
13	43.1[a]	—	—
14	28.0	1.35	m[e]
15 ∝		1.05	m[e]
	28.1		
15ß		1.91	m[e]
16∝		1.09	m[e]
	24.0		
16ß		1.51	m[e]
17	56.1	1.18	m[e]
18	13.1	0.71	s
19	13.0	1.08	s
20	55.9	1.33	m[e]
21	18.6	0.94	J = 6.6
22	39.4	1.13	m[e]
23	35.9	1.28	m[e]
24	23.8	1.37	m[e]
25	59.2	1.44	m[e]
26	22.7	0.87	J = 6.6
27	22.5	0.86	J = 6.6

[a] Assignments are interchangeable
[e] Overlapped with other signals

Various DEPT experiments identified the multiplicity of each ^{13}C NMR resonance. It indicated the presence of four quarternary carbons. Two of these were for two carbonyl functionalities while the remaining two were aliphatic in nature. The experiments also

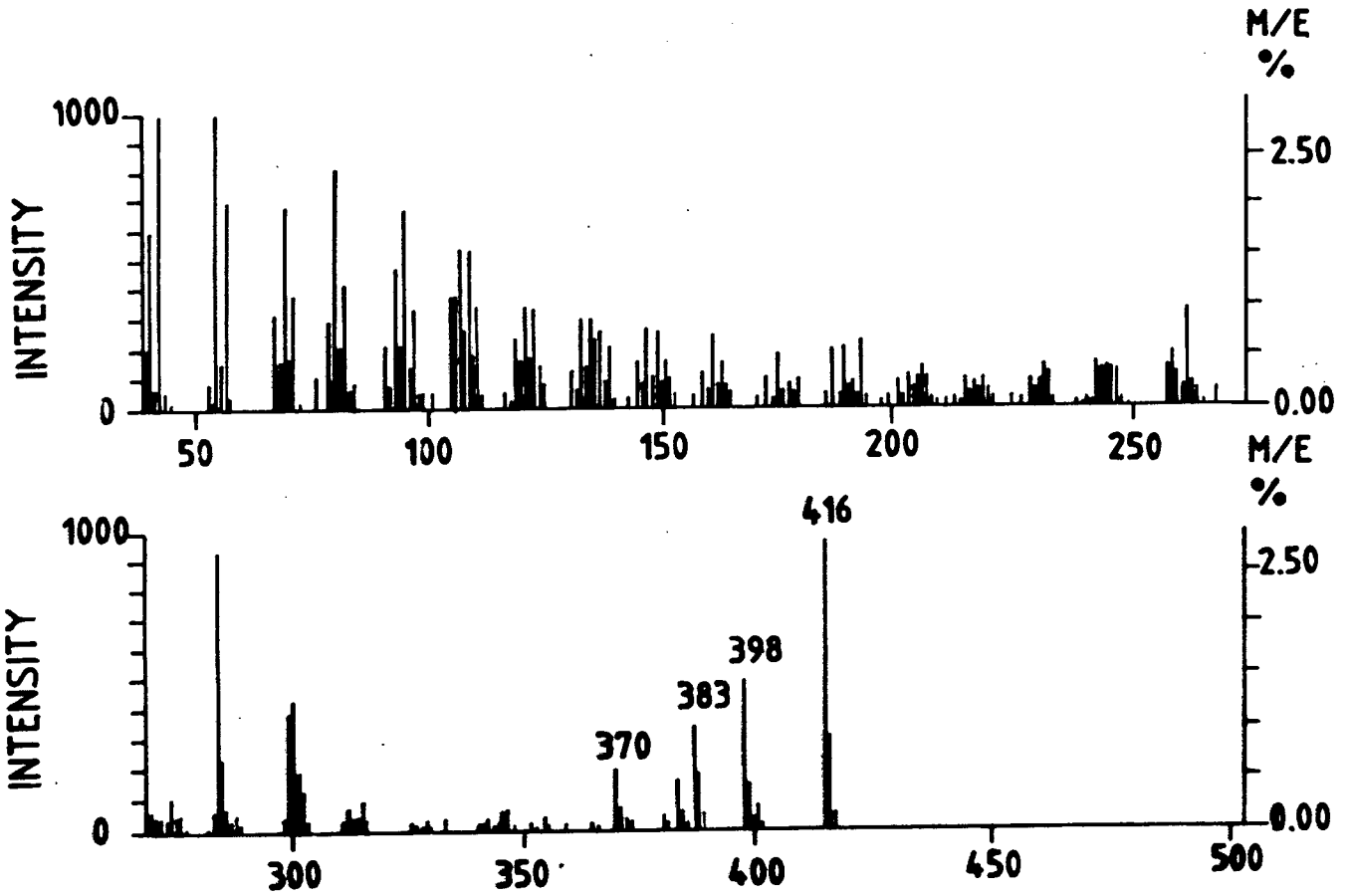


FIG.-22 MASS SPECTRUM OF 11∝- HYDROXY CHOLESTAN, 3,6 -DIONE

revealed the presence of seven aliphatic methines, one hydroxy methine, and ten methylenes and five methyl carbons in the molecule.

The nature of the carbons fully justify the structure of the molecule. However, the positions of the two carbonyl and one hydroxyl functions were still undecided. The positions of the functional groups in the molecule were finally fixed with the help of two dimensional NMR experiments as follows:

The signal at 4.01 in the ^{1}H NMR spectrum was unambiguously assigned to the spin system containing hydroxy function. It showed cross peaks(Fig.23) at 2.38, 1.41 and 1.31 only. The signal at 2.38 and 1.31 were due to a methylene signal from the 2D XHDEPT spectrum(Fig.24) whereas a methine signal was at 1.41. These evidences indicated that the carbon bearing hydroxyl group is flanked by a methylene and a methine carbon. Moreover, in the long range COSY spectrum(Fig.25), the Me-18 signal at 0.71 showed cross peaks with the signals at 2.38, 1.31 and 1.18 due to H-12ß, H-12α and H-17α(W- type couplings). Since H-12ß and H-12α were coupled to the proton bearing hydroxyl functionality, this could only be possible if the hydroxyl group is placed at C-11. The signal at 1.41 was for H-9 which further showed cross-peak at 1.83 for H-8. The signal gave cross peaks at 2.36 and 2.09(H-7ß,H-7α).

The H_2-7 protons did not show any cross peaks with any of the protons in the 2D spectrum, clearly suggesting that there are no adjacent protons which are scalar coupled to both the H_2-7 protons. Thus the locus of one of the carbonyl functions is at C-6. This was confirmed by the downfield shift of C-7 in the 2D^{1}H-^{13}C(XHDEPT) spectrum at 46.1 in the F_2 dimension and also by the coupling constants at H-7α in the ^{1}H NMR spectrum (Table 3).

The two methyl signals as doublets in the ^{1}H NMR spectrum at 0.87 and 0.86 were

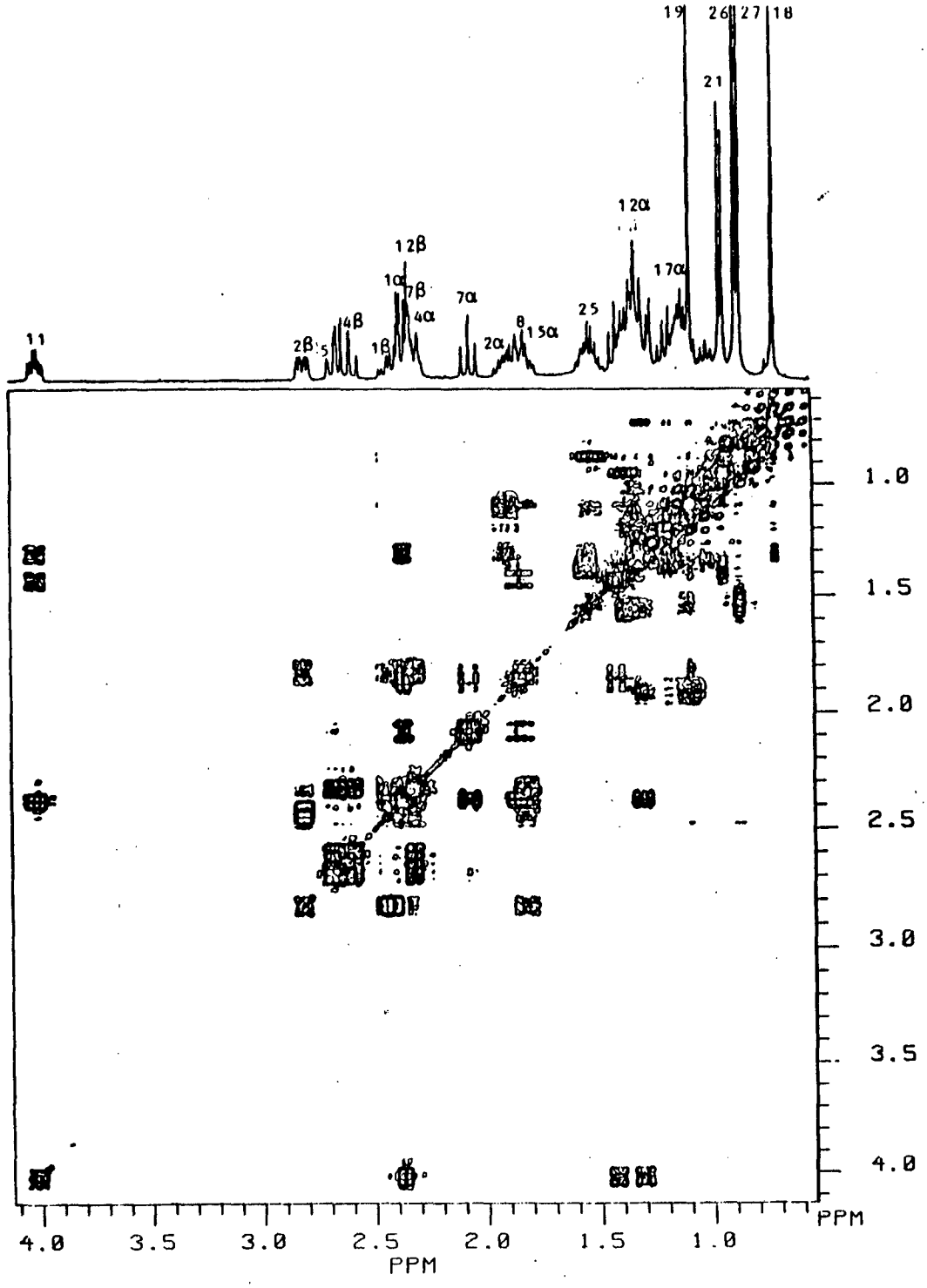


Fig. 23 ^{1}H-^{1}H COSY spectrum of IO underneath ID - ^{1}H NMR spectrum

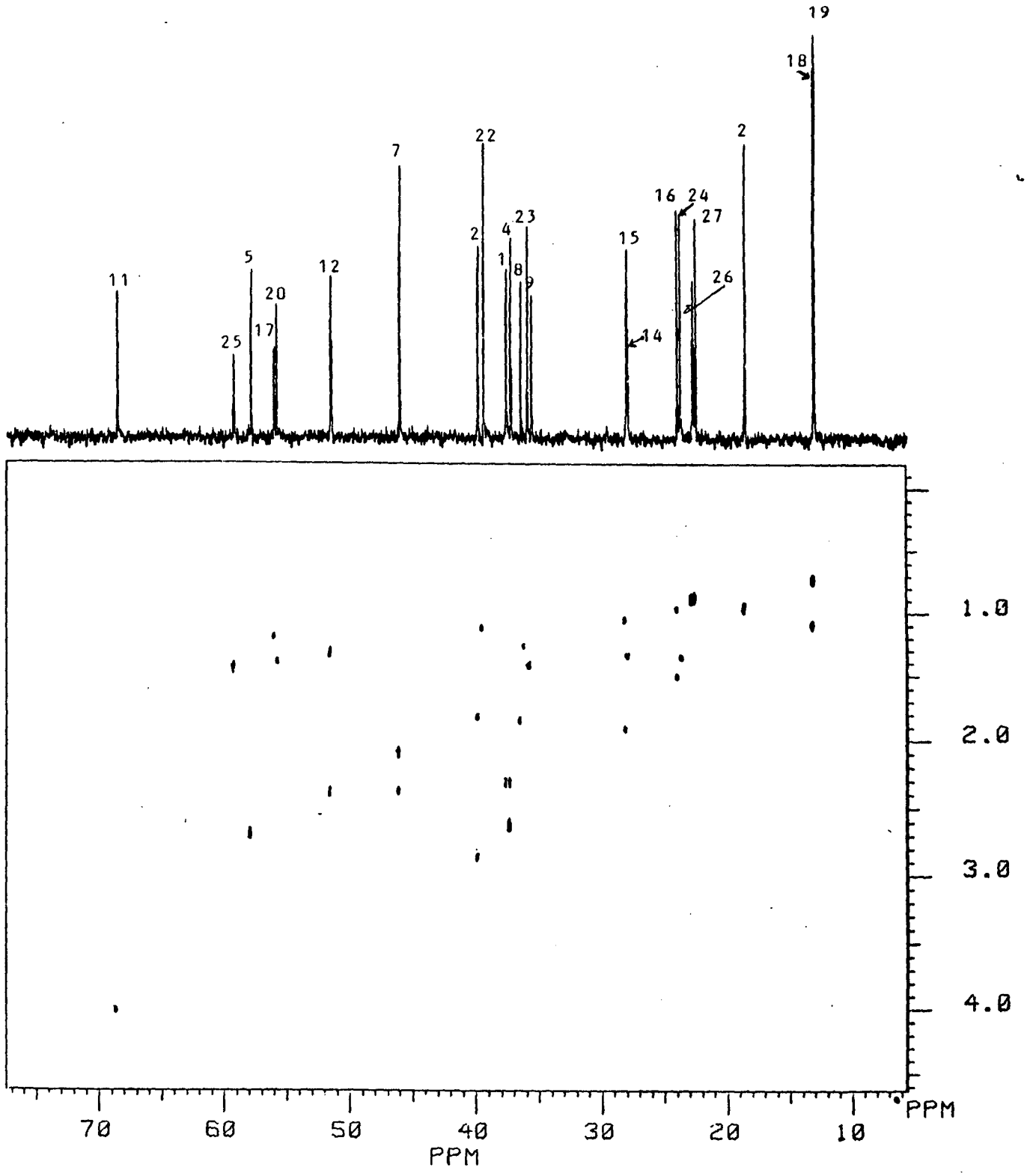


Fig. 24 $^{1}H-^{13}C$ COSY spectrum of 10

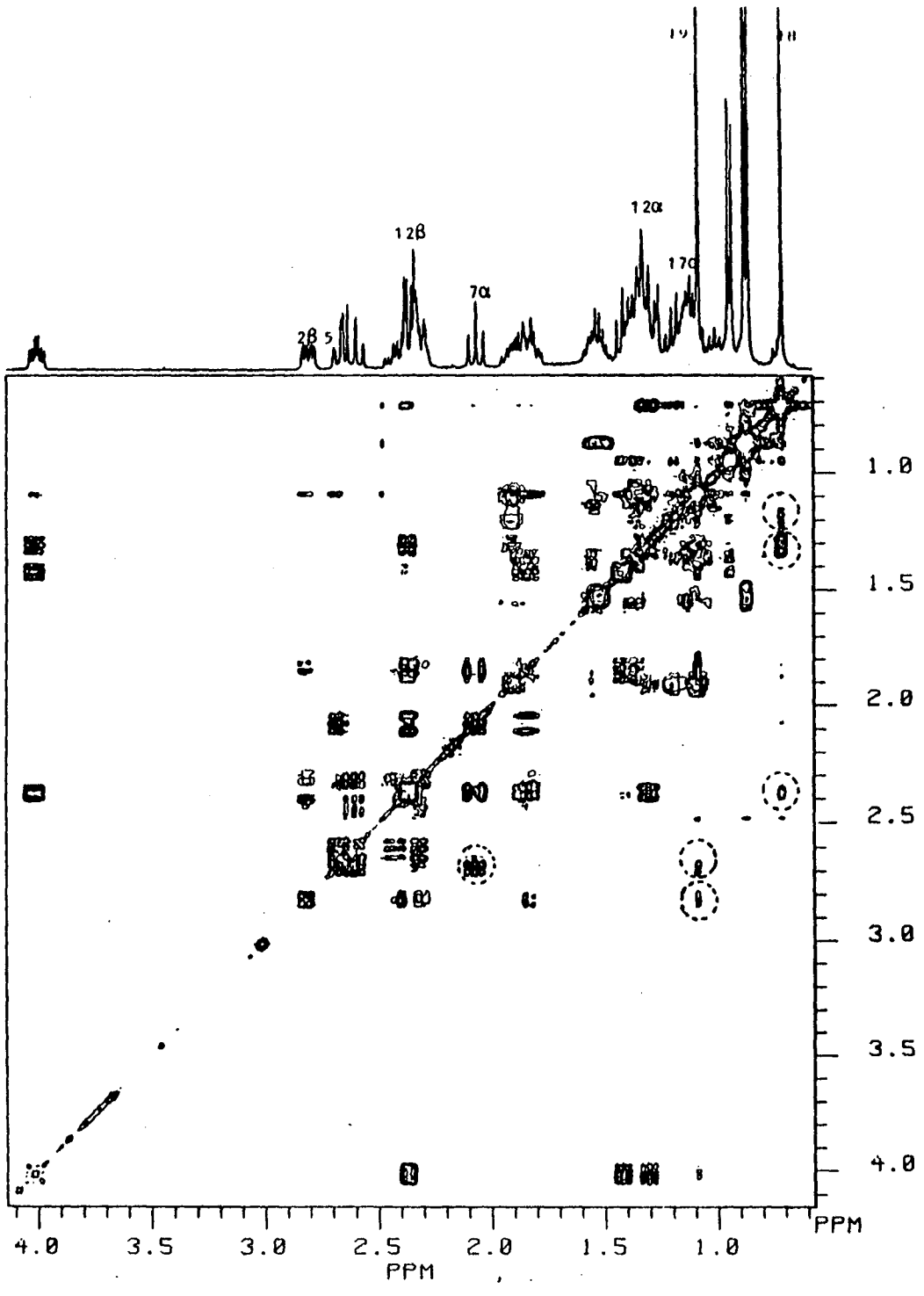
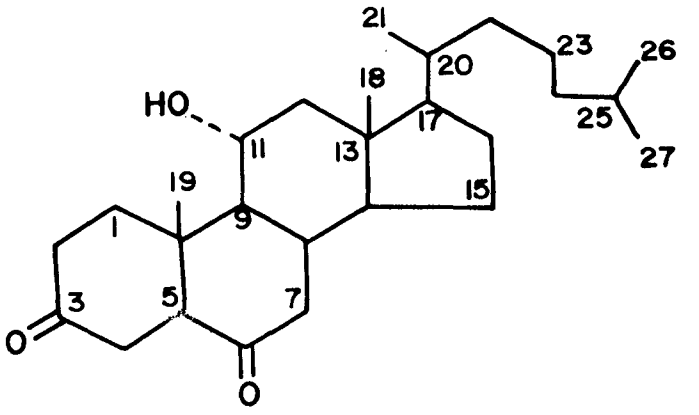


Fig. 25 $^{1}H-^{1}H$ COSY spectrum (long range) of IO underneath ID-^{1}HNMR spectrum

assigned to Me-26 and 27 and these signals showed cross peaks at 1.44 for H-25. Similarly connectivities were traced as an unbroken sequence from H-25 to H-14(protons of the side chain and D-ring in (10). The data discussed above suggested that the second carbonyl function is located in ring A in (10). The locus of this carbonyl function was at C-3, since in the COSYLR spectrum(Fig.25), the H-7 α signal showed cross peaks at 2.68 due to 'W' type ^4J coupling with H-5. The H-5 signal showed cross peaks (Fig.23) at 2.60 and 2.32 for both the H_2-4 protons. Both the H_2-4 protons did not show any cross peak indicating that there is an interruption at C-3. The assignment of H-5 was confirmed in the COSYLR spectrum(Fig.25) where it gave cross peak with Me-19 at 1.08. The signal at 2.81 was assigned unambiguously to H-2 (α to the carbonyl group), it showed cross peaks at 2.44, 2.37 and 1.82. The signal at 1.82 was due to the geminal proton of C-2 in the 2D-XHDEPT spectrum and was assigned to H-2α in ^1H NMR spectrum whereas the signals at 2.44 and 2.37 were assigned to both the H_2-1 protons. Thus, the ^1H and protonated ^{13}C NMR signals of the compound (10) were assigned. The signals for aliphatic quarternary carbons at 43.1 and 42.9 have not been assigned unambigously. These signals were due to C-10 and C-13 or vice versa.

The long range coupling interactions are known to be highly stereospecific in rigid systems. ^4J coupling follows empirical 'W' rule.[33-35] Larger long range ^4J coupling observed for a path having a zig-zag or W-like shape has been confirmed by double perturbation calculations.[36] These coupling interactions can be used in configurational and conformational analysis if their stereospecificity is clearly demonstrated. The Me-18 signal at 0.71 showed cross peaks(Fig.25) at 1.18, 1.32 and 2.36 for H-17α, H-12α and H-12β only. The Me-19 signal at 1.08 exhibited cross peaks at 2.68 for H-5, thus suggesting that the A/B ring junction is *trans*. Since theoretical and experimental studies[37] have shown that if one of the coupled protons is a part of a methyl group, appreciable values for ^4J H-C-5-C-10-Me are confined to the geometries where the dihedral angle between H-5, C-5, C-10 and

C-5, C-10, Me approaches 180°C. Irradiation of the Me-19 signal at 0.71 showed nOes with H-8 and H-11 whereas no nOe was observed for H-9 suggesting, thus that the B/C ring junction is *trans*. Similarly, irradiation of Me-18 showed no nOe for H-14 confirming thus that the C/D ring junction is *trans*. The couplings to H-11 of 11.0, 10.6 and 4.7Hz could be interpreted as $J_{11ax,\ 12ax}$, $J_{11ax,9ax,}$ $J_{11ax,12eq}$ respectively. Irradiation of H-11 gave nOes to Me-19, Me-18 and H-8, thus suggesting that the hydroxyl group at C-11 is ∝-equatorially oriented.



10

11 - ∝ - hydroxy cholestane - 3,6-dione

The new sterol on the basis of above studies was characterized as 11 ∝-hydroxy-5 ∝-cholestane-3,6dione,(10).

Cholest - 9 (11)-ene - 3, 6-diol

Further elution of the same column with 5% methanol in chloroform yielded one mg of a crystalline solid, m.p. 157°C. The molecular formula of (11), $C_{27}H_{46}O_2$, was determined by EIMS (Fig. 27), m/e 402 [M^+]. Its mass spectrum gave a base peak at m/e 384 and the molecular ion at m/e 402 besides major fragments at 369, 366, 355, 351, 331, 314, 301, 298, 287, 271, 261, 246, 229, 211, 149, 135, 121, 107, 95, 79, 69, 55 and 43. The presence of fragments at m/e 384 ($M^+ - H_20$) and 366 ($M^+ - 2H_2O$) indicated that the molecule contains two hydroxyl groups.

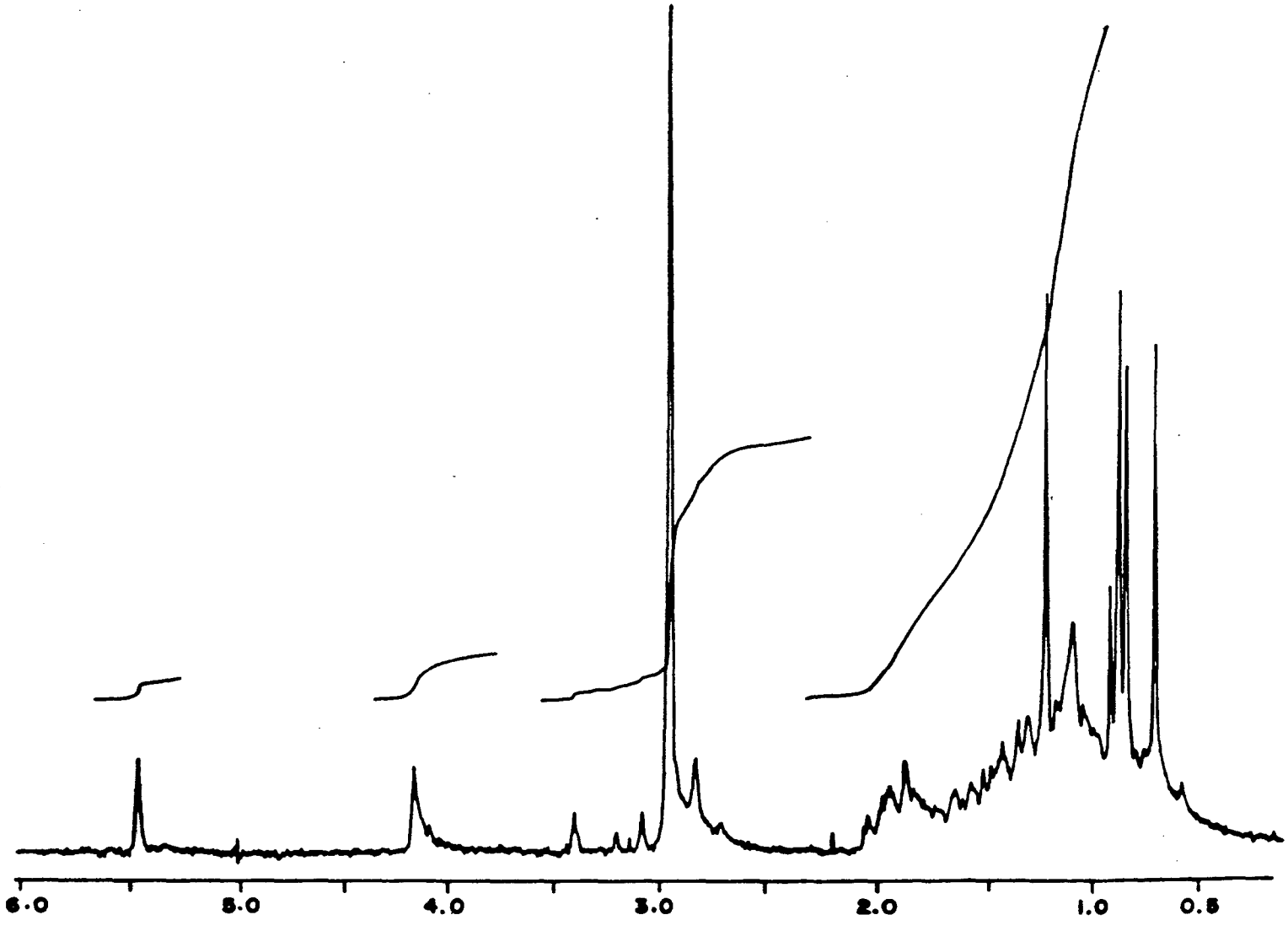


Fig.-26 ^{1}H NMR Spectrum of Cholest -9 (11) - ene - 3, 6 - diol

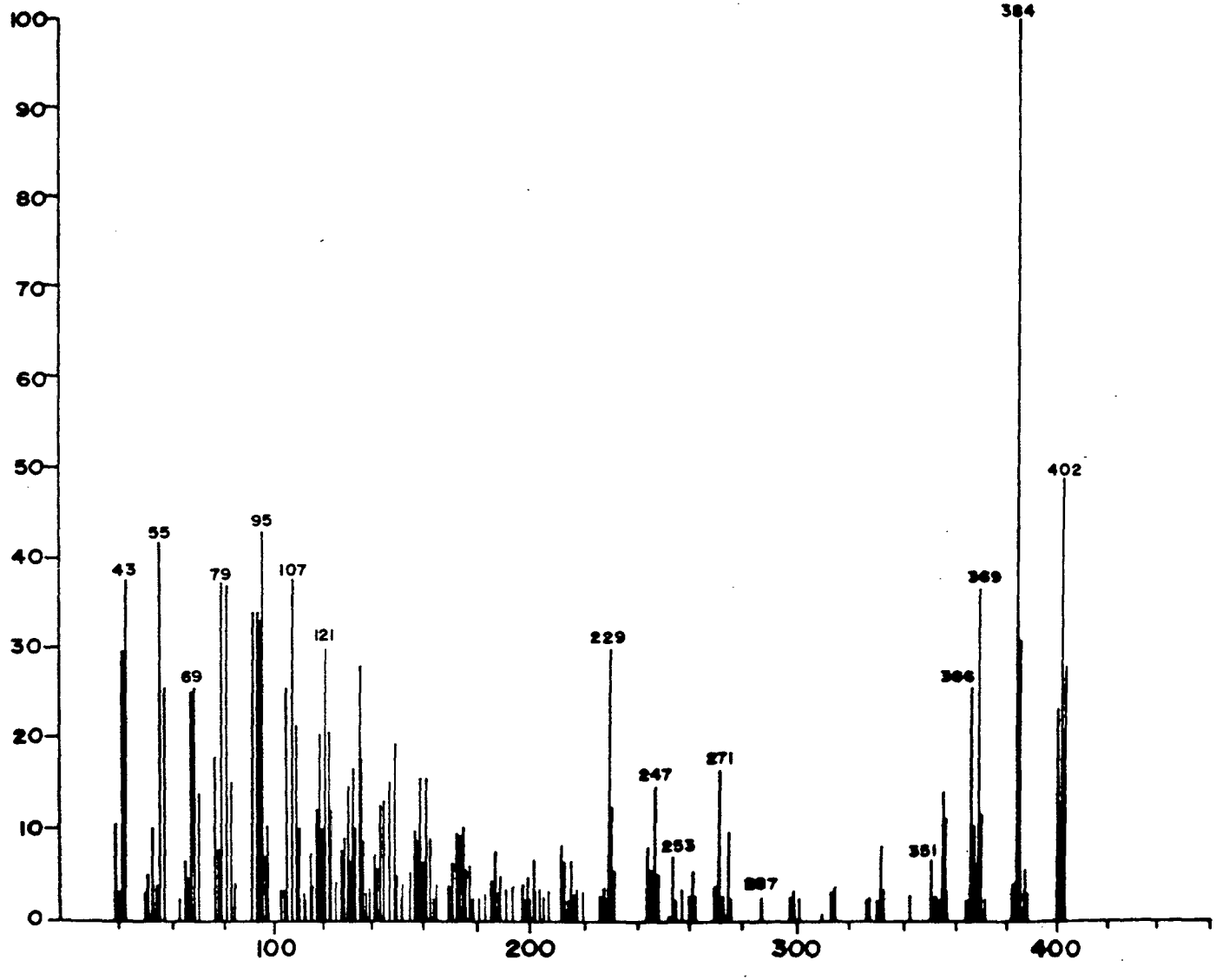
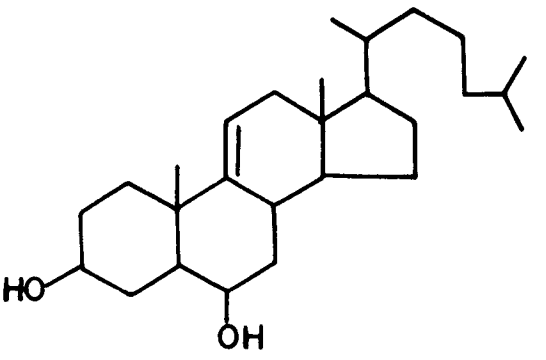


Fig.-27 Mass spectrum of cholest - 9 (11) - ene - 3 , 6 - diol

This was further reinforced in the ^1H NMR spectrum (200 MH_2, $CDcl_3$, Fig. 26) which displayed signal at 3.9 - 4.1 for two melthine protons, the presence of three secondary methyls at 0.85, 0.9 and 1.1 and two tertiary methyls at 0.7 and 1.2 alongwith the olefinic proton at 5.48. The spectral data given above revealed that the compound is a sterol belonging to the cholestane series with two hydroxyl groups and one double bond.

^1H NMR spectrum showed the presence of trisubstituted double bond at 5.4 (1H, S) aswell as terminal isoprohyl in the side chain at 0.85 (3H, d, J = 6.6Hz) and 0.91 (3H, d, J = 6.6Hz); singlets are also present at 0.73 and 1.23 due to C_{18} and C_{19} methyls respectively. Two proton multiplet between 3.9 and 4.2 were suggestive of the presence of secondary alcohol. The presence of OH group in ring A at C_3 is evident form the fragment in the mass spectrum at m/e 369 which involves elimination of water subsequent to the loss of an angular methyl group. The unusually high chemical shift of methine protons suggested the presence of perturbation in the vicinity of C_3. It was tempting to speculate that the additional secondary alcohol was located at positions 1,2 or 4. However the presence of a signal at 2.98 corresponding to 7H and assigned to protons $\overset{\alpha}{\wedge}$ to the carbinol carbon $\underline{HC}-\underset{OH}{\underset{|}{CH}}-\underline{CH}$ located the position of the double bond at $\Delta 9,11$ and second hydroxyl at C_6.



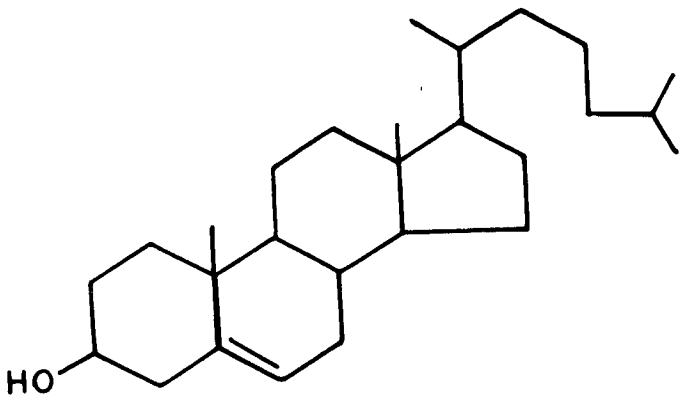
11

Cholest - 9 (11) - ene - 3 , 6- diol

Based on the above data, structure(11)was tentatively assigned to the steroid. On the basis of this structure, the genesis of some of the fragment in the mass spectrum could be explained as follows (Scheme 2); the peaks at m/e 384 ($M^+ - H_20$) and 366 ($M^+ - 2H_20$) are indicative of two — OH groups; m/e 287 loss of side chain indicates that the double bond is in the nucleus and not in the side chain. The presence of peaks at 261 and 246 arising from ring D cleavage indicates that the double bond and second -OH group are not present in the ring D.

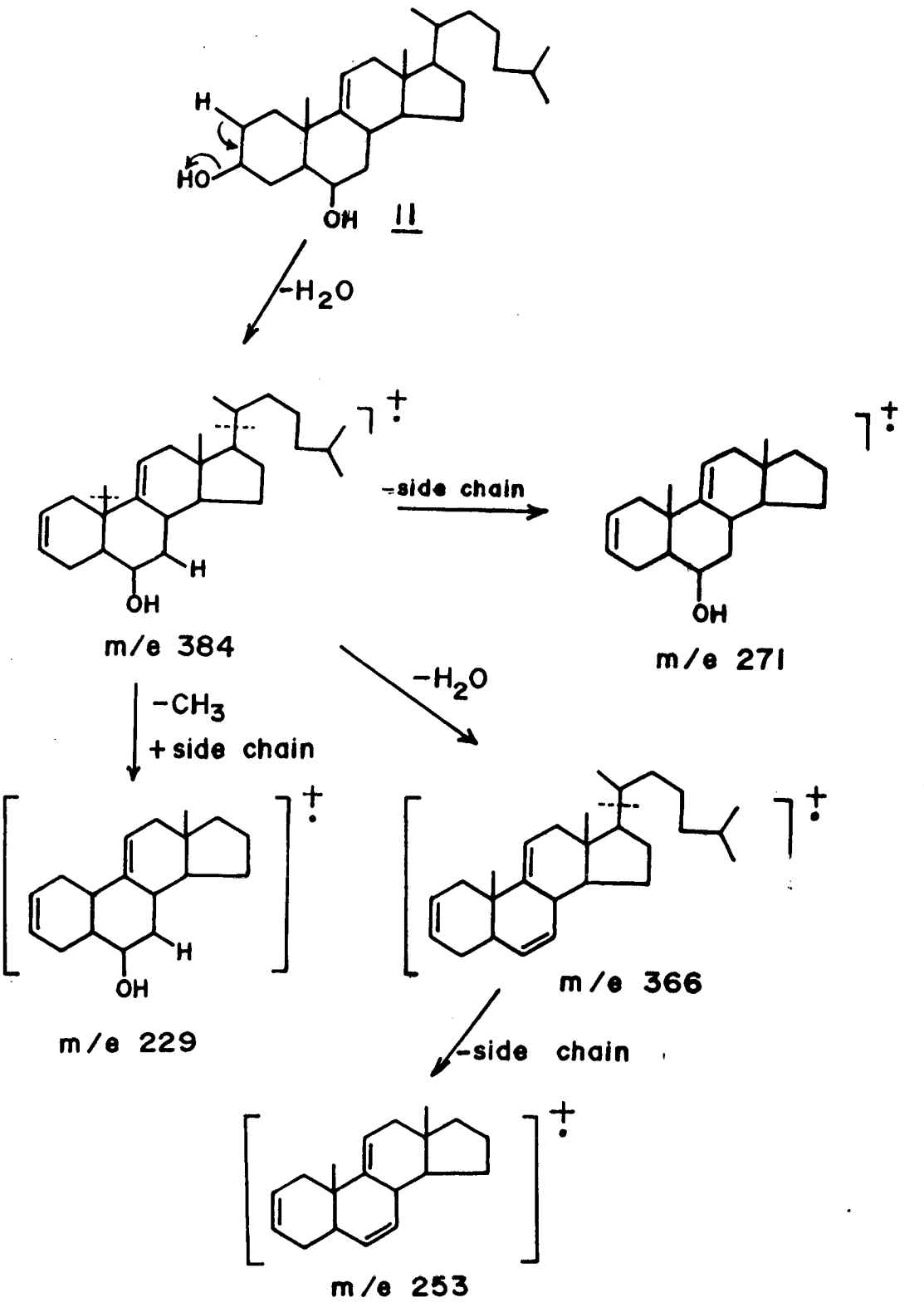
Besides the diketone, diol and dipeptides, known constituents like cholesterol, methyl palmitate and fatty acids, stearic and palmitic acids characterized as their methyl esters have also been isolated and identified.

Extensive silica gel chromatography of petroleum ether: ethyl acetate(90:10) eluate gave the steroid cholesterol(12) as crystalline solid, m.p.146°C and answered tests characteristic of sterols. Treatment with acetic anhydride/pyridine gave acetate with a m.p. of 113°C. IR spectrum showed bands at 3430, 2952, 2870, 1475, 1380 and 1070cm^{-1}. ^1H NMR spectrum showed signals at 0.7, 0.88, 1.0, 1.25, 2.32, 3.45, 5.35 and 7.76. Mass spectrum showed the molecular ion peak at m/e 386. Its identity was confirmed by determination of the mixed m.p. with an authentic sample and comparison of spectral data.



12

cholesterol


H
HO
OH
II
-H2O
-side chain
H
OH
m/e 384
m/e 271
-H2O
-CH3
+side chain
H
OH
m/e 229
m/e 366
-side chain
m/e 253

Scheme-2

The same eluate also yielded a liquid, IR spectrum of which showed bands at 1730, 1000, 1110, 1160, and 1180cm^{-1}. ^{1}H NMR(200MHz,$CDCl_3$) showed a triplet at 0.9($3H_1$-CH_3 group of aliphatic chain), multiplet centered at 1.3(24H,12--CH_2 group of the aliphatic chain), multiplet centered at 1.6(2H, ß-protons in the acid portion of the molecule), triplet centered at 2.3(2H, α -protons in the acid portion of the molecule) and a singlet at 3.68(3H—CH_3 group in the alcohol portion of the molecule). Mass spectrum showed molecular ion peak at m/e 270 corresponding to the molecular formula $C_{17}H_{34}O_2$, a base peak at m/e74, characteristic of straight chain methyl esters which arise by McLafferty rearrangements. Besides, there are also fragments of the general formula [$(CH_2)_nCOOCH_3^+$] indicated by m/e 87, 143, 199, 185, 213 and 227(M^+-43). It was characterized as methyl palmitate.

$H_3C-(CH_2)_{16}-COOH$

stearic acid

$H_3C-(CH_2)_{14}-COOCH_3$

Methyl palmitate

$H_3C-(CH_2)_{14}-COOH$

Palmitic acid

Elution of the column with glacial acetic acid yielded a mixture of fatty acids: stearic and palmitic acids which were separated by careful column chromatography and esterified with diazomethane. Stearic acid, m.p. 69°C, characterized as its methyl ester was obtained as a colourless solid to which was assigned the molecular formula, $C_{18}H_{36}O_2$, based on the mass spectra of acid (M^+-284) and its methyl ester(M^+-298). The mass spectrum showed base peak m/e74 formed by McLafferty rearrangement. The mass also showed the separation of most of the fragments by 14 mass units. IR spectra displayed characteristic peaks found for saturated long chain acids. The ^{1}H NMR spectrum (60MHz) of its ester provided confirmatory evidence regarding its paraffinic nature. The -CH_2- methylene protons appeared as a singlet

at 1.2(28H), $-CH_3$ group of aliphatic chain appeared at 0.84, multiplet centered at 2.2(4H) was assigned to the α and ß-protons in the acid portion of the molecule and singlet at 3.4 corresponding to the $-CH_3$ groups in the alcohol portion of the molecule.

Palmitic acid was obtained as a solid, m.p. 63°C. Its mass spectrum gave the molecular formula, $C_{16}H_{32}O_2$, (M^+-256). IR showed bands at 3420, 2875, 2858, 2835, 1700, 1325, 1295, 1270, 1248, 1230, 1210 and 1185cm^{-1}. ^1H NMR spectra of its methyl ester displayed signals at the same position as in the spectra of methyl stearate, the only exception being that the singlet at 1.2 corresponded to (24H) as compared to the (28H) in the ^1H NMR spectra of methyl stearate.

EXPERIMENTAL

Antifertility testing:

Testing procedure has been given in Section II, Chapter I.

The crude methanolic extract of *Acantophora spicifera* prevented implantation in 100% of the animals at a dose of 200mg/Kg body weight and the petroleum ether and chloroform fractions showed 60% anti-implantation activity at a dose of 50mg/Kg of body whereas the remaining n-butanol and water soluble fractions were found inactive at the same dose. Based on the screening results, petroleum ether and chloroform soluble fractions were taken up for the isolation and identification of active principle.

Collection, Extraction and Isolation :

Acantophora spicifera (7Kg) was collected at low tides during postmonsoon periods(Nov-Jan), air dried and extracted three times with choloroform. The combined extract was evaporated under reduced pressure to give a residue (130g, 1.9% dry wt.) which was chromatographed over silica gel and eluted with mixtures of increasing polarity of petroleum ether : ethyl acetate, 100ml fractions were collected. Fractions eluted with petroleum ether : ethyl acetate (80:20) yielded three crystalline compounds.

The least polar, giving a yellow spot turning into orange spot with 2:4 DNP on TLC (solvent system:- petroleum:ether:glacial acetic acid, 70:30:6) was characterized as 11α-hydroxy-5 α -cholestane3,6-dione (10), next in polarity in the same TLC solvent system but visualized by exposure to iodine vapours were the dipeptides, acetates of aurantiamide(1) and dia-aurantiamide (2), the later being the most polar.

Elution of the same column with MeOH:CHcl3 (5:95) yielded yet another crystalline solid, cholest-9,11-ene 3,6 diol, m.p. 157°C.

The less polar eluate (petroleum ether : ethyl acetate, 90:10) of the active chloroform soluble fraction yielded sterol, cholesterol and ester, methyl palmitate. Elution of the same column with glacial acetic acid yielded fatty acids which were characterized as methyl esters. Some of the compounds isolated from these fractions are being tested for the activity observed.

Aurantiamide acetate

UV (Fig. 1)

λ_{max}^{EtOH} 202nm with sh at 225nm.

IR spectrum (KBr, Fig.2)

It showed bands at 3330, 3090, 3050, 1730, 1660, 1635, 1535, 1450, 1370, 1255, 1040, 735 and 690 cm^{-1}.

1H NMR spectrum (CDCl3, 200MHz, (Fig. 3)

6.85(1H,d,J=7.5Hz); 6.1(1H,d,J=7.5Hz); 7.12-7.78(15H,m); 2.03(3H,s); 3.07(1H,dd,J=14.5,8Hz); 3.22(1H,dd,J=15,6Hz); 2.74(2H,d,J=7Hz); 3.84(1H,dd,J=11,5Hz); 3.95(1H,dd,J=11,5Hz); 4.2-4.47(1H,m) and 4.78(1H,m).

13C NMR spectrum (Fig. 4)

170.75; 170.31; 167.13; 136.70; 136.60; 133.66; 131.89; 129.28; 129.10; 128.6; 128.55; 128.22; 127.11; 127.05; 126.72; 64.56; 54.97; 49.45; 38.41; 37.42; 20.76.

Mass spectrum (70eV Fig.6),m/e (rel.int.)

43(9,$COCH_3$); 77(22,C_6H_5); 91(12,C_7H_7); 105(100,$C_6H_5CO^+$); 120(4,$C_6H_5CH{=}NH_2$); 131(6,$C_6H_5CH{=}CHCO^+$); 176(3,$C_6H_5CH_2{=}CHOAc$); 224(17,$C_6H_5CO\text{-}NH\text{-}CHCH_2Ph$); 252[23,$C_6H_5CONHCH(CO^+)CH_2C_6H_5$]; 293[6,$M^+$-($CH_3COOH$+$C_6H_5CH_2$)]; 353[10,($M^+$-$C_6H_5CH_2$)]; 384(18-$M^+$-$CH_3COOH$); 444(10,$M^+$). The fragments m/e 134(4,$C_6H_5CH_2CH{=}CHOH$) and 311(10, M^+-$C_6H_5CH_2$) are indicative of the presence of the corresponding amide (3).

Dia-aurantiamide acetate:

1H NMR spectrum (CDCl3,200MHz, Fig.10)

6.9(1H,d,J=7.5Hz); 6.31(1H,d,J=8Hz); 7.1-7.72(15H,m); 20(3H,s) 2.99(1H,dd,J=13.6,9Hz); 3.12(1H,dd,J=14.1,6.5Hz); 2.79(1H,dd,J=14,8); 2.85(1H,dd,J=13.8,5.6); 3.90(1H,dd,J=11,6.5); 3.96(1H,dd,J=11,5.5); 4.28-4.31(1H,m); 4.81(1H,m).

Mass spectrum (70 eV, Fig. 9); m/e(rel.int.)

43(24); 77(51); 91(30); 105(100); 131(15); 134(10); 177(8); 176(7); 224(56); 252(77); 269(13); 293(5); 311(8); 323(4); 353(6); 384(15); 444(54).

Synthesis of RS-isomer of aurantiamide acetate:

[N-(N'-benzoyl-R-phenyl alaninyl)-S-phenylalaninol acetate](9c)

1. Benzoylation of R-phenylalanine

Benzoyl chloride (8ml) was added in batches to a solution of R-phenylalanine (4.5gms) in 10% NaOH (50ml) and shaken vigorously until all the chloride has reacted. The solution was transferred to a beaker containing crushed ice and acidified with conc HCl. The mixture of benzoylphenylalanine and benzoic acid was separated by boiling with CCl_4 for 10 minutes. Filtered residue consisting mainly of R-N-benzoylphenylalanine was recrystallised from a mixture of acetic acid : benzene (1:1), m.p. 185°C, yield 4.7 gms.

IR spectrum (Fig.11, KBr)

It showed bands at 3400, 1715, 1600, 1560, 1520, 1480, 1430, 1345, 1330, 1260, 1220, 1185, 750, 720, 690, and 470 cm^{-1}.

1H NMR spectrum (CDcl3 + DMSO), 90MHz, Fig. 12)

3.1(2H,m); 4.65(1H,d,m); 7.1-7.75(10H,m); 8.3(3H, broad).

Mass spectrum 70eV, m/e (rel.int.), Fig.13)

55, 77, 91, 105(100%), 122(8.1), 148(89.4), 224(9.8), 251(3.9) and 269(8.3).

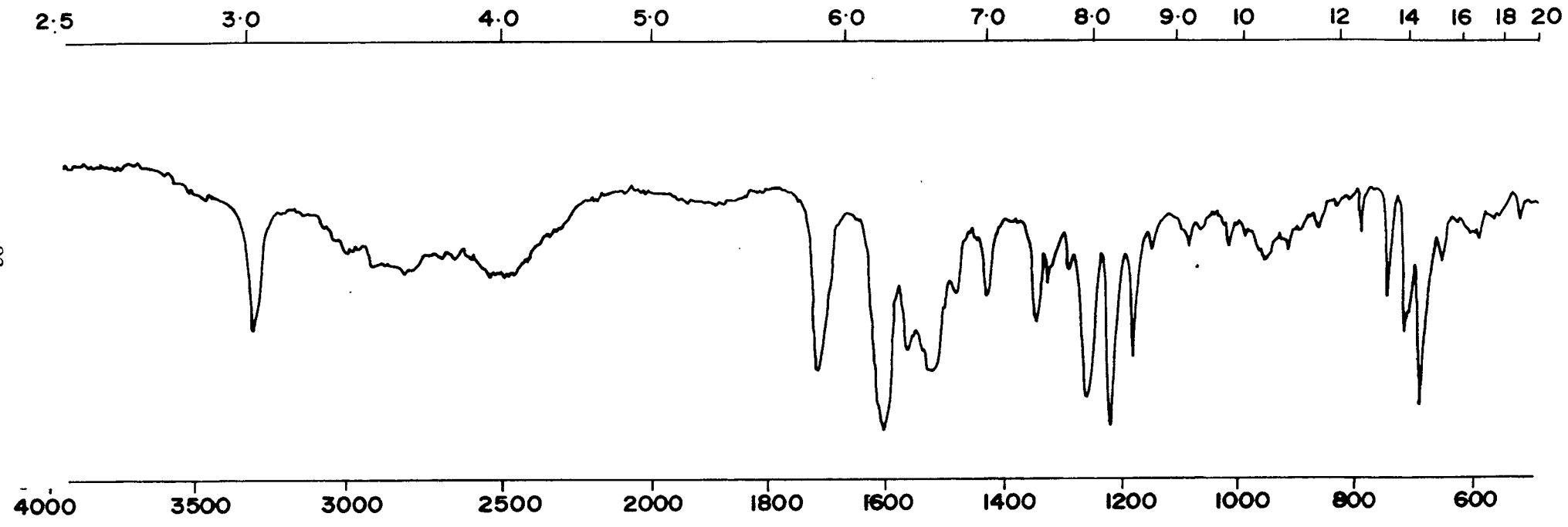


Fig. 11 R – N – Benzoyl phenyl alanine

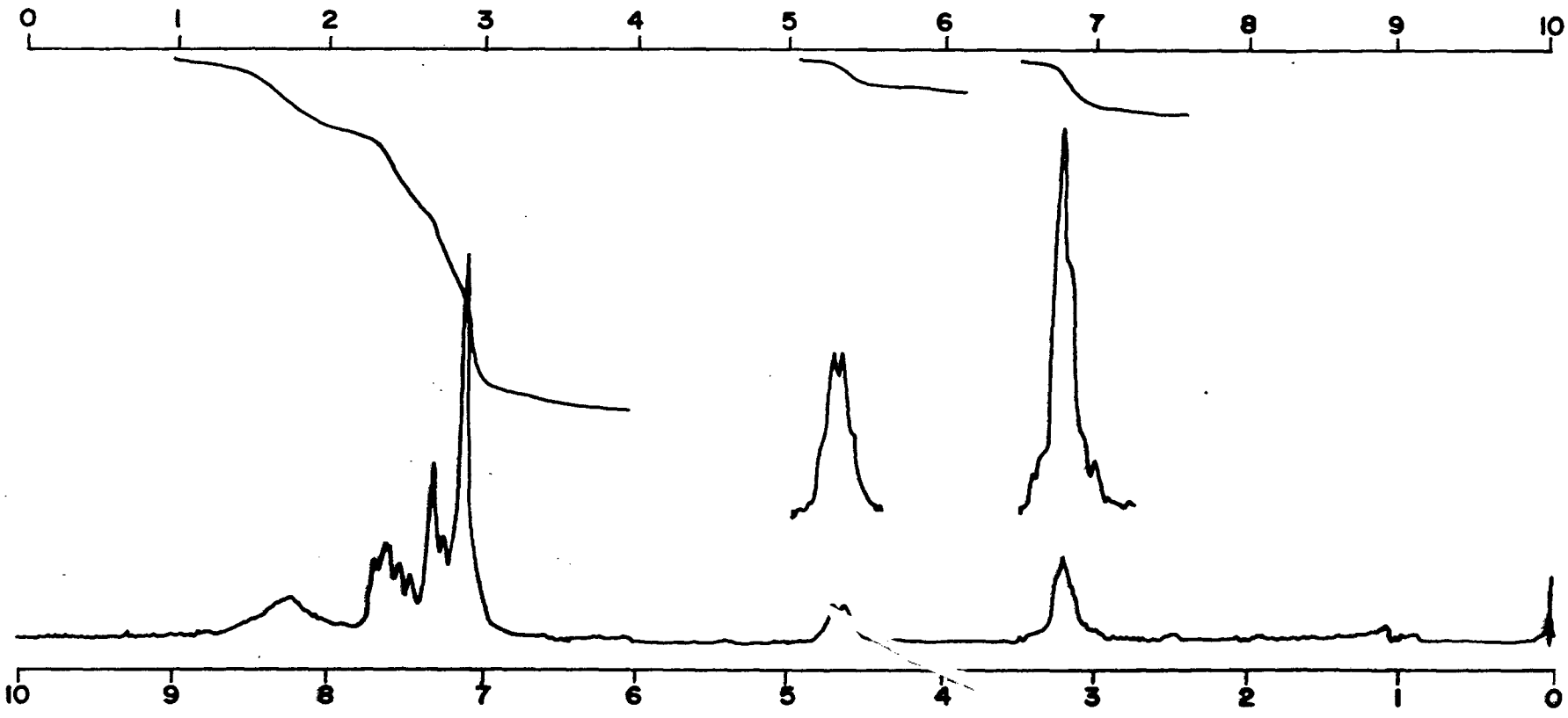


Fig.12. R - N - Benzoyl phenyl alanine

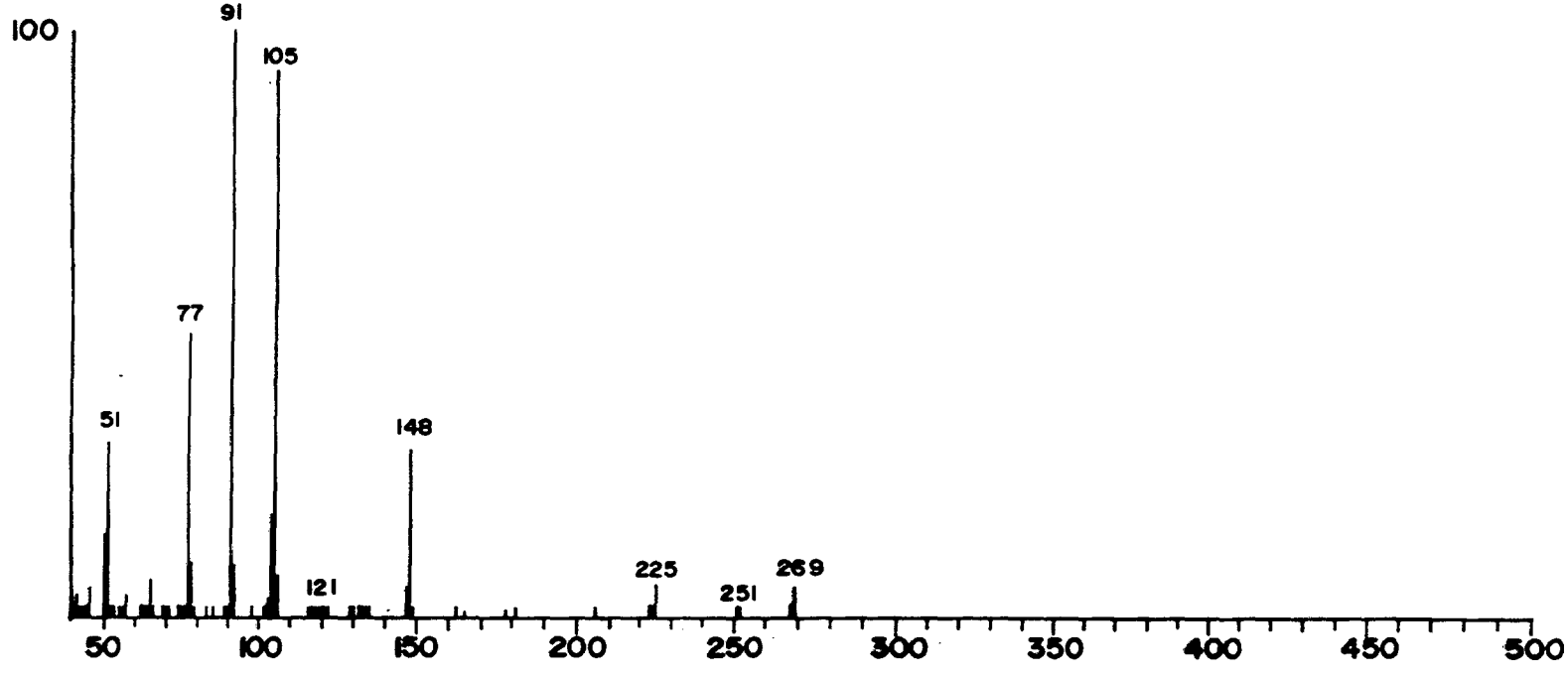


Fig. 12 Mass spectrum of
R - N - Benzoyl phenyl alanine

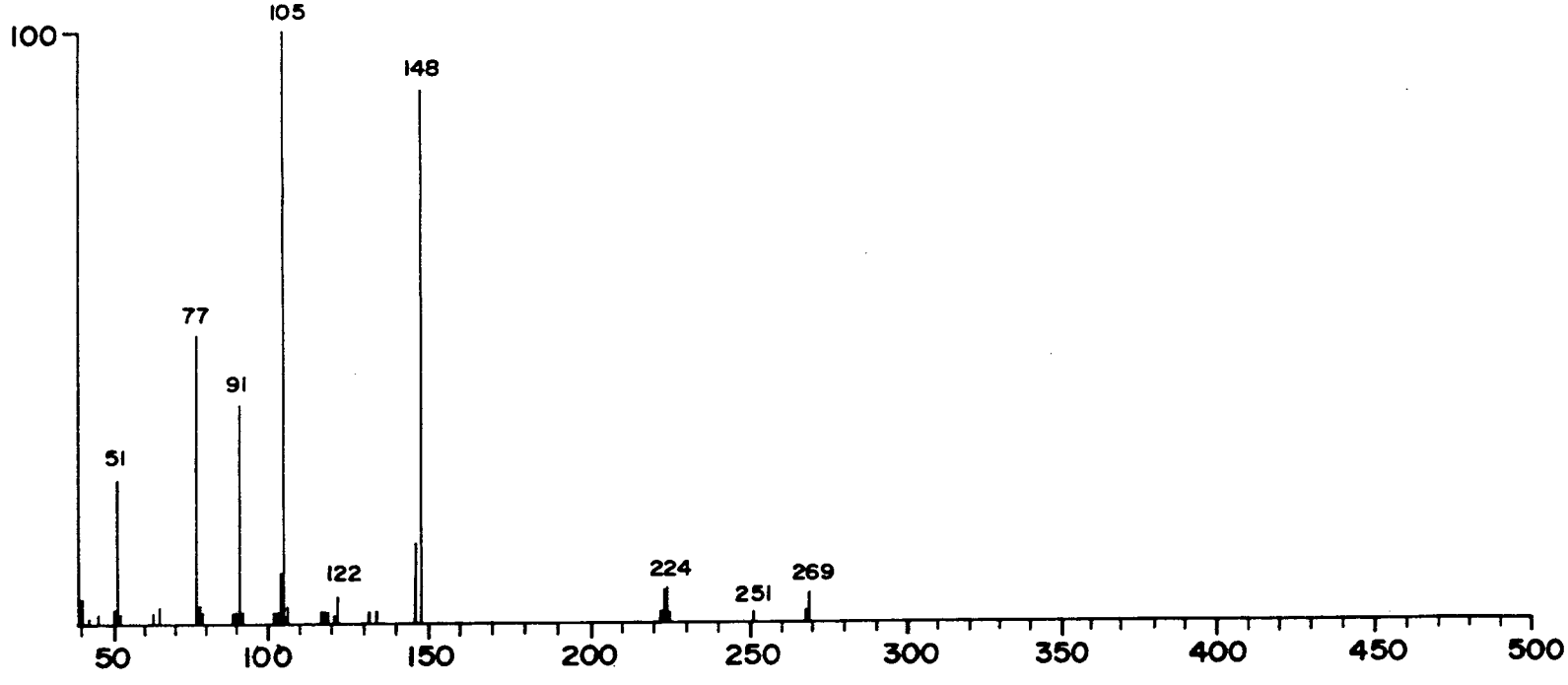


Fig. 13 Mass spectrum of
R - N - Benzoyl phenyl alanine

2. Condensation of R-N-benzoylphenylalanine with S-phenylalaninol:

R-N-benzoylphenylalanine (3gms) and S-phenylalaninol (2gms) were dissolved in dry THF (70ml), cooled to 0°C using ice and salt mixture and DCC (3.6gms) in THF (20ml) added dropwise to the stirred solution. Stirring was continued for 4 hours at 0°C and for 2 hours at room temperature. A few drops of acetic acid were then added, stirred for 30 minutes and filtered. The filtrate was concentrated and THF was replaced by ethyl acetate and a further amount of di-cyclohexylurea which precipitated was filtered. The solution was successively washed with dil HCl, H_2O, dil $NaHCO_3$ solution and H_2O. During the condensation reaction there is a chance of racemization which was minimized by using DCC[19] and carrying out reaction under mild conditions for a short time. Nevertheless, the aurantiamide formed was contaminated with a small amount of its diastereoisomer. The two were separated by column chromatography over silica gel using increasing concentration of methanol in chloroform as the eluant. Fractions having the same TLC profile were mixed and concentrated to yield N-(N^1-benzoyl-R-phenylalaninyl)-S-phenylalaninol crystals (800mg), m.p. 184°C. TLC solvent system — petroleum ether : ether : acetic acid (70 : 30 : 8), detecting agent used were iodine vapours.

IR spectrum (KBr, Fig. 14)

It showed bands at 3330 (NH,OH); 3020; 2930; 1645 and 1620 (-CONH); 1570; 1595; 690; 735(monosubstituted benzene).

1H NMR spectrum (DMSO, 90MHz, Fig.15)

8.28(1H,d,J=7.9Hz); 8.14(1H,d,J=10Hz); 7.7(2H,dd,J=2,6Hz); 7.3-7.5(3H,m); 7.15(10,m); 4.7(2H,m); 3.32(2H,d,J=6Hz); 2.6-3.1(4H,m).

Mass spectrum 70eV, m/e (rel.int.), Fig. 16)

402(1.6,M^+); 372(2); 311(4.8); 269(9.2); 252(50.4); 224(37.9); 190(6.1); 131; 120; 105(100); 91; 77 and 60.

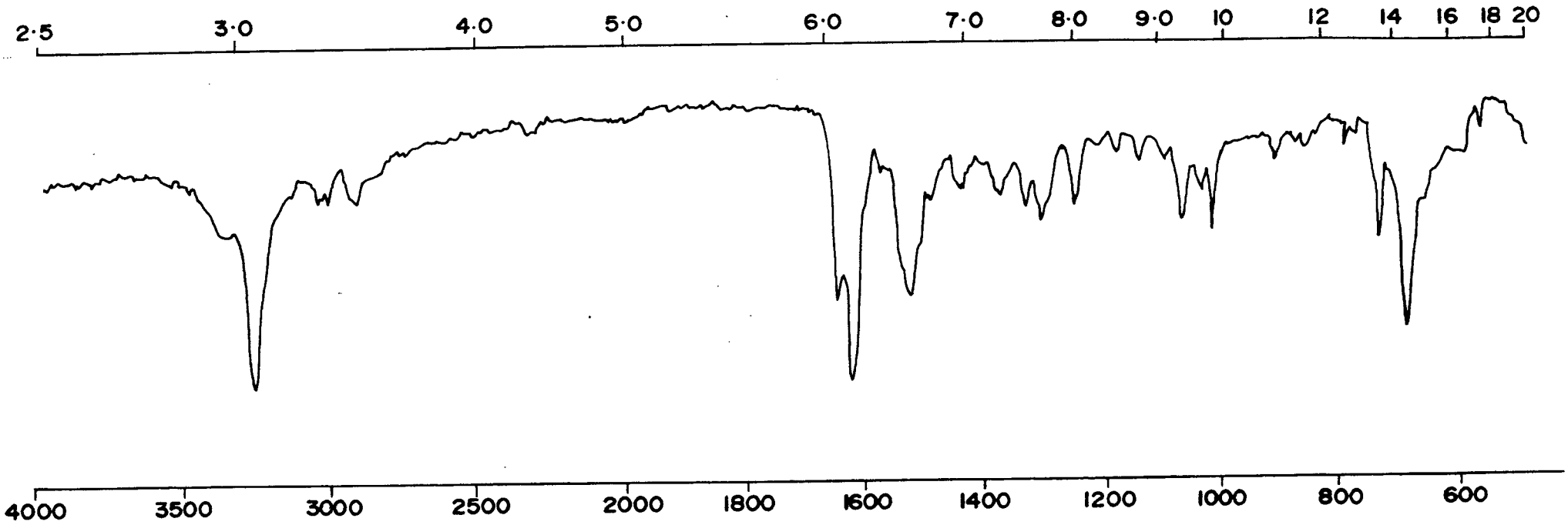


Fig.-14 N - (N'- Benzoyl R-phenyl alaninyl) S-phenyl alaninol

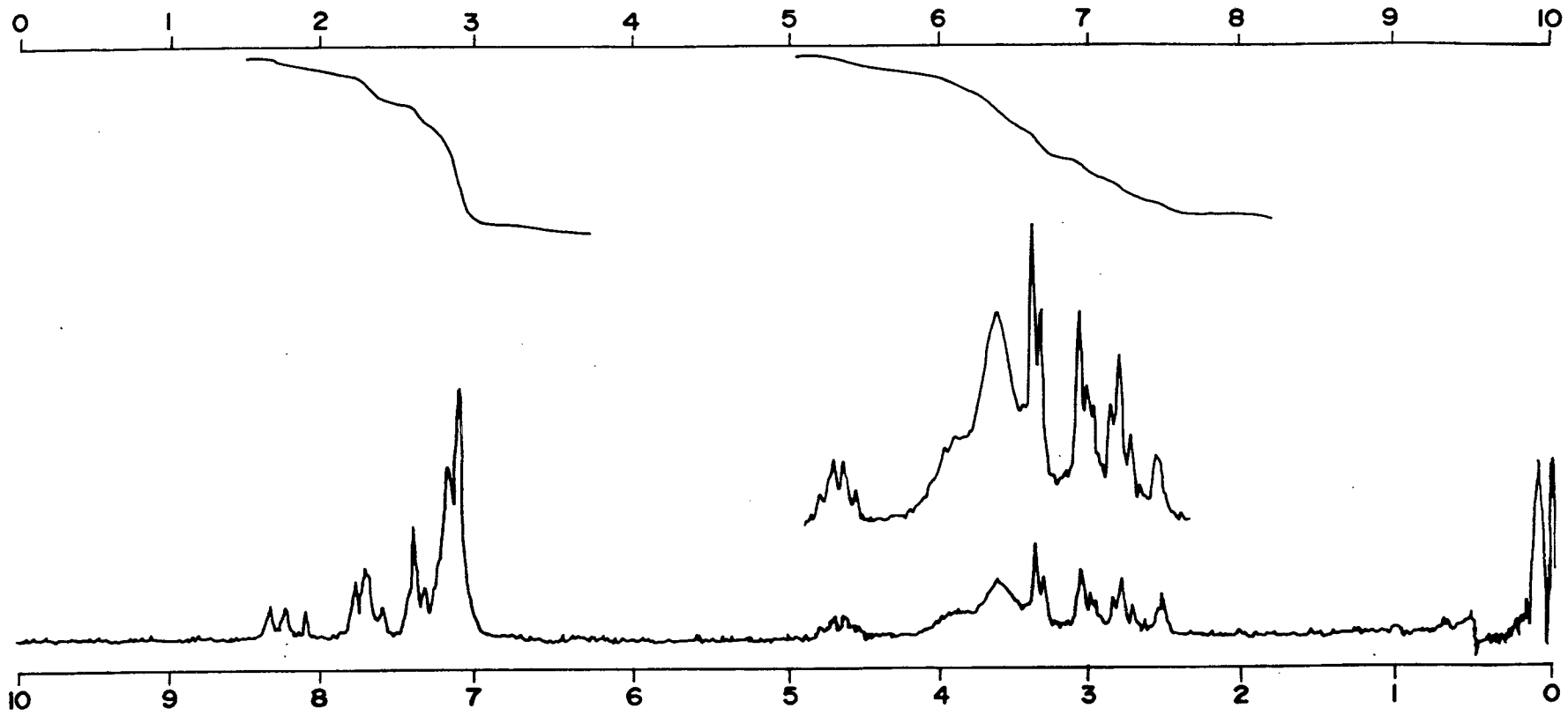


Fig.-15 N - (N' - Benzoyl R - phenyl alaninyl) S - phenyl alaninol

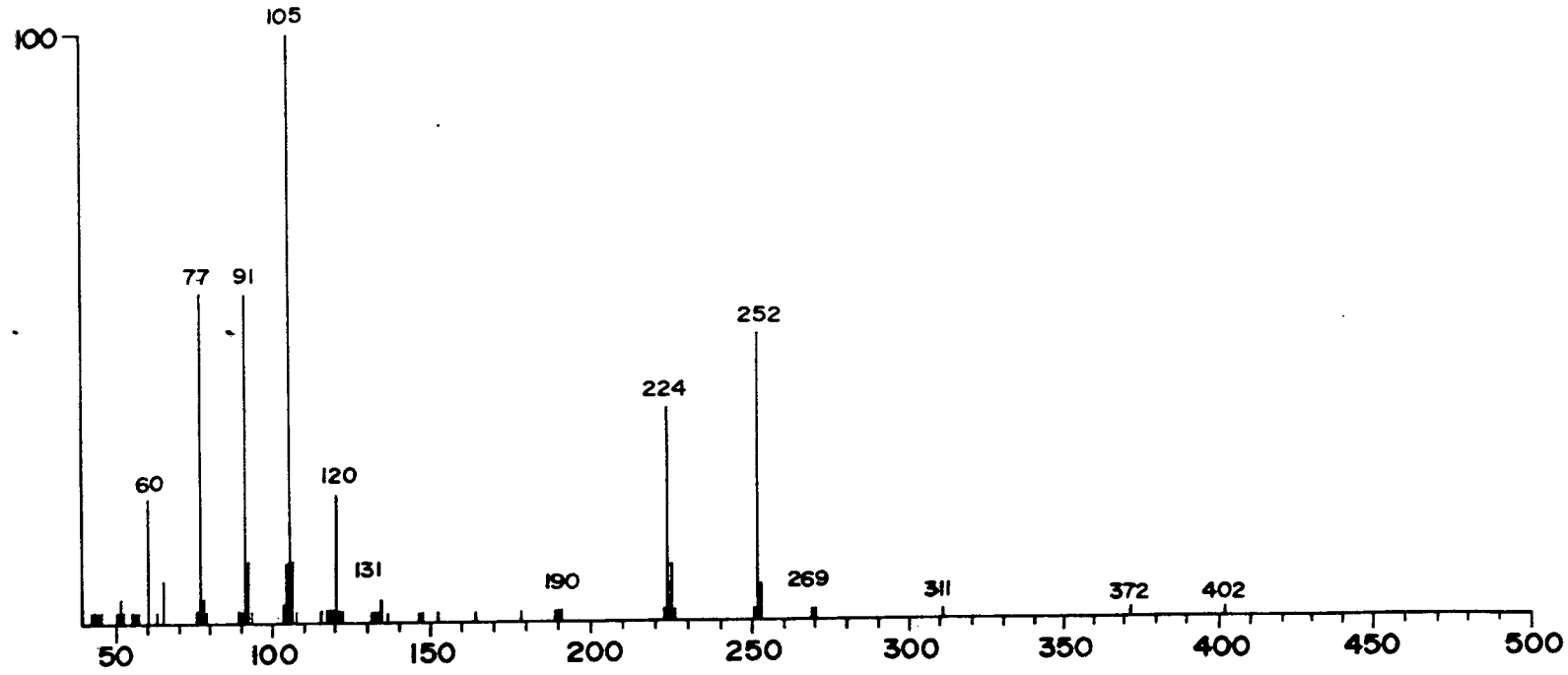


Fig.-16. N-(N'-Benzoyl R-phenyl alaninyl) S-phenyl alaninol

3. Acetylation of aurantiamide

A mixture of condensation product (100mgs) and acetic anhydride in dry pyridine was kept overnight at room temperature. The reaction mixture taken in choloroform was washed first with dil HCl and then with water. Removal of solvent gave aurantiamide acetate, yield 83mgs, m.p. 179°C.

IR spectrum (KBr, Fig.17)

It displayed bands at 3310, 3030, 3015, 1730, 1660, 1635, 1580, 1525, 1490, 1370, 1320, 1260, 1050, 730 and 690 cm^{-1}.

1H NMR spectrum (CDcl3, 400MHz, Fig. 18)

1.9(3H,s); 2.62(1H,dd,J=8,13Hz); 2.7(1H,dd,J=7,13Hz); 3.02(1H,dd,J=8,14Hz); 3.15(1H,dd,J=6,14Hz); 3.76(1H,dd,J=6,10Hz); 3.85(1H,dd,J=5.6,12Hz); 4.3(1H,m); 4.75(1H,dd,J=6,12Hz); 6.17(1H,d,J=9Hz); 6.82(1H,d,J=7.2Hz); 6.95-7.12(5H,m); 7.13-7.24(5H,m); 7.35(2H,t,J=8Hz); 7.45(1H,m); 7.65(2H,d,J=8Hz).

Mass spectrum (70 eV, Fig. 19) m/e (rel.int.)

444(4.6); 353(1.3); 311(11.1); 293(1.3); 269(15.3); 252(80.6); 224(70.1); 176(9.3); 134; 131; 105(100); 77 and 44.

Remaining three isomers N(N'-benzoyl-S-phenylalaninyl)-R-phenyl alaninol acetate, m.p. 179°C,(9d); N(N'-benzoyl-S-phenyl alaninyl)-S-phenyl alaninol acetate, m.p. 169°C, (9a) and N(N'-benzoyl-R-phenyl alaninyl)-R-phenyl alaninol acetate, m.p. 170°C, (9b) were synthesized by following the same procedure as described above. IR and ^{1}H NMR spectra of (9b) were identical to (9a) and the IR and ^{1}H NMR spectra of (9c) and (9d) were also identical to each other.

11 — α -hydroxy cholestane-3,6-dione (10)

IR spectrum (KBr, Fig. 20)

It displayed bands at 3460, 2930, 2850, 1700, 1680, 1450, 1405, 1375, 1250, 1100, 1050, 1020 and 950cm^{-1} .

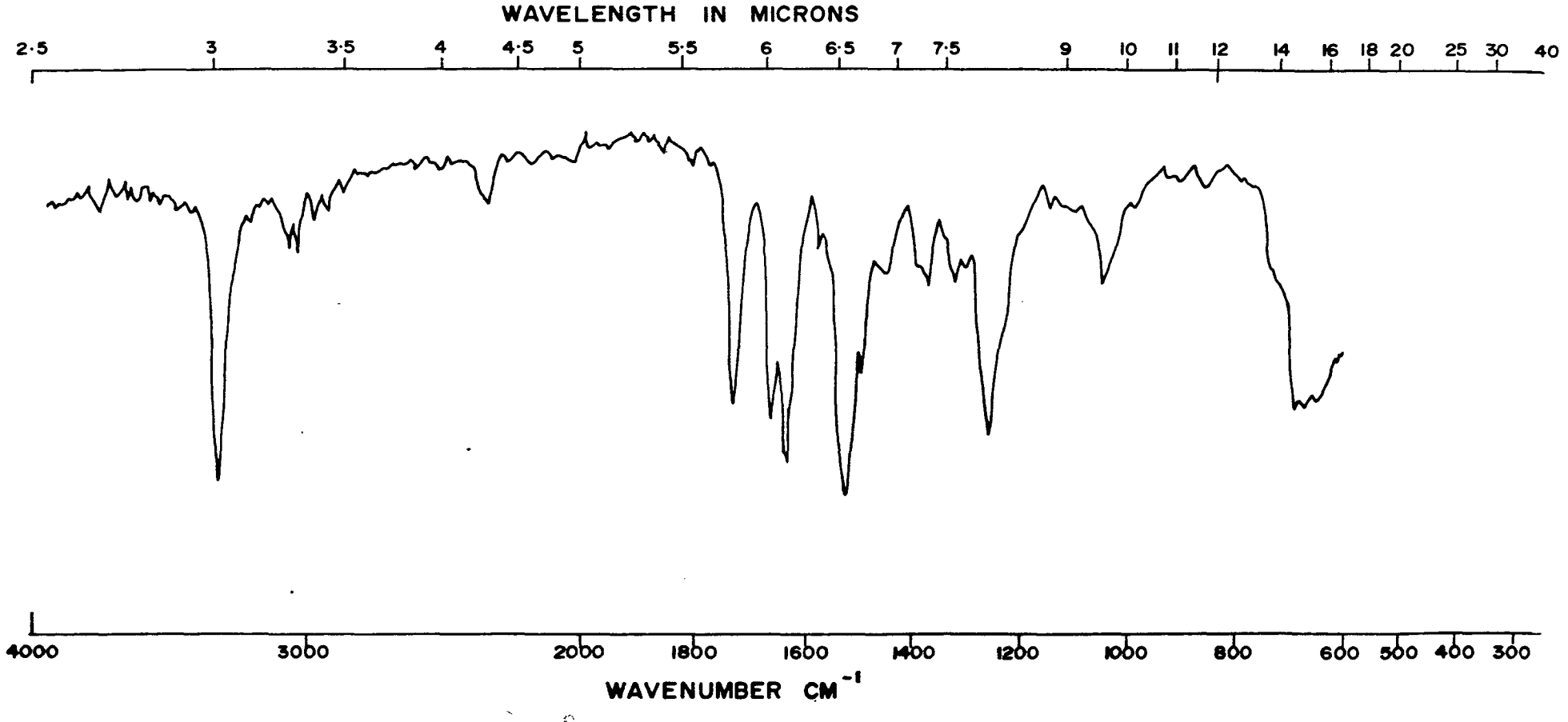


Fig. 17. N - (N' - Benzoyl R - phenyl alaninyl) S - phenyl alaninol acetate

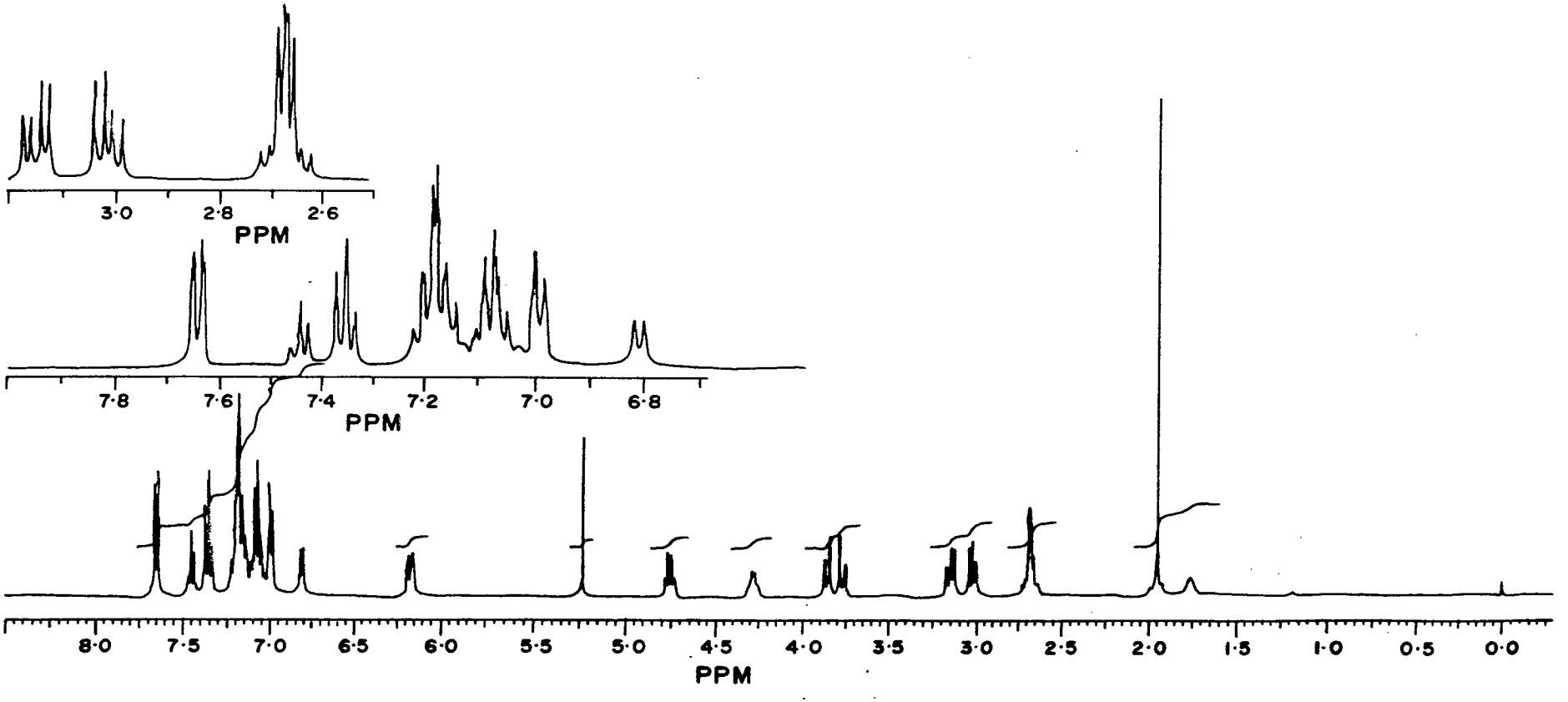


Fig.-18 N - (N'- Benzoyl R - phenyl alaninyl) R- phenyl alaninol acetate

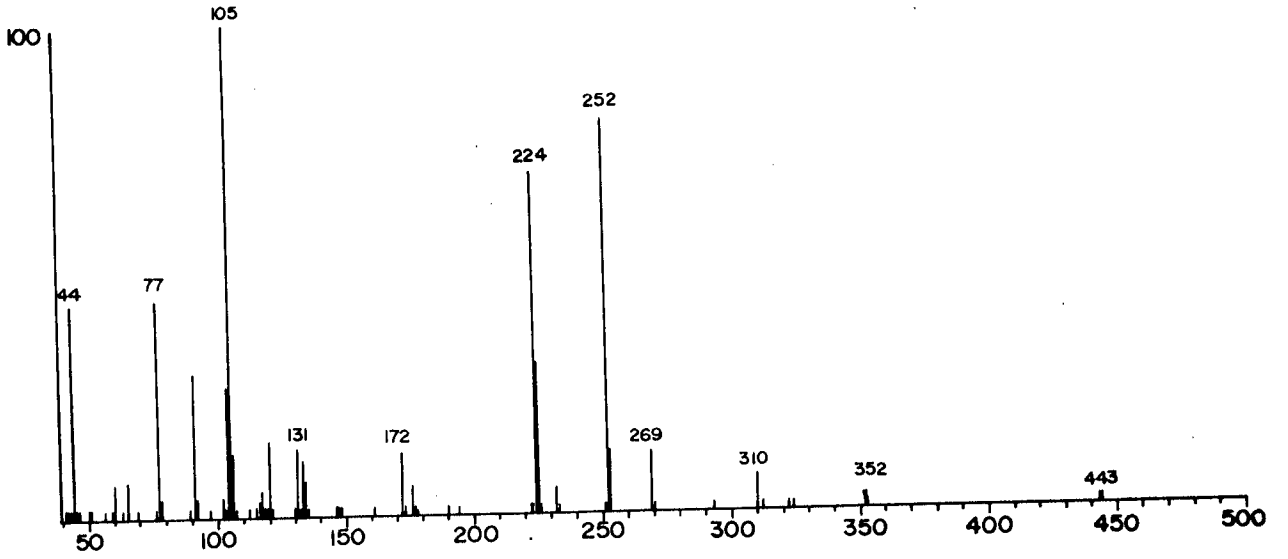


Fig.19 N-(N'-Benzoyl R-phenyl alaninyl) S-phenyl alaninol acetate

1H NMR spectrum (90MHz, Fig. 21, CDCl3)

0.71(3H,s); 1.08(3H,s); 0.94(3H,d,J=6Hz); 0.86(6H,d,J=6Hz); 4.05(1H, septet); 2.0-2.85(7H,m).

Mass spectrum (EI, 70 eV, Fig.22)

m/e 416, 398, 387, 383, 370, 355, 301, 300, 285, 259, 243, 105, 81, 69 and 55.

1H NMR spectrum (200 MHz, CDcl3, Fig. 26)

5.4 (IH,S); 0.85 (3H, d, J = 6.6 Hz); 0.91 (3H, d, J = 6.6 Hz); 0.73 (3H, S); 1.23 (3H, S); 3.9 - 4.2 (2H, m); 2.98 (7H, m); 5.48 (IH).

Mass spectrum (70eV, Fig. 27)

m/e 402, 384, 369, 366, 355, 351, 331, 314, 301, 298, 287, 271, 261, 246, 229, 211, 149, 135, 121, 107, 95, 79, 69, 55 and 43.

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CHAPTER III

SECTION I

BENZOXAZINONE AND BENZOXAZOLINONE FROM *ACANTHUS ILLICIFOLIUS*

Mangrove forest is an important component of subtropical and tropical estuarine eosystem and forms a natural transition between the land and the sea. In India, these ecosystems, occur both along the east and the west coasts. They have a high rate of primary productivity and provide substantial input of detrital material into the surrounding ecosystem.

The importance of mangroves is increasingly realized all over the world. It is more so because they support a large number and variety of marine animals and plants in near shore waters. The utility of this ecosystem is very wide. In India, many local inhabitants depend upon these mangroves for several uses, one such use being the medicinal use of mangrove plants. Some of the common mangrove plants are *Rhizophora mangle, R. mucronata, Avicennia marina (A. nitada), Sonneratia caseolaris, Nypa fruticans* and *Acanthus illicifolius.*

Acanthus illicifolius, a plant belonging to the family Acanthaceae and known as Kandar locally occurs extensively fringing the tidal creeks of the Mandovi-Zuari estuarine system in Goa. This evergreen spiny herb is also known to occur along the sea coasts of India, Ceylon, Malaysia, Phillipines, Australia and South Africa. In the traditional folk medicines of India, this plant enjoys a reputation of being effective in the treatment of paralysis and asthma. Its leaves are recorded to be used for fomentation in rheumatism and neuralgia. The leaves and tender roots are also useful in the treatment of snake bite.[1]

Due to its medicinal properties different parts of the plant have been subjected to chemical investigations by different workers. Thus Ghosh *et al*[2] have reported isolation of two sterols and five triterpenoids, there being no record of any work on bezoxazinones. Ramchandra and Pouchname[3] have isolated flavone glycosides — Methylapigenin 7-0-ß-D-Glucoronate and apigenin-7-0-ß-D-glucopyranuroside and its methyl esters from the leaves of *Acanthus illicifolius.* Minocha and Tiwari report a number of known chemical constituents[4] besides a new

alkaloid[5], Acanthicifolin, from the root of this spiny herb. Udom Kokpol *et al*[6] have also worked on the roots of this plant. Murty *et al*[7] have independently reported the isolation of 2-benzoxazinones. Intrigued by these medicinal properties in addition to its traditional use of leaves for relief from rheumatic pains by villagers in Goa, it was decided to take up a detailed study of the pharmacological aspect of this plant. During screening it was observed that its crude methanolic extract exhibited marked analgesic effect when tested with methods which are conventionally employed for narcotic as well as non-narcotic analgesics.

ED_{50} values have indicated that the analgesic potency of the extract is of high order as compared to the potency of standards, phetidine and sodium salicylate. Experiments had also indicated absence of addiction liability. The anti-inflammatory activity against the inflammation induced by carrageenan was found not to be dose dependant as there was not much change in the activity observed when the dose increase was ten fold, which means that maximum effect is manifested at low dose level. This is in contrast to phenyl butazone where the effect is dose dependant.

The follow up studies resulted in the location of the analgesic activity in chloroform soluble and anti-inflammatory in both chloroform and water soluble fractions. The isolation and identification of the 1,4 benzoxazime derivative-2-0-ß-Glucopyranosy l-1,4-(2H)-benzoxazine-3-one (HBOA-Glc) and dimer of benzoxazoline-2-one, 5,5' bis-benzoxazoline 2,2'dione from the pods of *A. illicifolius* has been reported here.

Cyclic hydroxamic acids e.g.(1)are present in the cereal grasses, maize, wheat and rye and have been reported to be important in resistance of these plants to fungi and insects.[8] They are found in these plants mainly as glucosides, which are enzymatically transformed into the aglycones by the action of ß-glucosidase upon crushing the plant tissue.[9]

Compound(2)was isolated from the active water soluble fraction of *A. illicifolius*. Chromatography over silica gel of this active fraction and elution with methanol : chloroform (20:80) afforded a solid, m.p. 221°C. It showed UV absorption at λ max. 255 with sh at 286nm. Its IR (Fig.1) showed an intense absorption at 860 cm^{-1} indicative of ß-linkage between the aglycone and sugar moiety. The presence of three absorptions at 1000, 1025 and 1060 cm^{-1} suggested pyranose structure of glucose, amide carbonyl was evident from the absorption at 1690. Its ^1H NMR (Fig. 2) showed a multiplet in the region 3.1-3.9 for 6H of glucose, a doublet at 4.8 with a coupling constant of 7.7Hz was assigned to the anomeric proton. This coupling constant requires that $C_{1'}$ and $C_{2'}$ protons of the hexose to be trans diaxial suggesting a ß-glucopyranoside. ^1H NMR also showed singlet at 5.9 assigned to the CH methine proton at C_2 and a broad multiplet at 7.1 integrated for 3H alongwith a broad singlet for 1H at 7.4 were attributed to the aromatic protons. A sharp singlet at 4.6 is due to HDO.*

Its ^{13}C NMR spectrum (fig. 3) showed signals which were assigned (Table 1) to different carbons in(2)on the basis of co-relation with the reported value for related compound. The additional signal observed at 171.7 has been assigned to C-3 of the tautomeric form of (2).

Table 1

^{13}C NMR data of compounds(2)and(3)in $CDcl_3$

C	(2)	(3)
2	164.75	115.62
3	173.99	170.58
5	119.18	117.84
6	127.61	124.3169

* D_2O was used as solvent

7	126.54	124.9614
8	104.59	100.6270
9	120.42	123.4370
10	142.50	140.1447
1'	97.95	96.0649
2'	75.54	71.0678
3'	79.01	72.5151
4'	71.88	68.1104
5'	78.18	72.3606
6'	63.28	61.81
7'		170.1782
8'		
9'		169.3069
10'		
11'		20.6207
12'		20.4478
13'		
14'		
$N = C\text{-}0\text{-}\underline{C}(=O)\text{-}CH_3$		160.4431
$N = C\text{-}0\text{-}C(=O)\text{-}\underline{CH_3}$		53.34
$N = \underline{C}\text{-}0\text{-}H$	171.7	

The EIMS of compound(2)(Fig. 4) is characterized by a molecular ion peak at m/e 327 and a number of fragments. The probable genesis of some of the major fragments is shown

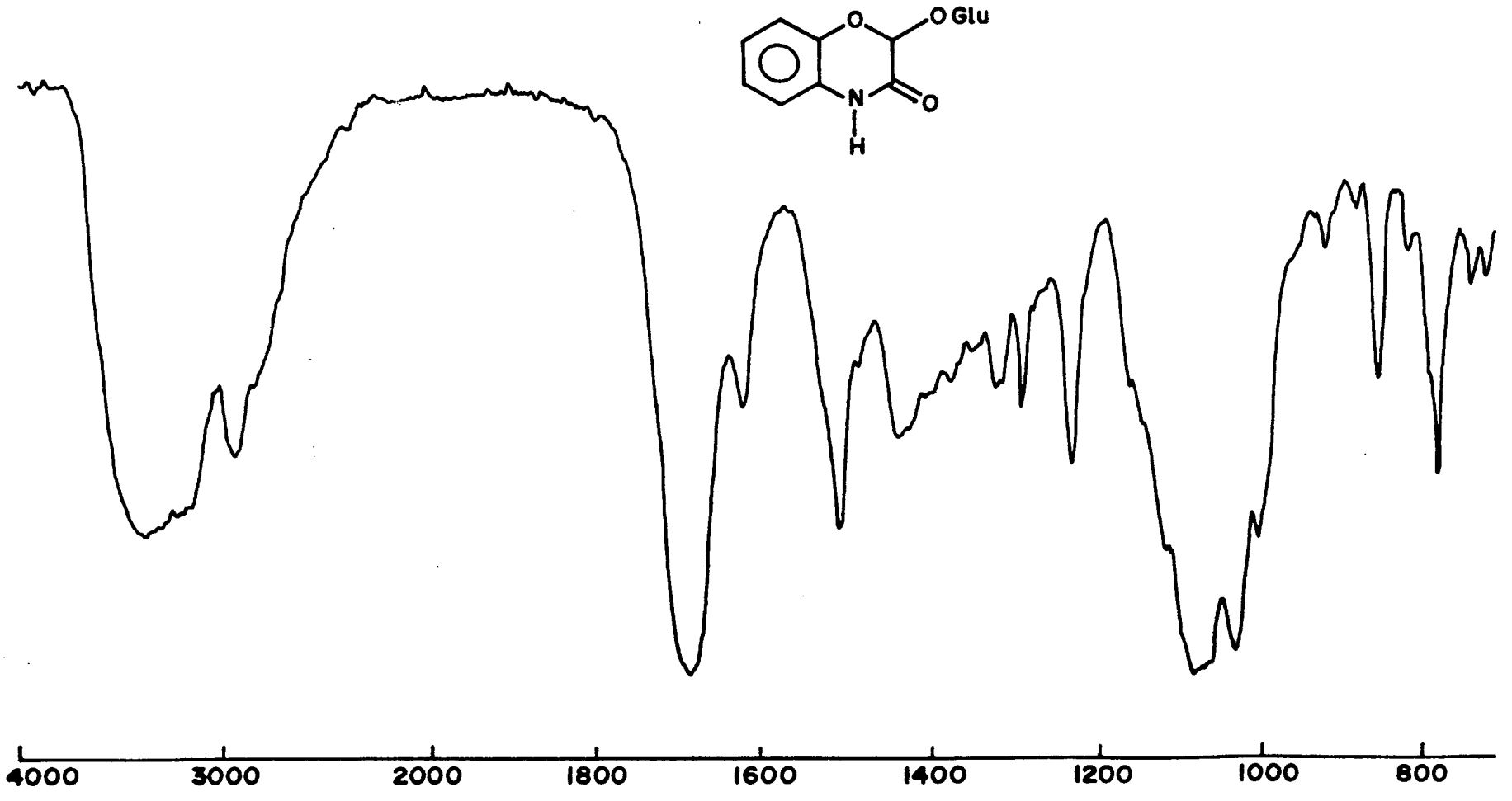


Fig. 1 IR Spectrum of HBOA – Glc

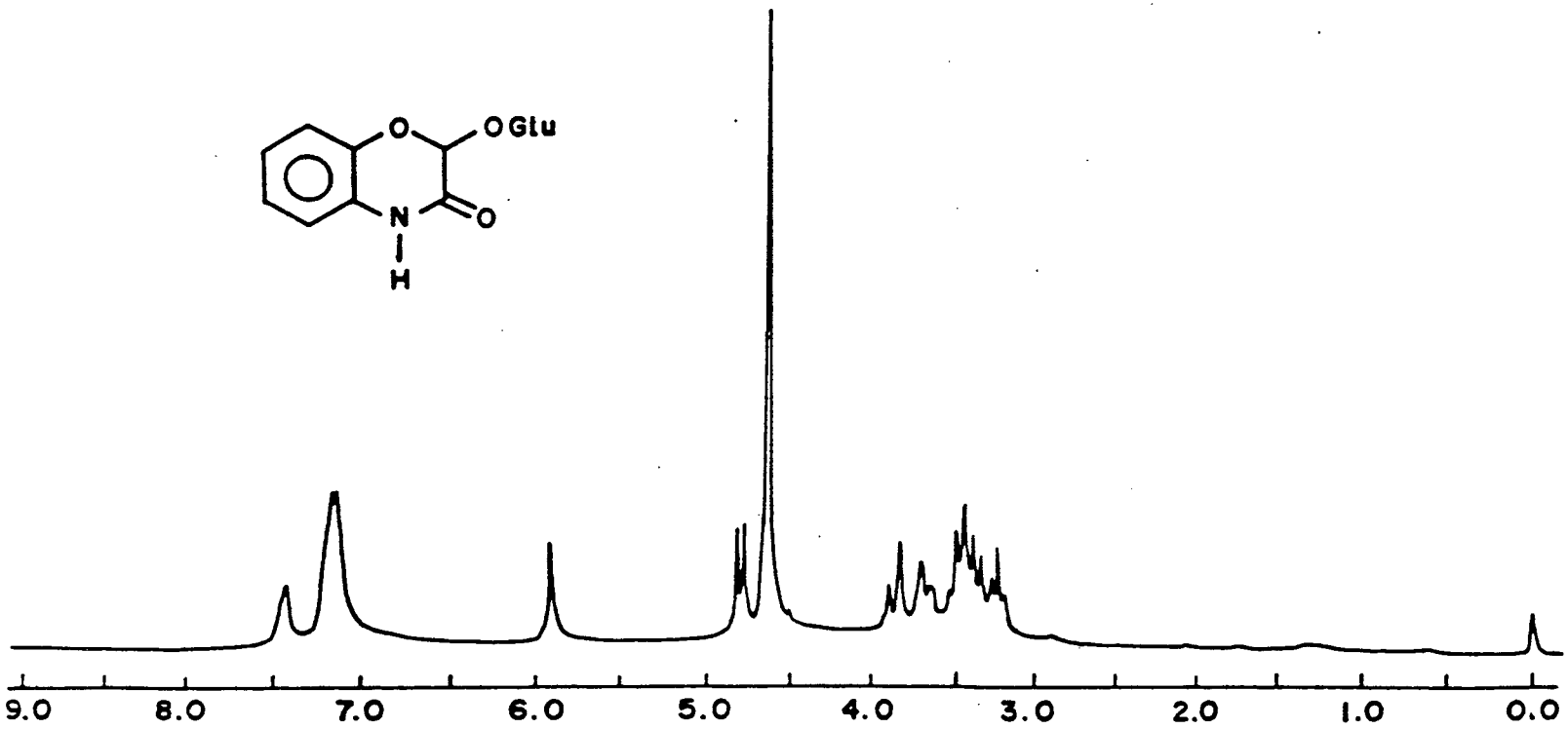
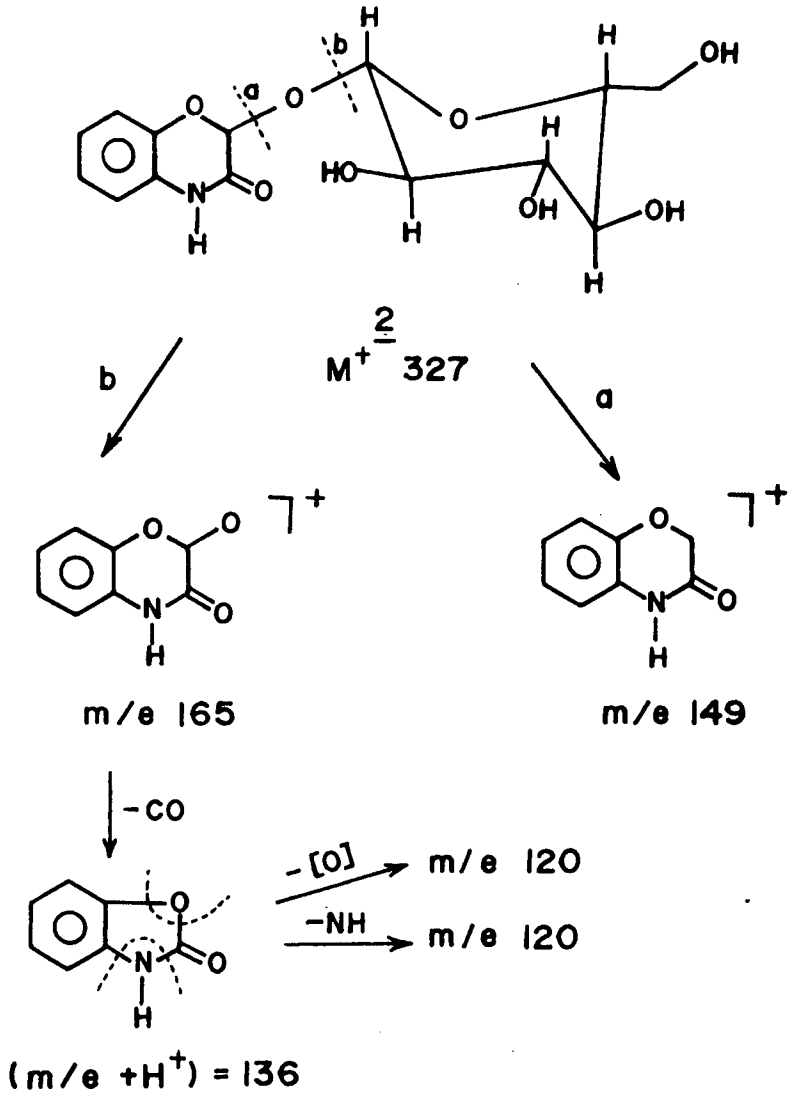


Fig. 2 ^{1}HNMR Spectrum of HBOA - Glc

Scheme 1



This work was communicated to the Journal of Natural Products and as per referee's comments that the compound could be rhamnosyl derivative on the basis of the base peak at m/e 136 observed in the mass spectrum of the compound (2) the structure was reinvestigated.

Hydroxamic acids are known to decompose to benzoxazolinones with liberation of

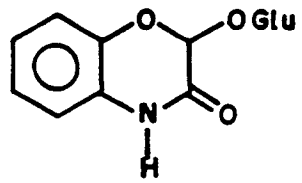
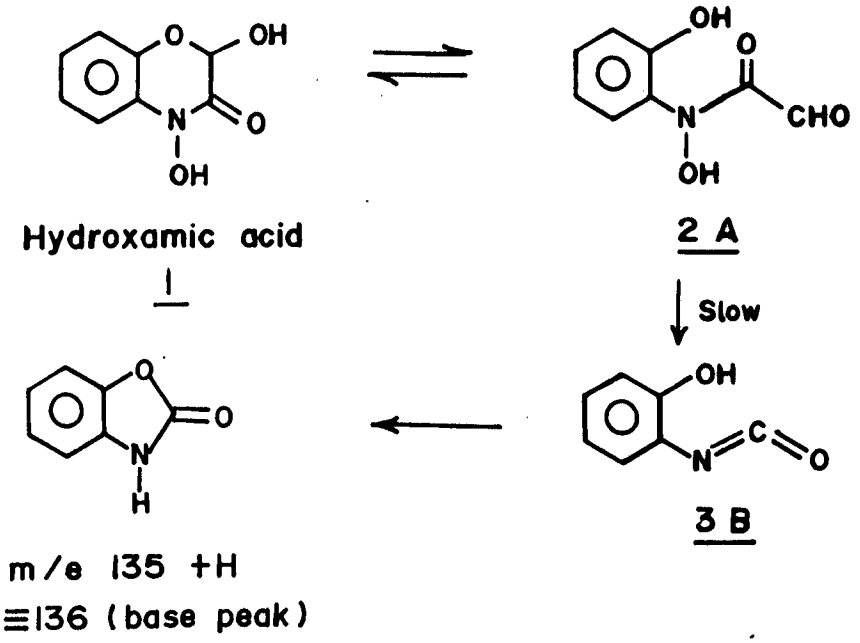


Fig.-3 ^{13}CNMR Spectrum of HBOA – Glc

formic acid. The mechanism of the reaction appears to involve the fast formation of aldol A, and the rate limiting formation of isocyanate B as shown in Scheme 2.

Scheme 2



This mechanism is supported by studies in aprotic solvent, where the aldol has been quantified,[11] and the participation of the hydroxamic oxygen atom as a nucleophile has been confirmed[12] and in alcoholic solvents, where the participation of hydroxamic carbonyl group in the rate limiting step has been demonstrated.[13]

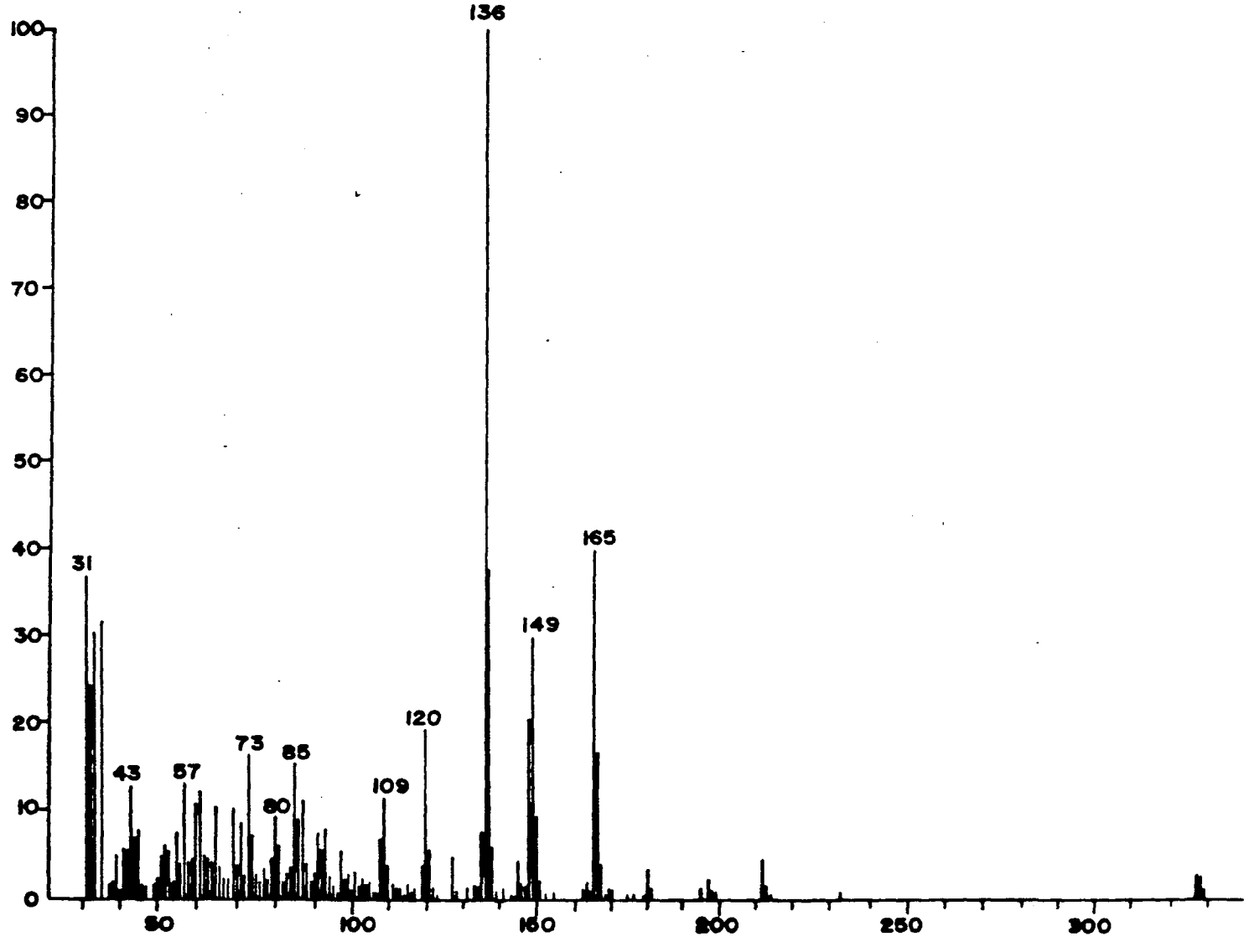


Fig. 4 Mass spectrum of HBOA – Glc

Additionally, higher pH values of the decomposing media,[14,15] as well as solvents of higher electron donating properties[15] increase the reaction rate as well as yields of benzoxazolinone as expected for the mechanism proposed.

Alternative mechanisms have been proposed on the basis of higher reactivity of some of the analogues, methylated at the hydroxamic oxygen with respect to their corresponding parent compounds and the lack of reactivity of compounds with an alkylated phenolic OH group with respect to dihydroxybenzoxazolinone. These facts have been rationalized by the proposed mechanism by taking into account the higher nucleophilicity of a methoxyl oxygen atom as compared to a hydroxyl oxygen atom, and the possibility of aldol (A) adopting a conformation with an intramolecular hydrogen bond between the phenolic OH and the hydroxamic carbonyl group which exposes the aldehyde carbonyl group to attack by the hydroxamic OH group, thus leading to a low energy transition state.

Since the mass spectrum of compound (2) (Fig.4) indicated base peak m/e 136 which is expected to be formed by the decomposition of the parent compound to benzoxazolin-2-one led to comment that (2) is a DIBOA -Rhamnosyl and not HBOA-Glucoside.

Compound (2) on acetylation with acetic anhydride and pyridine at room temperature yielded at tetra-acetate (3). EIMS (Fig.7) indicated a molecular ion at 496. The IR spectrum (Fig. 5) of (3) displayed bands at 1750, 1230 and 1710 cm^{-1}, characteristic of the carbonyl stretching vibration of acetate and the amide. 100MHz ^1H NMR (Fig.6) spectrum of (3) showed the presence of acetate group at 1.92 (3H,s); 1.96 (6H,s) and 2.2 (3H,s). In addition to the signals which are due to amide 9.35 (s), the singlet at 5.6 could only be assigned to H-2. The spectrum also revealed the presence of four aromatic protons. The signal at 6.8 was assigned to H-6 and the 3H multiplet between 6.9-7.0 were assigned to H-5, H-7 and H-8. ^1H NMR signals due to glucose appeared in the region 3.7-5.3. The anomeric H-1' proton of glucose was

Fig. 5 IR Spectrum of HBOA – Glc acetate

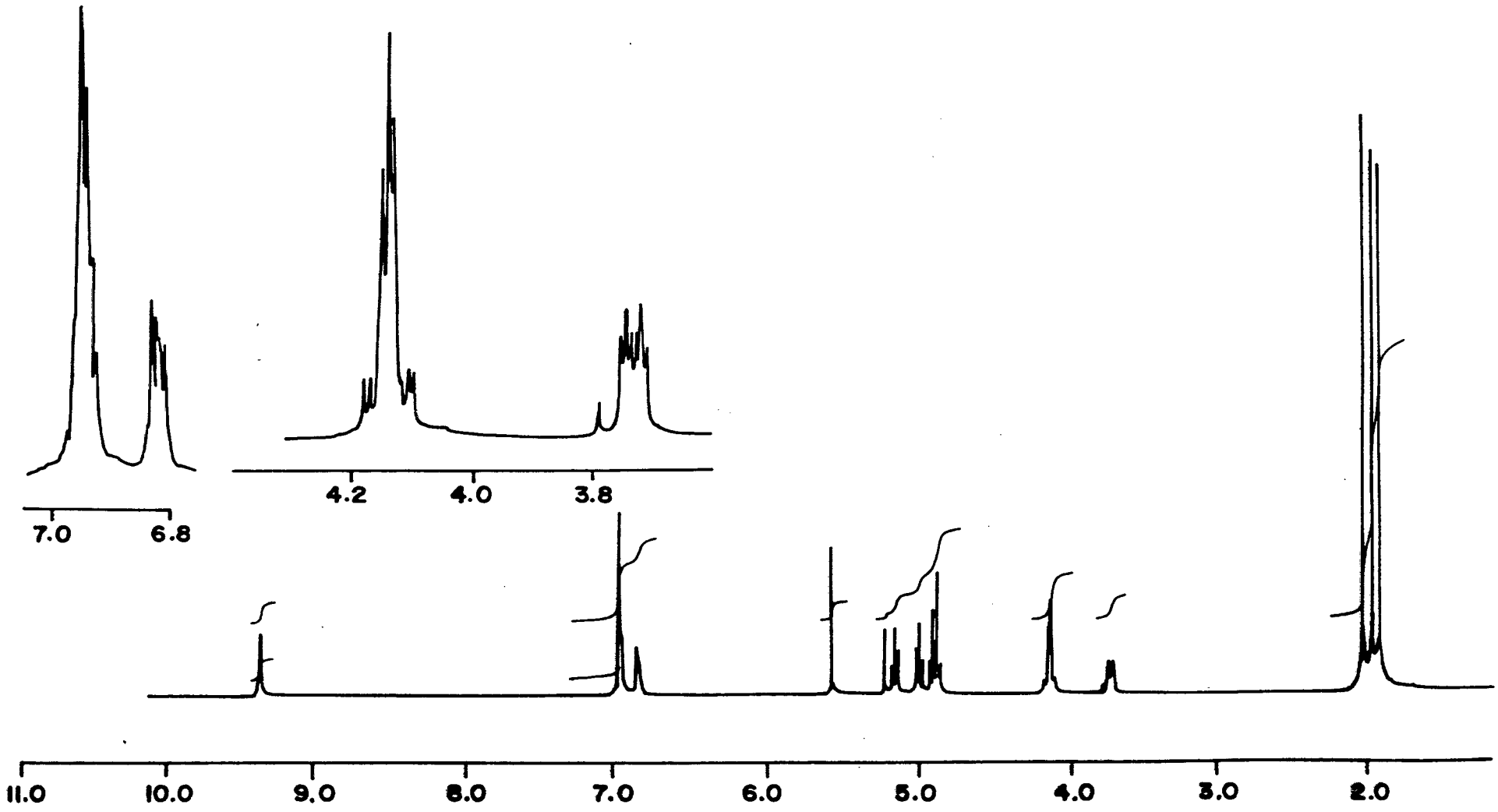


Fig. 6 ^{1}H NMR Spectrum of HBOA−Glc acetate

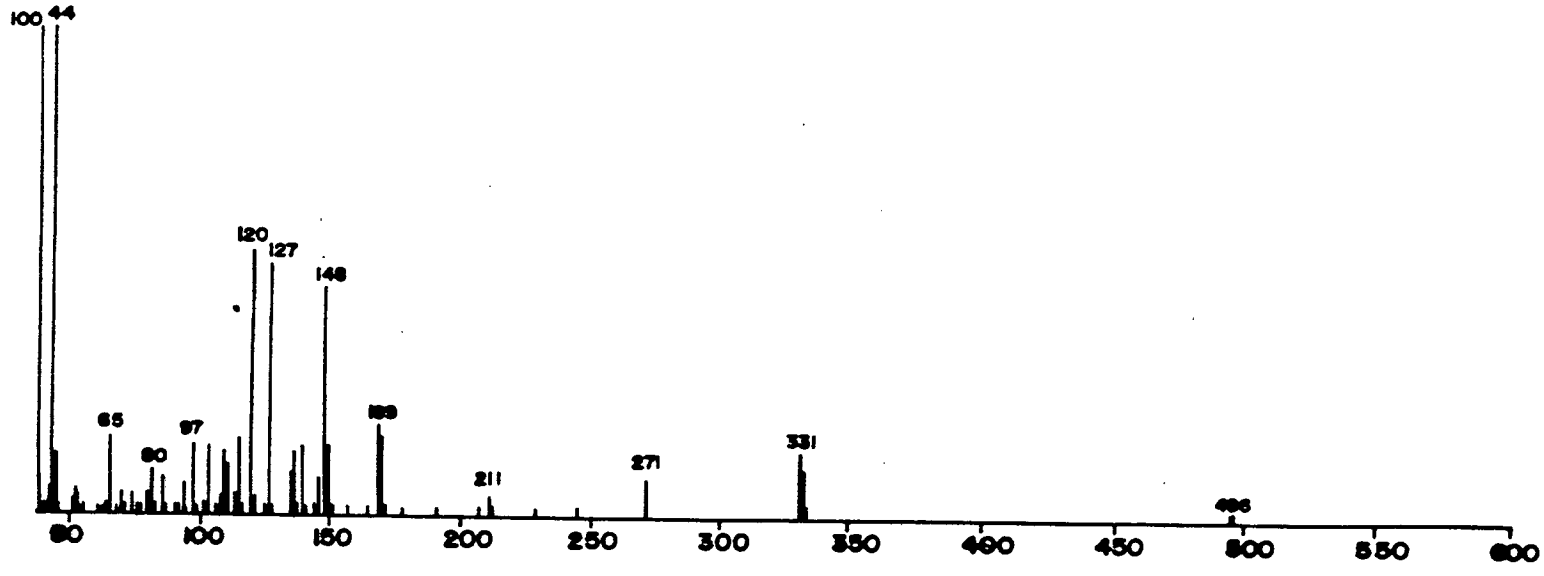


Fig. 7. Mass Spectrum of HBOA—Glc. acetate

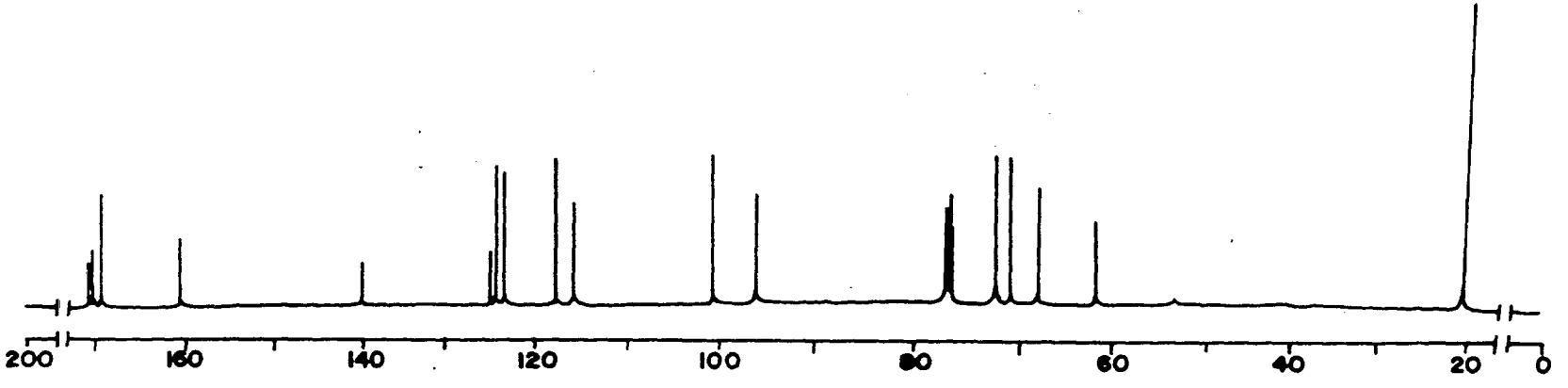
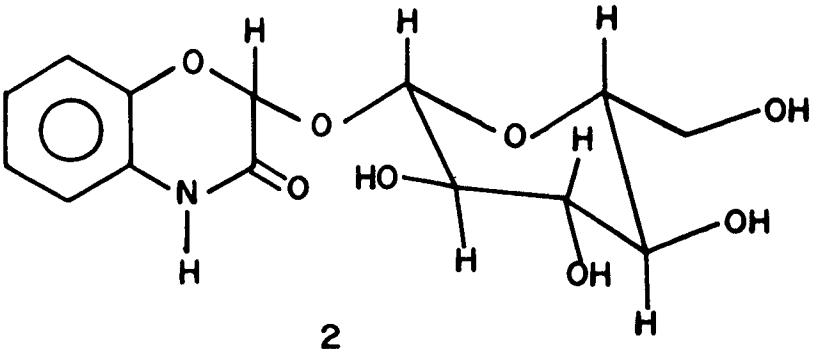


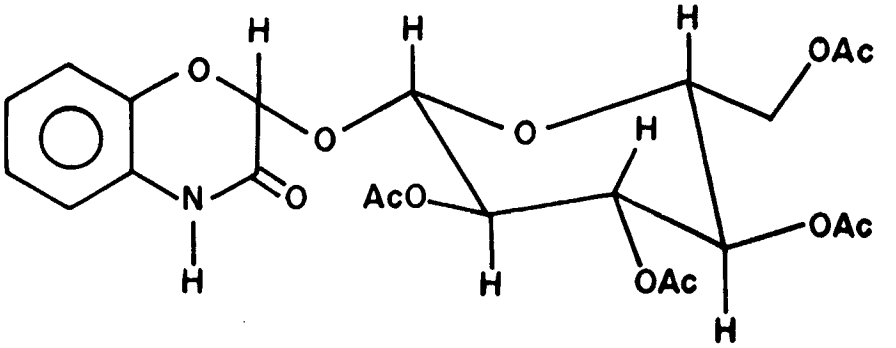
Fig. 8. ^{13}C NMR Spectrum of HBOA-Glc. acetate

assigned a doublet at 5.15 (d,J=8Hz) and all vicinal coupling constant of glucose was confirmed as ß-D-Glucopyranoside. H-5' proton of glucose appeared at 3.73. H-6' as 2H multiplet centered at 4.12. The glucose protons H-2', 3', and 4' appeared as doublet of doublet with some overlapping in the region 4.8-5.2.



2

HBOA-Glc



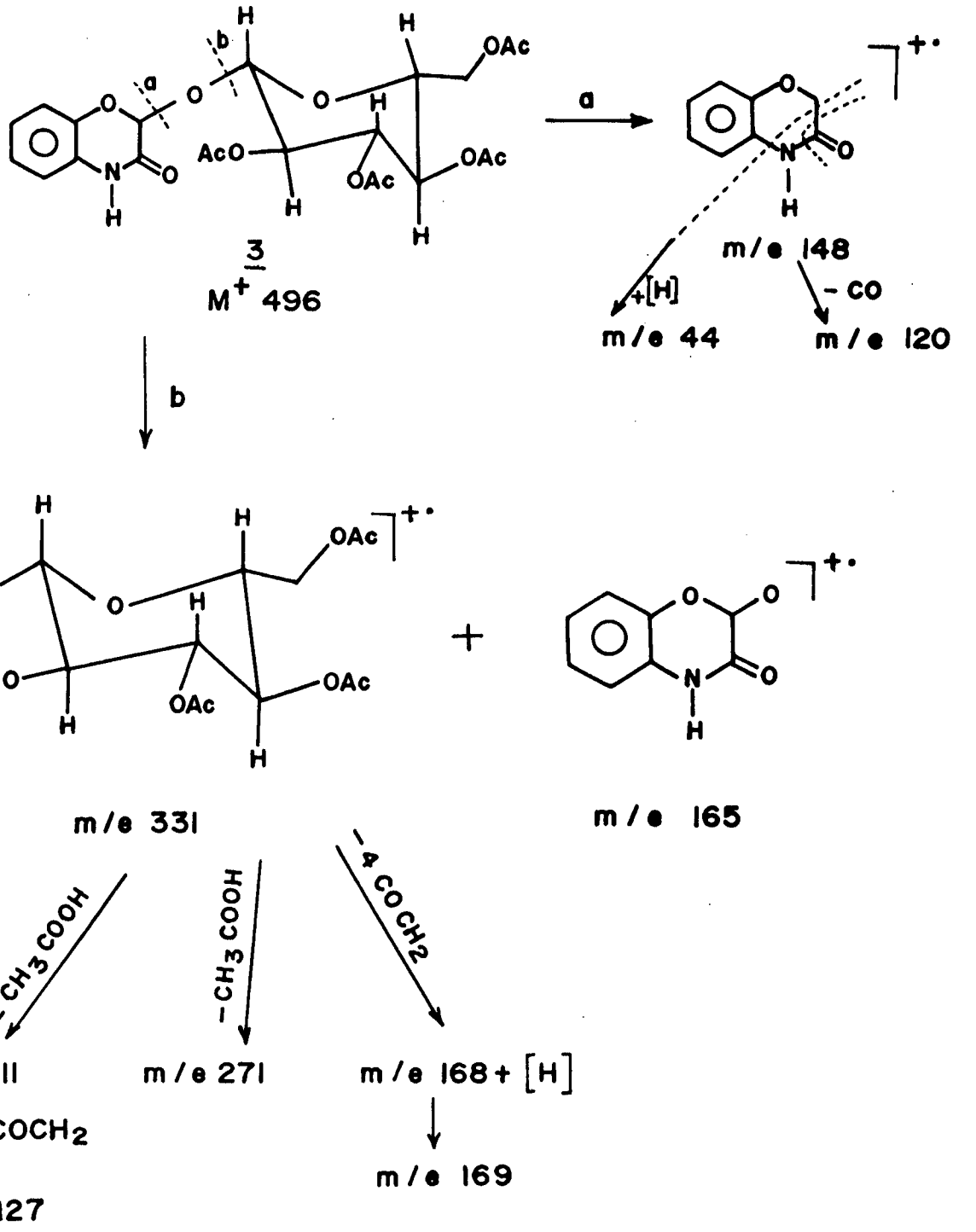
3

HBOA-Glc acetate

The ^{13}C NMR Fig. 8 (Table 1) data was consistent with the structure (3) proposed for the acetylated compound and hence the compound isolated from *A.illicifolius* is a glucoside and not rhamnoside.

The probable genesis of some of the major fragments is shown in Scheme 3.

Scheme 3


a
b
OAc
AcO
OAc
OAc
3
M+ 496
m/e 148
+[H]
- CO
m/e 44
m/e 120
m/e 331
m/e 165
-2 CH3COOH
-CH3COOH
-2 COCH2
m/e 211
m/e 271
m/e 168+ [H]
-2 COCH2
m/e 169
m/e 127

This fragmentation pattern further supports glucose to be the sugar moiety of the glycoside (formation of tetra-acetate, rhamnosyl is expected to give tri-acetate). All evidences led to the confirmation of structure (3) for the acetate and hence structure (2) for the parent compound.

Methanol: Chloroform (5:95) eluate yielded yet another crystalline solid, (4), m.p. 122° C. Its IR spectrum (Fig. 9) was indicative of the presence of an amide carbonyl (1730 and 1770 cm^{-1}). It also showed an intense band at 3240cm^{-1} due to NH grouping, 1250 (C-0-C stretching) cm^{-1} and absorption characteristic of oxazole ring system at 1480, 1390 and 1035 cm^{-1}. ^1H NMR spectrum of (4) (Fig. 10) in $CDCl_3$ displayed only two signals: one characteristic broad singlet at 9.9 due to NH and a multiplet at 7.0-7.18 due to aromatic protons in the intensity ratio of 1:3 respectively. Its ^{13}C NMR revealed the presence of seven signals for carbon atoms in the molecule.

The signals in the ^{13}C NMR spectrum (Fig.11) were very similar to those of benzoxaline-2-one (BOA) (Table 2). Its mass spectrum (Fig. 12) with a molecular ion peak at m/e 268 (M^+) indicated that it could be a dimer of BOA.

Table 2

Table 2 : ^{13}C NMR spectral data of compound (4) and BOA

C	4	BOA
2	156.2664	156.233
4	110.1016	110.11
5	110.2204	110.197
6	124.8121	124.160
7	122.6986	122.709
8	143.9279	144.998
9	129.4731	129.519

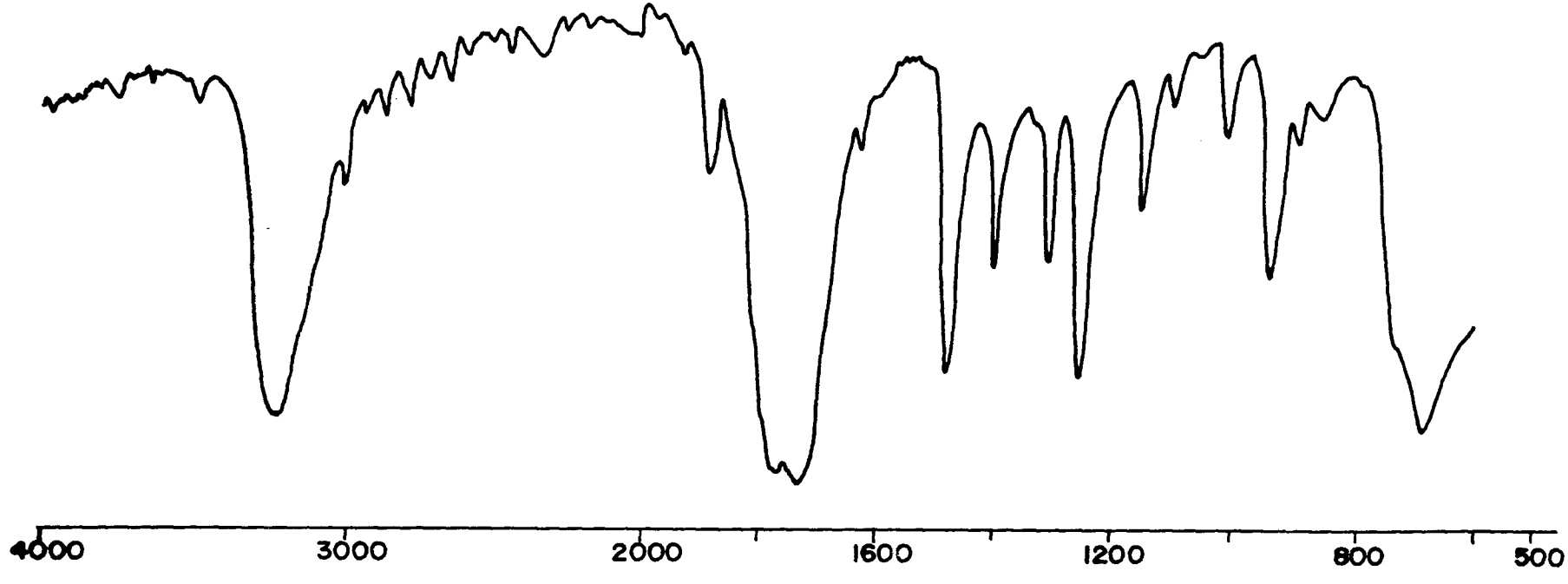


Fig. 9. IR Spectrum of Dimer of BOA

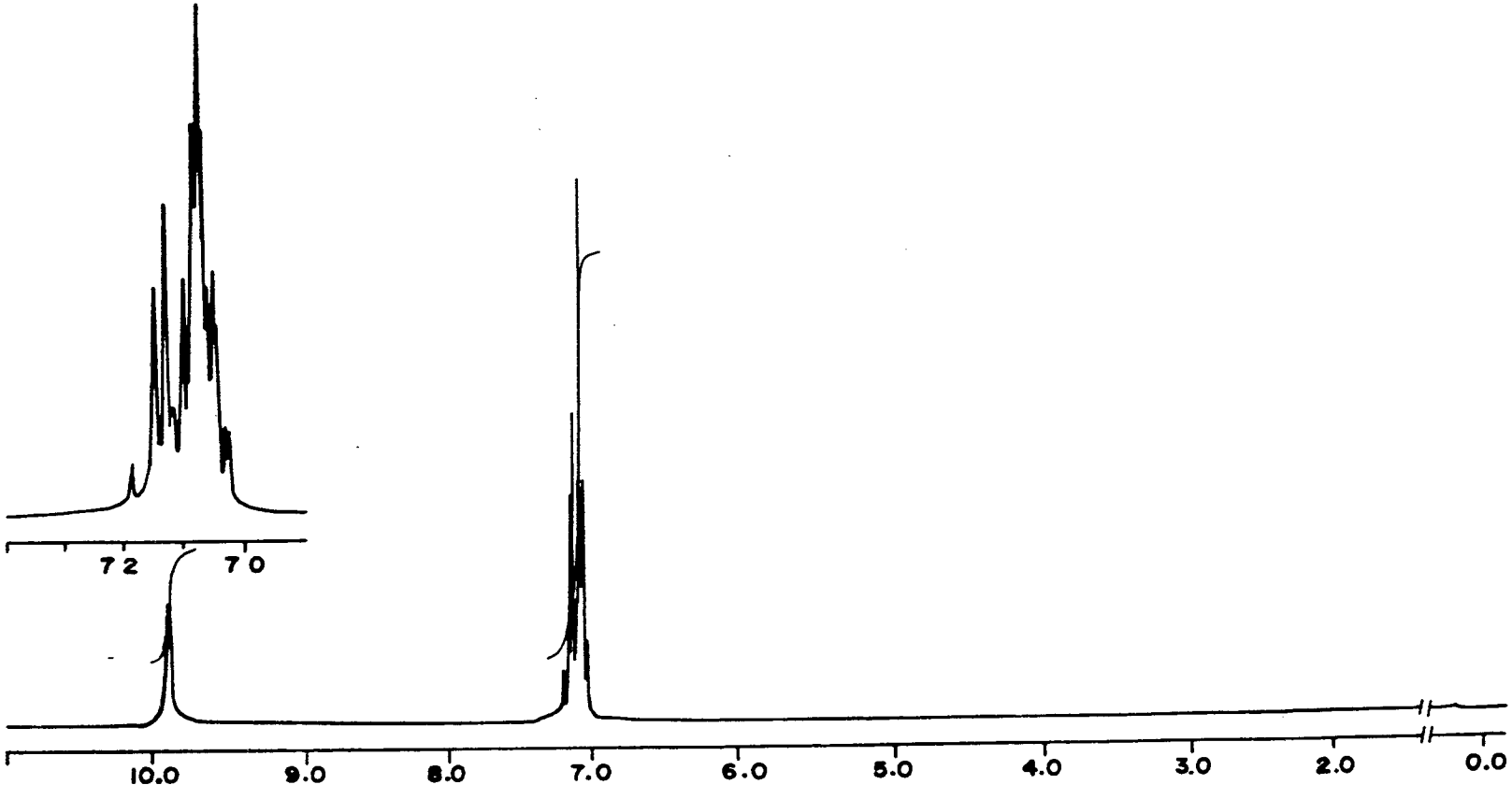


Fig. 10 ^{1}H NMR Spectrum of Dimer of BOA

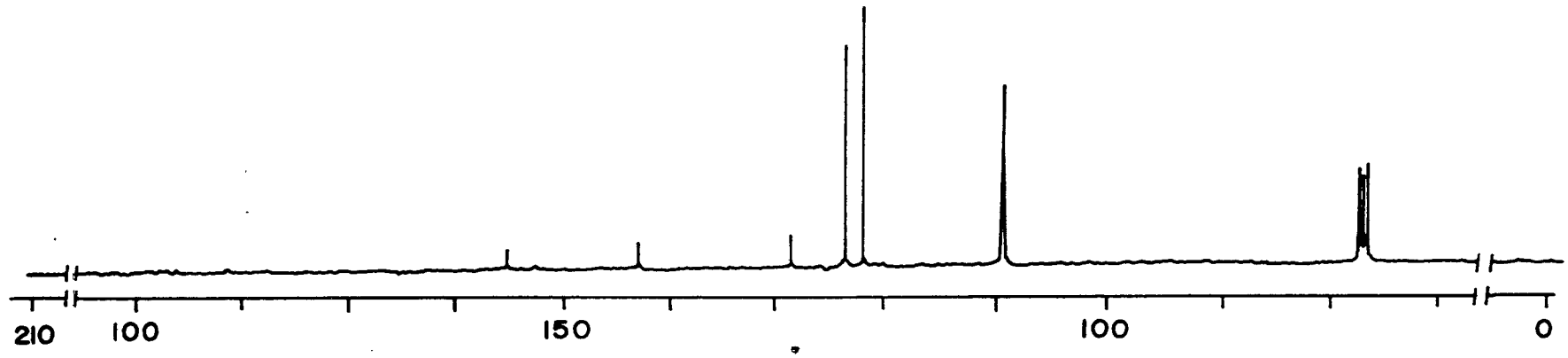


Fig. 11. ^{13}C NMR Spectrum of Dimer of BOA

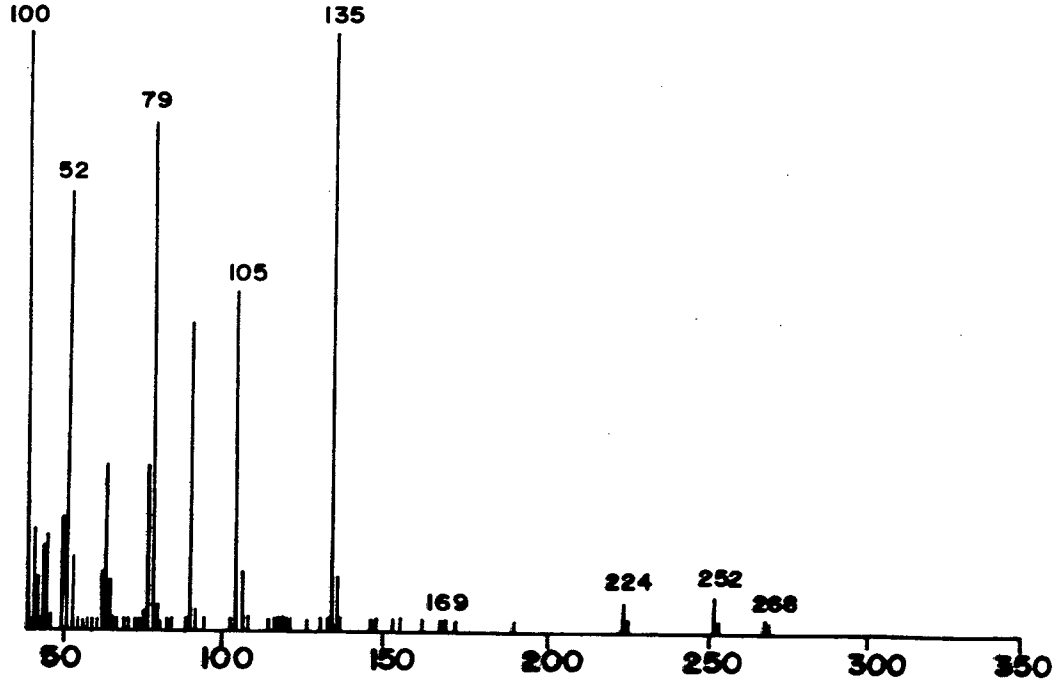
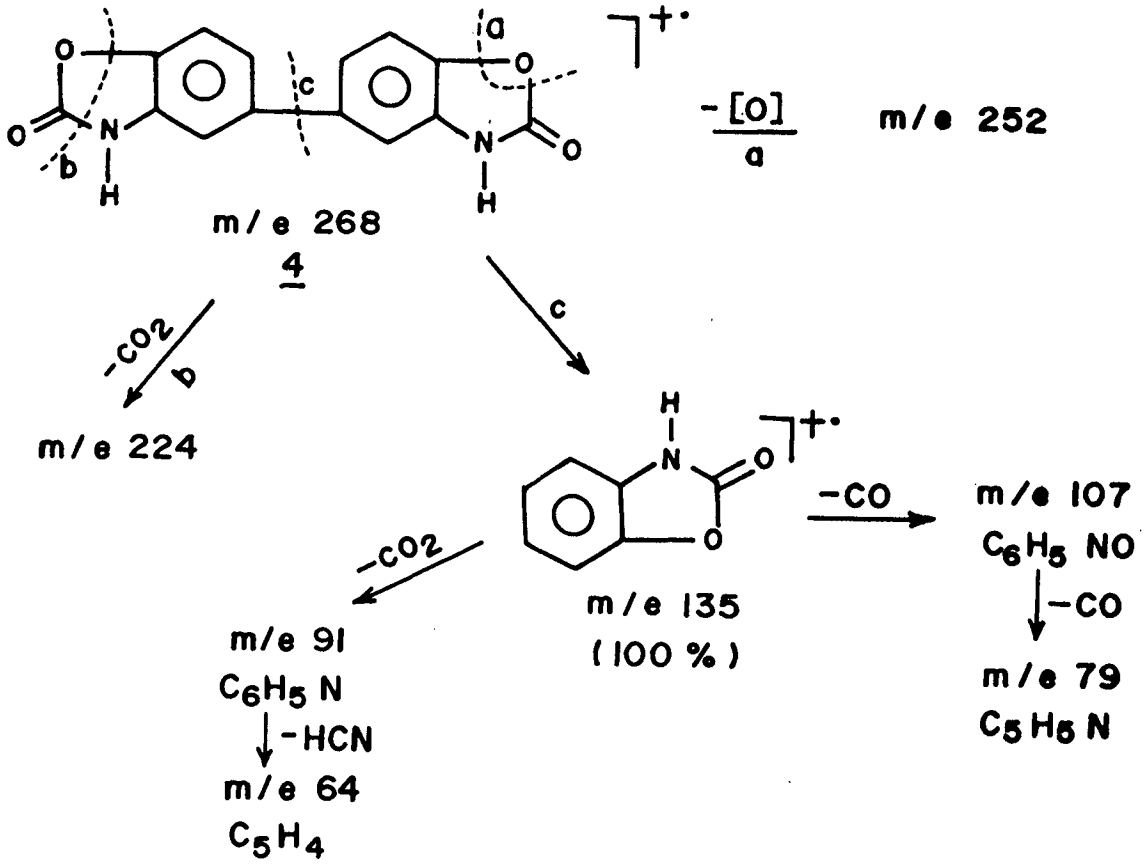


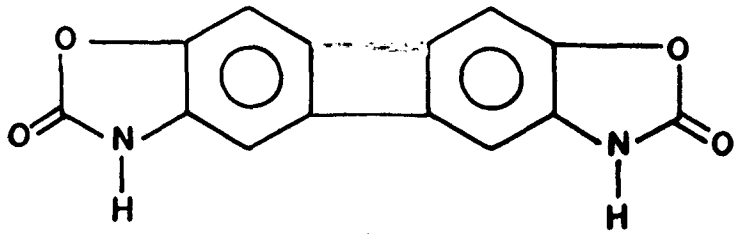
Fig.-12. Mass Spectrum of Dimer of BOA

Scheme 4



The observation of only seven carbon signals in the ^{13}C NMR spectrum (Fig. 11) of (4) indicated that the two BOA units must be linked through same elements of symmetry. Evidences for the linkage at position 5' was obtained from the observation of changes in the intensity of C-5 signal of BOA in ^{13}C NMR of dimer of BOA (there is a decrease in the intensity of this signal with the substitution). Additional evidence was obtained by the presence in the ^{1}H NMR spectra of a doublet at 7.14(H) and a triplet at 7.07(2H). Two of

the protons are ortho to each other and the third is meta. Infra red bands at 850 and 890 also indicated the presence in aromatic rings of adjacent hydrogens and an isolated hydrogen respectively.



4

Dimer of BOA

On the basis of spectral evidences, structure (4) was assigned to the dimer of BOA.

The genesis of the various fragments in the mass spectrum of the compound (4) is shown in Scheme 4.

EXPERIMENTAL

Preparation of the extract

Fresh *Acanthus illicifolius* pods (5 kgs) were collected from the banks of Cumbarjua canal connecting the two estuaries of the river Mandovi and Zuari during the month of May. The pods were soaked in 90% aqueous methanol at room temperature for 72 hours. This process was repeated thrice and the combined extracts were concentrated to dryness under reduced pressure at 50°C. The concentrated extract was kept in a vacuum descicator for final drying, before testing for the pharmacological activity.

Analgesic activity

This activity was studied in albino mice (Haffine strain) of either sex weighing 25-30g. Six mice fasted overnight were used per group.

Tail clip method

An artery clip with the jaws covered with thin rubber tubing was applied to the base of the tail of the mouse. Only those animals which made repeated attempts to remove the clip within 15 seconds were used. Absence of any attempt to remove the clip within 30 seconds was taken as criterion of analgesia. Pethidine hydrochloride was used as standard for comparison. Different doses of the extract or pethidine were administered intraperitoneally (IP) 30 min before application of the clip.

Writhing method[16]

An aqueous solution (v/v) of hydrochloric acid 0.5% was administered IP in the dose of 0.2ml per mouse. The animals were observed continuously for 20 minutes for writhing syndrome. Different doses of the extract or sodium salicylate were administered IP 30 minutes before the injection of HCl and the percent protective effect was noted.

Anti-inflammatory activity

Testing procedure given in Chapter I, Section II.

Fractionation of the methanolic extract

The crude methanolic extract was fractionated into petroleum ether, chloroform, n-butanol and water soluble fractions. The follow-up investigations led us to locate the analgesic activity in chloroform soluble fraction and water soluble fraction.

Isolation of 2-O-ß-Glucopyranosyl-1,4-(2H)-benzoxazine-3-one (HBOA-Glc) (2)

The aqueous fraction was chromatographed over silica gel filled in chloroform and eluted with chloroform, methanol : chloroform and water : methanol. The methanol: chloroform eluant was rechromatographed, being eluted with increasing concentration of methanol in chloroform. The fraction eluted in methanol : chloroform (20:80), gave a crystalline solid, (1) yield 1.567g, m.p. 221°C.

The less polar eluate MeOH : $CHCl_3$, (5:95) yielded a crystalline solid,(5), m.p. 122°C TLC solvent system used was $CHCl_3$: MeOH (98:2) and ethyl acetate : petroleum ether (80:20) and the spots were visualized with iodine vapours.

The TLC solvent system used was $CHCl_3$: MeOH : H_2O (65:25:4) and the spots were visualized with iodine vapours and vanillin : sulphuric acid.

HBOA-Glc (2)

UV MeOH λmax 225, 286 nm

IR spectrum (KBr, Fig. 1)

3600, 2900, 1690, 1640, 1610, 1510, 1440, 1320, 1290, 1230, 1060, 1025, 1000, and 860 cm-1

^{1}H NMR Spectrum (200MHz, D_2O, Fig.2)

7.4 (1H,s); 7.1 (3H,m); 5.9(1H.s); 4.8(1H,d,J=7.7Hz) and 3.1-3.9 (6H,m)

^{13}C NMR spectrum (200MHz, D_2O, Fig. 3)

173.99, 171.7, 164.75, 142.50, 127.61, 126.54, 120.42, 119.18, 104.59, 97.95, 79.01, 78.18, 75.54, 71.88 and 63.28.

Mass Spectrum (EIMS, 70eV, Fig.4)

M^+ 327, m/e 166, 165, 150, 149, 148, 136, 135, 120, 119, 109, 108, 85, 80, 73, 57, 43, and 31.

Acetylation of HBOA-Glc

HBOA-Glc (50mg) was dissolved in pyridine (1ml) on slight warming. To this solution acetic anhydride (2ml) was added and the mixture was allowed to stand at room temperature for 68 hours. On usual work up it gave a tetra-acetate as crystalline solid, m.p. 153°C, yield 58 mg.

HBOA - Glc acetate (3)

IR spectrum (KBr, Fig. 5)

It showed bands at 3240, 2965, 1750, 1710, 1660, 1620, 1500, 1425, 1375, 1230, 1040, 1010, 900, and 748 cm^{-1}.

^{1}H NMR spectrum (100MHz, $CDCL_3$, Fig 6)

9.35 (1H,s); 6.9-7.0 (3H,m); 6.8 (1H,m); 5.6 (1H,s); 5.15(d,J=8Hz, H-1'); 4.8-5.2(3H,dd); 4.12(2H,m); 3.73(1H,dt); 2.2 (3H,s); 1.96 (6H,s) and 1.92 (3H,s)

^{13}C NMR spectrum ($CDCl_3$, Fig 8)

170.58; 170.1782; 169.3069; 160.4431; 140.1447; 124.9614; 124.3169; 123.4370; 117.84; 115.62; 100.6270; 96.0649; 72.3603; 72.5151; 71.0678; 68.1104; 61.81; 53.34; 20.6207 and 20.4478.

Mass Spectrum (EIMS, 70eV, Fig. 7) m/e

496(M^+), 331, 271, 211, 169, 148, 127, 120, 115, 103, 97, 80, and 65.

5, 5' bis-benzoxazoline-2,2'-dione (4)

IR spectrum (KBr, Fig.9)

3250, 3230, 3010, 1885, 1770, 1730, 1620, 1480, 1400, 1305, 1250, 1145, 1090, 1005, 935, 890, 850 and 685.

^{1}H NMR spectrum (100MHz, $CDCl_3$, Fig. 10)

9.9 (2H, bs) and 7.0-7.18 (6H, m)

^{13}C NMR spectrum ($CDCl_3$, Fig. 11)

156.2664; 143.9279; 129.4731; 124.8121; 122.6986; 110.2204 and 110.1016.

Mass spectrum EIMS, 70 eV, Fig. 12) m/e

268(M^+), 252, 224, 190, 169, 136, 135 (base peak), 107, 105, 91, 79, 77, 64 and 52.

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SECTION II

CONSTITUENTS OF THE BROWN ALGA *STEOCHOSERMUM MARGINATUM*

Stoechospermum marginatum (C. Agardh) Kuetzing, a brown seaweed, belonging to the family Dictyotaceae is found in abundance along the Anjuna coast (Goa) only during the premonsoon period. During the search for the biologically active constituents, the crude methanolic extract of *Stoechospermum marginatum* exhibited spasmolytic activity.[1] The extract of *Stoechospermum marginatum* has also been reported to be antibacterial inhibiting the growth of **Staphylococcus aureus.** The active constituents were found to be a mixture of monoacetates belonging to the spatane diterpenoids.[2]

Marine algae of this family are prolific producers of interesting secondary metabolites, consisting of C_{11} acetate derived compounds,[3] compounds of mixed biogenesis,[4,5] sesquiterpenoids[6,7] and diterpenoids.[8-11] The isolation and identification of two new diterpenoids,[9,12] stoechospermol and stoechospermol acetate belonging to the recently characterized spatane group of diterpenoids[2] have been reported earlier from this laboratory.

In this section, the isolation and identification of phthalate esters, dibutyl phthalate (M^+278), bis(2,6-dimethyl hexyl) phthalate (M^+390) and another ester (M^+418), sterols fucosterol, dihydrofucosterol, 24-ketocholesterol, a fatty ester, ethyl palmitate and fatty acids from the brown alga *Stoechospermum* marginatum has been dealt with.

Extensive column chromatography over silica gel of the neutral fraction of the methylene chloride extract of the seaweed and gradient elution with petroleum ether and increasing concentration of ethyl acetate in petroleum ether yielded several compounds. Petroleum ether eluate gave ethyl palmitate, the least polar, followed by the phthalate esters A, B and C, in polarity, C being the most polar. Ethyl acetate : petroleum ether (10:90) eluate yielded the sterols 24-ketocholesterol, fucosterol and dihydrofucosterol.

Ethyl palmitate: This compound obtained as liquid, its IR spectrum, (Fig.1),

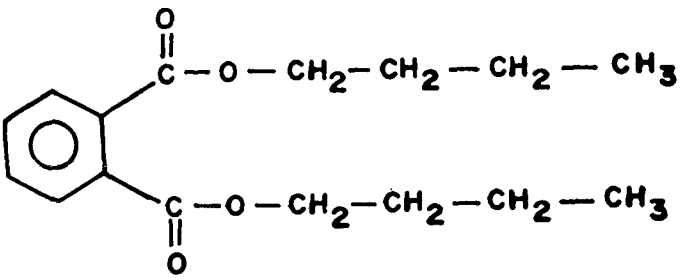
revealed the presence of >C=O at 1730, 1165, 1110, 1030 and 715cm^{-1}. The mass spectrum showed molecular ion peak at M^{+}284, (Fig. 3), corresponding to molecular formula $C_{18}H_{36}O_2$, base peak at m/e 88, characteristic of ethyl ester and the separation of units of the fragments by 14 mass units. Its ^{1}H NMR, (Fig.2) showed signals at 0.75, 1.2, 3.5 and a multiplet centered at 4. All this data indicated that the compound was ethyl palmitate.

Compounds A, B and C were obtained as light yellow viscous mass. Their mass spectra gave the molecular ion peaks at M^{+}278 (Fig.4), M^{+}390 (Fig. 4) and M^{+}418 (Fig.5) corresponding to molecular formulas $C_{16}H_{22}O_4$, $C_{24}H_{38}O_4$ and $C_{26}H_{42}O_4$ respectively. The base peak in the mass spectra of all the three compounds at m/e149 was indicative of phthalate esters.

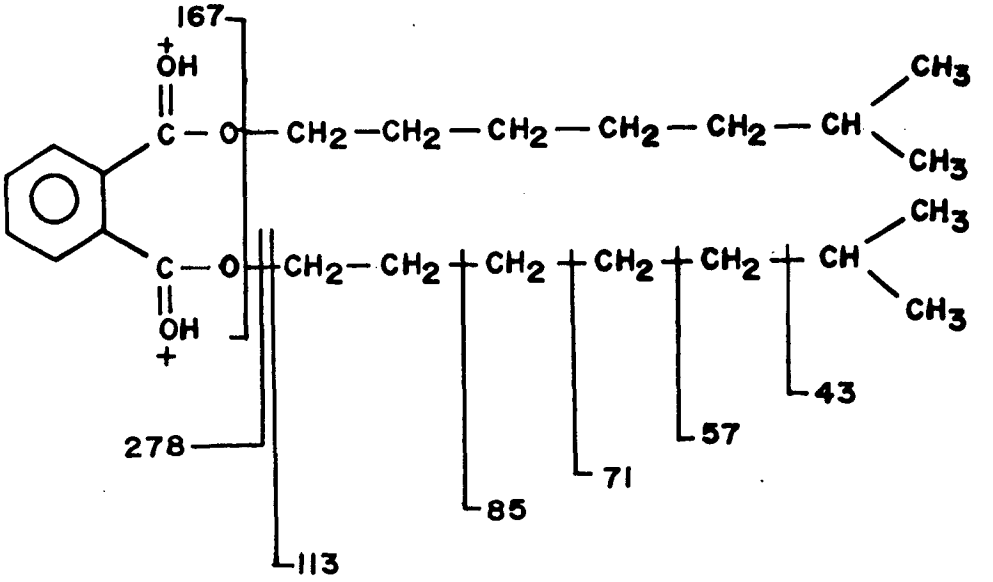
^{1}H NMR spectrum of A (Fig. 6) showed a signal for four aromatic protons at 7.5 while the signal for 6H of methyl appeared at 0.89. The existence of an aliphatic side chain was evident from the absorption in the region 1.0-1.8 integrated for 8H. The ester methylene groups (4H) appeared at 4.12 as doublet (J=6Hz).

^{1}H NMR spectrum of B (Fig.6) was similar to that of A, the only difference being that the absorption at 0.9 corresponded to 12H which indicated the presence of 4 methyls; the absorption in the region 1.1-2.3 was assigned to the 8H of the aliphatic side chain. The ester methylene (4H) appeared again as a doublet at 4.1.

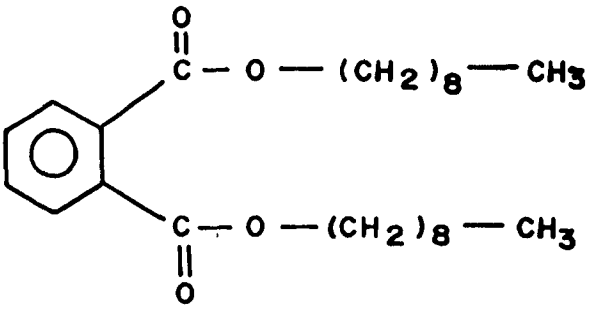
Their complete structures were, however, elucidated on the basis of critical mass spectral analysis. Mass spectrum of A exhibited prominent molecular ion peak at M^{+}278 followed by prominent peaks at 167 and the base peak at m/e 149 arising by the loss of alcohol residue (Scheme 1).



A



B



C

Scheme 1

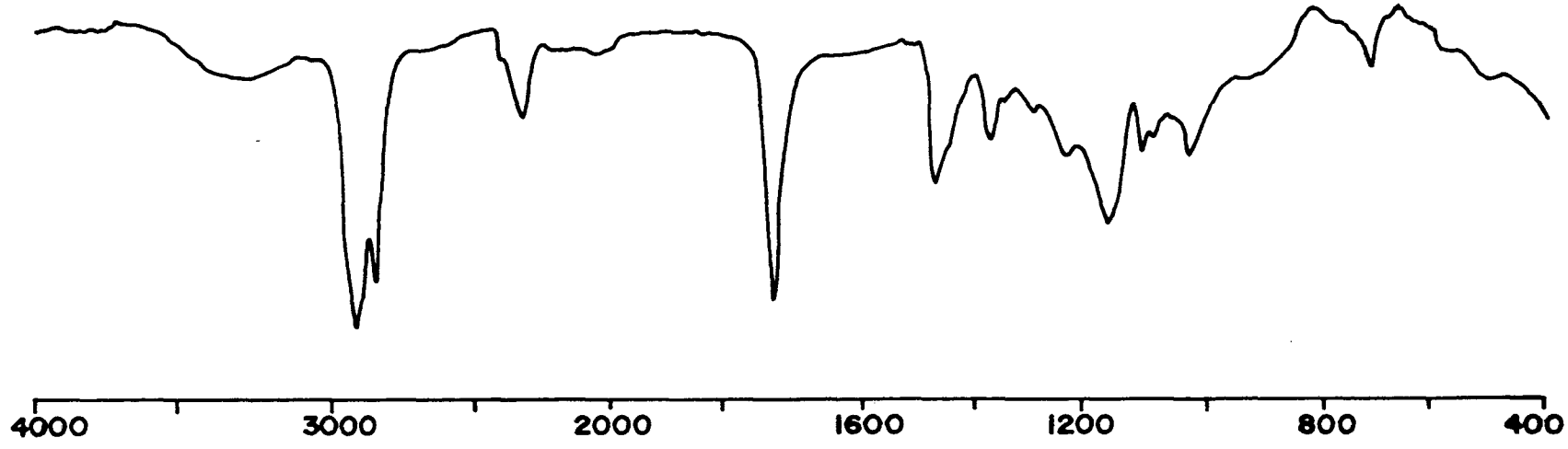


Fig.-1 IR spectrum of ethyl palmitate from stoechospermum marginatum

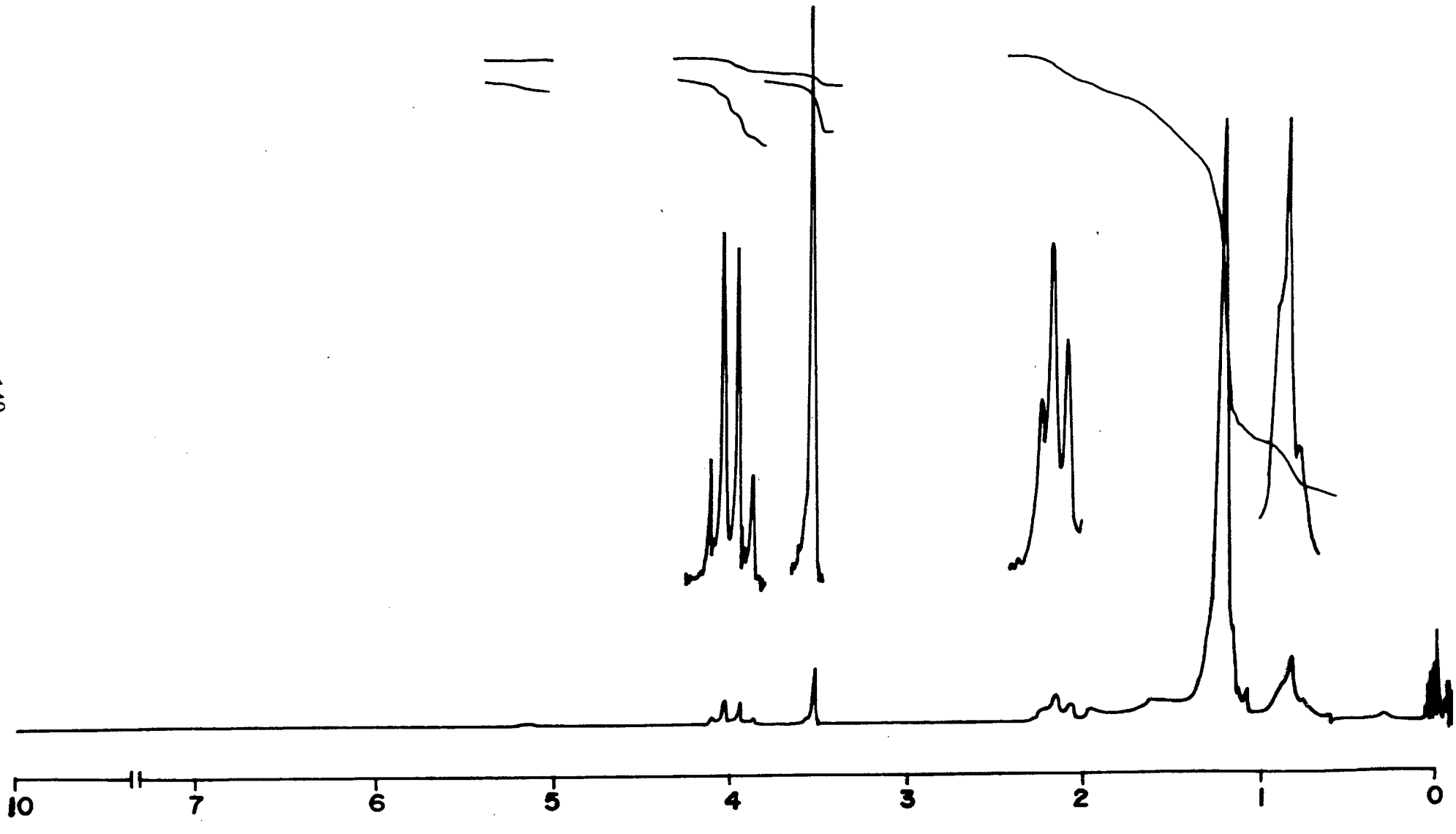


Fig.-2 ^{1}H NMR spectrum of Ethyl palmitate from Stoechospermum marginatum

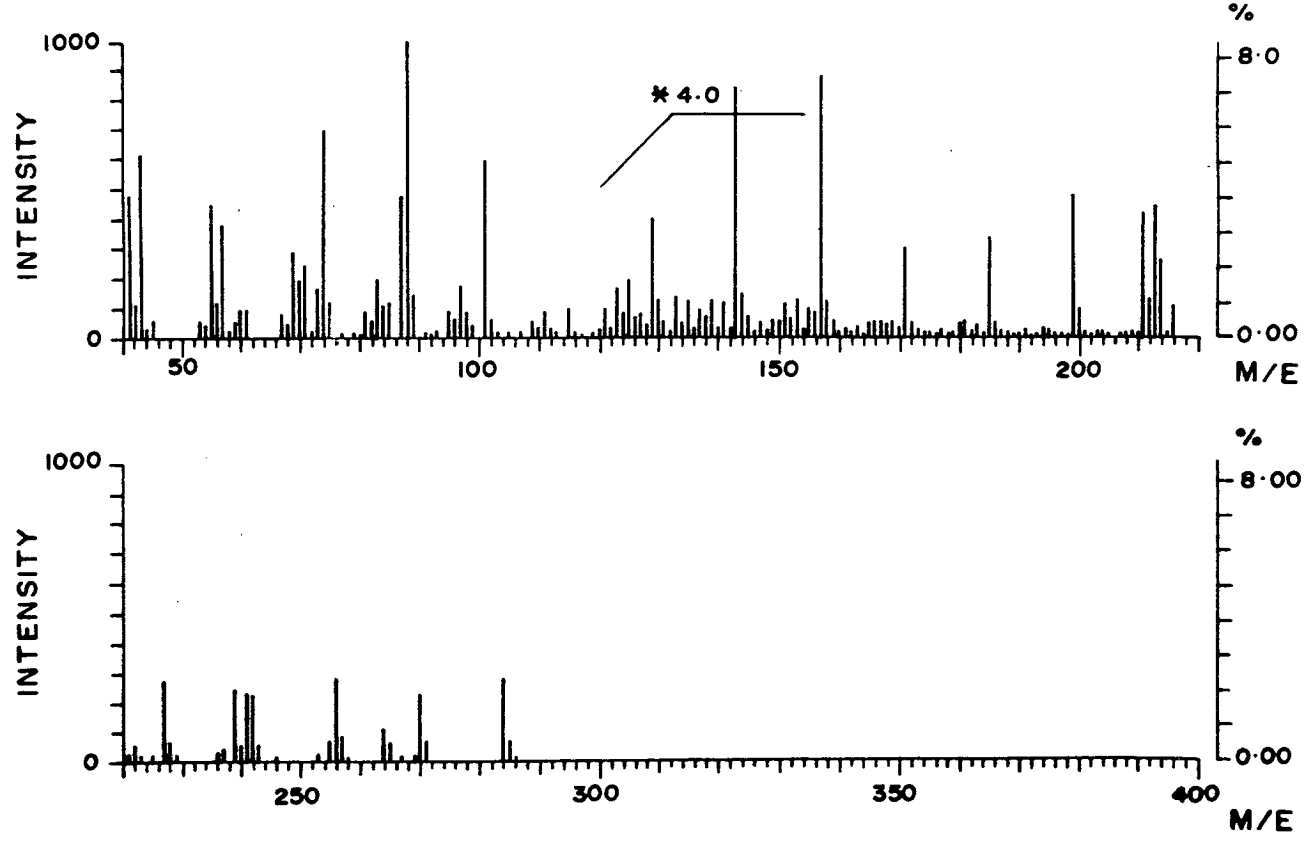


FIG.-3 MASS SPECTRUM OF ETHYL PALMITATE FROM STOECHOSPERMUM MARGINATUM

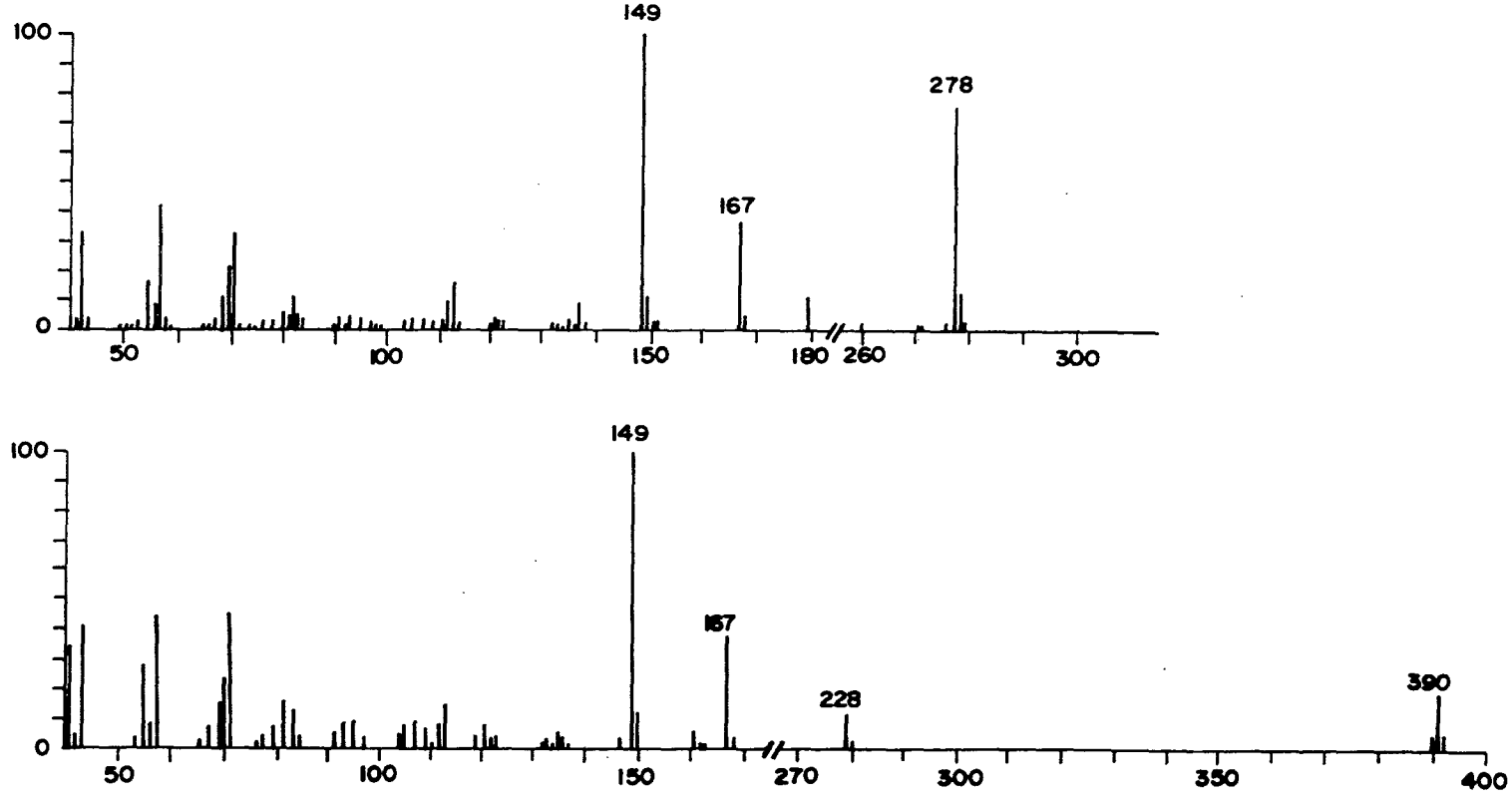


FIG.-4 MASS SPECTRA OF PHTHALATE ESTERS ISOLATED FROM STOECHOSPERMUM MARGINATUM

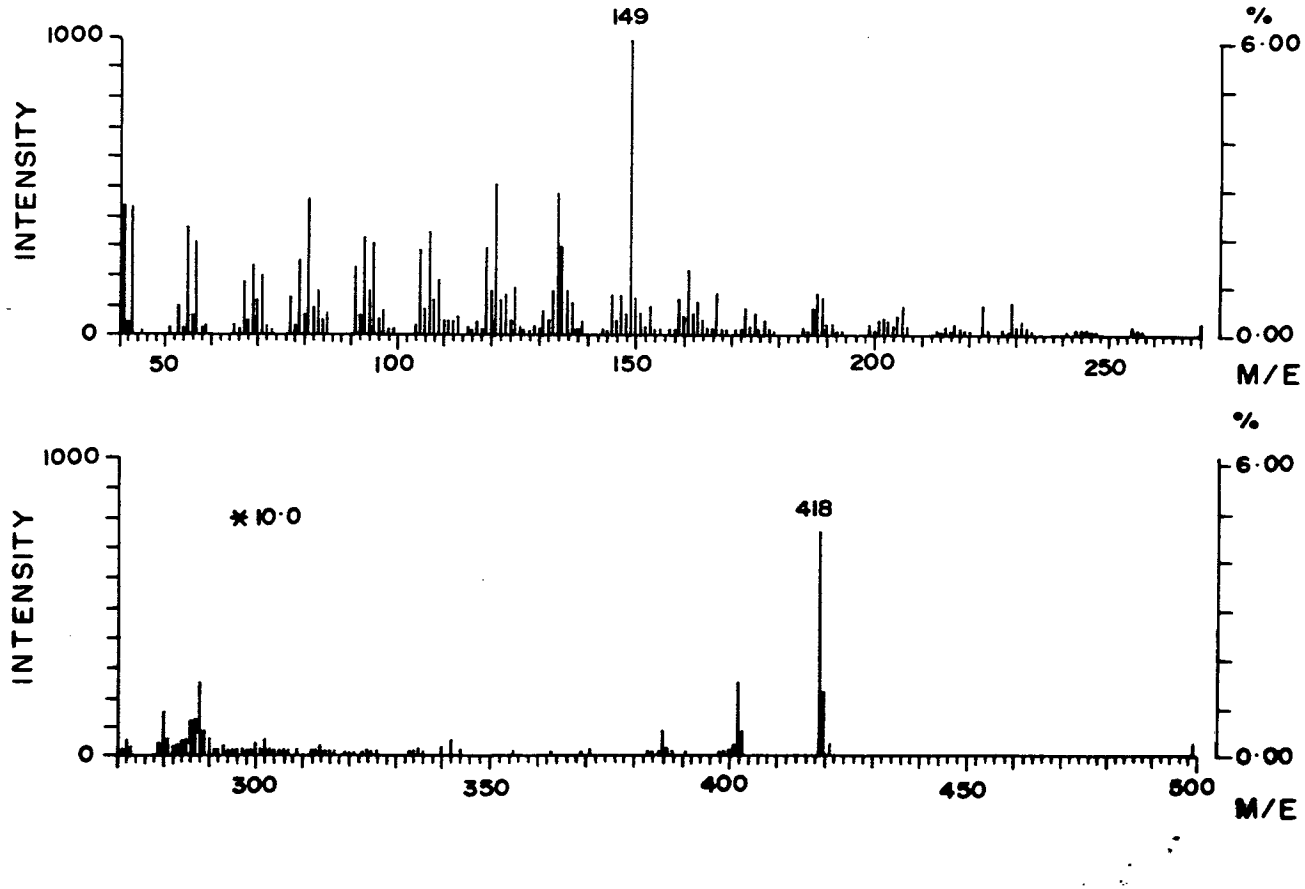


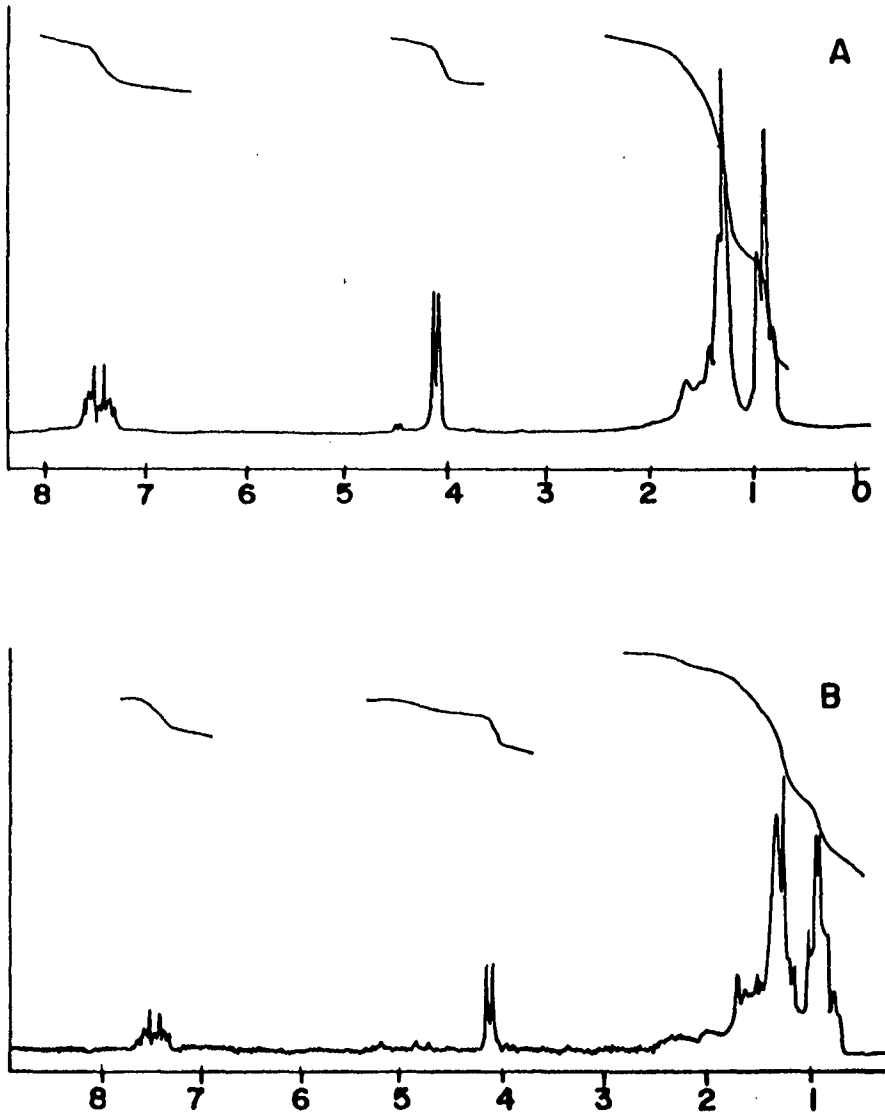
FIG.-5 MASS SPECTRUM OF PHTHALAE ESTERS ISOLATED FROM STOECHOSPERMUM MARGINATUM

Similarly mass spectrum of compound B exhibited molecular ion peak at m/e 390 followed by two prominent peaks at m/e 278 and 167 arising by the sequential loss of alcohol residue. This was confirmed by the presence of counter fragments at m/e 112 and 113 corresponding to C_8H_{16} and C_8H_{17} respectively. The structure of side chain could readily be deduced as 2-methyl-n-heptyl on the basis of the peaks at m/e 113, 85, 71, 57 and 43 as shown in Scheme 1. Hence on the basis of foregoing evidences structure of compound B has been established as phthalic acid bis(2-methyl-n-heptyl)-ester and that of compound A as dibutylphthalate.

Due to paucity of material, only the mass spectrum of compound C could be obtained. It has been identified as phthalate ester on the basis of base peak at m/e 149. Besides, the base peak, its mass gave a molecular ion peak at M^+ 418, (Fig. 5). It also showed peak at m/e 167, typical of phthalate ion, further confirming it to be a phthalate ester. Based on the mass spectrum, it has been tentatively identified as dioctyl phthalate.

There are numerous reports in the literature of the occurence of phthalate esters in micro-organisms, plants and animals[13] but regarding its occurence in marine algae there is a solitary reference[14] on the presence of bis-2-ethyl hexyl in the red alga *Ceramium rubrum* and very recently there is a report[15] of dimethyl terephthalate pollution in the three red algae *Phyllophora nervosa*, *Acantophora delilei* and *Hypnea musciformis*.

How these substances get incorporated into the algae or what is the genesis of these compounds is not known but certainly they must have been taken up from the aquatic environment where they are known to get accumulated either from industrial wastes, as decomposition products of certain algae like *Macrosystis aeruginosa* which is known to give bis(2-methyl propyl)-o-phthalate or from dispersants which are chemicals used to disperse the oil spills, and these esters are used as solvent for the emulsifiers.



A,B HNMR SPECTRA OF PHTHALATE ESTERS ISOLATED FROM STOECHOSPERMUM MARGINATUM

Fig.-6

Fucosterol

White solid, m.p. 124°C, gave positive Liebermann Burchard test; acetate m.p. 118°C analysed for $C_{29}H_{48}O$, M^+ 412, and its IR and PMR spectra showed the following bands: IR- 3450 and 1035 cm^{-1} (-OH), 1350cm^{-1} and 1370cm^{-1} (isopropyl group), 790 and 830 cm^{-1} (^{5} bond in sterols) and 810 (H atom on a trisubstituted double bond); NMR bands at 5.3(m, C_6-H); 0.66(s,3H,C_{18}); 0.9(s,3H,C_{19}); 1.59(d,6H,3H,C_{29}); 5.1(1H,J=6Hz,9), 2.2(1H,allylic,C_{25}); 0.95(d,6H,>C<); 3.4(C_3 , 1H,m); mass m/e 412, (M^+), base peak at 314 and triplet at m/e 299, 300 and 301 (indicative of $^{24(28)}$ double bond). These are well in agreement with the data reported for fucosterol,[16] and hence it was characterized as fucosterol.

The mass spectrum of fucosterol also showed the presence of dihydrofucosterol 414 (M^+). Fucosterol, a major sterol of S. marginatum, has been reported to be non-toxic and has the ability to reduce blood cholesterol level.[17] It has also been known to induce sexual reproduction in the Ascomycetes fungus, **Phytopthora cactorum.**[18] This steroid may become significantly important as a base material for the manufacture of sex hormones and for steroid synthesis.[19]

24-ketocholesterol

IR bands at 1710 cm^{-1} (>C=O), m/e at 400 (M^+), 43(base peak), 382, 314, 271, 255, 213, 159, 145, 133, 119 and 107, mass spectral data agreed with the data reported for 24-ketocholesterol.[20] It is possible that this steroid may be an artifact as reported by Knights,[21] since the sample was air-dried prior to extraction and is present in trace quantities.

This is the first report of the isolation of three steroidal constituents from *S. marginatum*.

Glacial acetic acid eluate of the column yielded a mixture of fatty acids (2.8g). The esterified acid mixture (diazomethane) on comparative GC analysis, (Fig. 7), with authentic samples (relative retention times) indicated the presence of myristic (30.6%), palmitic (60%), stearic (1.6%), oleic (0.097%) and heptadecanoic (0.132%) acids as their esters.

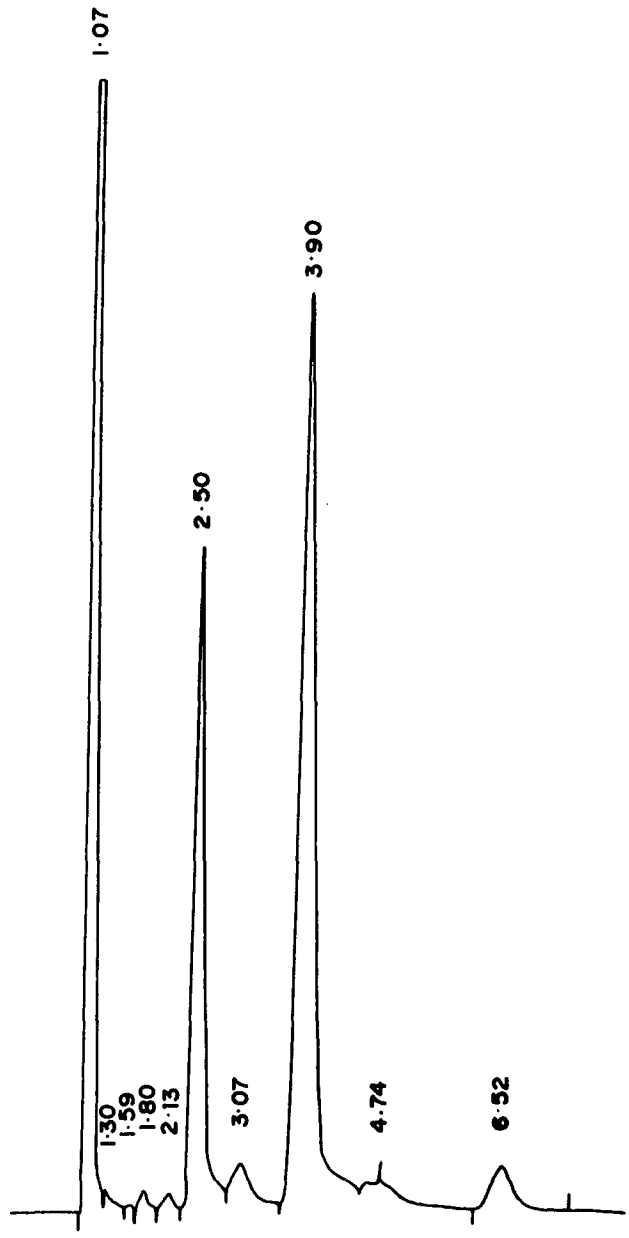


FIG.-7 GC CHROMATOGRAM OF ESTERIFIED ACID MIXTURE FROM STOECHOSPERMUM MARGINATUM

EXPERIMENTAL

Stoechospermum marginatum, 4Kg., a brown seaweed, was collected in April at Anjuna coast, air dried at room temperature after washing with running water and powdered. The dried, powdered alga was extracted thrice with methylene chloride, each extraction lasting three days. The combined extracts were evaporated at reduced pressure at 50°C to leave a dark green viscous oil (70gms). The crude extract was freed from pigments by passing it over a column of alumina in benzene, 500ml fractions were collected using the following elution scheme: benzene, ethyl acetate, methanol and glacial acetic acid. Benzene eluate was further chromatographed as a concentrated petroleum ether solution on a column containing silica gel in petroleum ether, 20ml fractions were collected employing gradient elution with a mixture of petroleum ether : ethyl acetate; fractions exhibiting similar TLC profiles were combined. Fractions eluted with petroleum ether and 1% ethyl acetate in petroleum ether were found to contain phthalate esters besides ethyl palmitate. They were purified by repeated chromatography over silica gel. Phthalate esters were characterized and designated as A, B and C.

Further elution of the column gave fucosterol, dihydrofucosterol and 24-ketocholesterol.

The fatty acids obtained from glacial acetic acid eluate were esterified and subjected to GC analysis. A Hewlett Packard Model 5840A gas chromatograph, equipped with flame ionisation detector and containing 6ft x 1/8" stainless steel column packed with 10% silar on 100-200 mesh chromosorb WHP maintained at 200°C was used for analysis.

Esterification of fatty acids

The cooled acidic fraction (2.8g) in dry ether was esterified with an ethereal solution of diazomethane in small portions until gas evolution ceases and the solution acquires a pale yellow colour. The coloured solution was tested for excess of diazomethane by removing a few drops into a test tube and introducing into it a glass rod moistened with glacial acetic

acid. Immediate evolution of gas indicated the presence of excess of diazomethane. Removal of the solvent gave a mixture of esters (2.51g), which was subjected to GC analysis.

Ethyl palmitate

IR spectrum (liquid film, Fig. 1)

It showed bands at 2900, 2840, 2320, 1730, 1460, 1360, 1240, 1165, 1110, 1030 and 715 cm^{-1}.

1H NMR spectrum ($CDCl_3$, 90MHz, Fig. 2)

0.75(3H,t); 1.2(20H,m); 4.0(4H,dd); 3.5(3H) and 2.15(t,6H).

Mass spectrum (70 eV, Fig. 3)

m/e 284, 270, 256, 242, 238, 210, 198, 184, 170, 156, 142, 138, 100, 88, 74, 55 and 43.

Phthalate esters

Mass spectrum of A (70 eV, Fig. 4)

m/e 278, 179, 167, 149, 113, 70, 69, 68, 56, 54 and 43.

Mass spectrum of B (70 eV, Fig. 4)

m/e 390, 228, 167, 149, 113, 112, 71, 57, 55 and 43.

Mass spectrum of C (70 eV, Fig. 5)

m/e 418, 402, 386, 229, 167, 149, 134, 121, 107, 95, 93, 81, 59, 57, 55 and 43.

1H NMR spectrum of A (90MHz, $CDCl_3$, Fig. 6)

7.5(4H,m); 0.89(6H); 1.08-1.0(8H,m); 4.12(4H,d,J=6Hz).

1H NMR spectrum of B (90MHz, $CDCl_3$, Fig.6)

0.9(12H); 1.1-2.3(8H); 4.1(4H,d,J=6Hz).

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SECTION III

OXYTOCIC PRINCIPLE OF THE RED ALGA *AMPHIROA FRAGILISSIMA*

Amphiroa fragilissima (LINNAEUS) Lamouroux, a red alga belonging to the class Florideophyceae, order Cryptonemiales and family Corallinaceae is the only calcified alga growing in the lowest mid-littoral zone along the Anjuna coast in Goa (West coast of India) besides *Cheilosporum spectabile, Jania rubens* and *Padina gymnospora.* It occurs in the shallow rock pools throughout the year and is associated with the zoanthid, *Gemmaria sp.* This alga has been reported to be densely associated with the animal community, the density being partly due to its high adsorption coefficient.[1] Literature survey indicated that no chemical work has been done on this marine alga except on its halogen* content[2,3] and few reports on it being an efficient producer of calcium carbonate.[4]

The broad based screening results given in section II, chapter I, have indicated that the crude alcoholic extract of this calcareous alga exhibited oxytocic, spasmogenic and antiviral activities. The follow up investigations located the oxytocic activity in the water soluble fraction. The isolation and identification of the oxytocic principle from the active water soluble fraction has been dealt with in this section.

Table 1: Relative **in vitro** potency of *Amphiroa fragilissima* in comparison to oxytocin in rat uterus.

Amphiroa fragilissima	Dose ug/ml	Rat uterus Own response mm	Oxytocin contraction (0.0002ug/ml)	
Crude extract	10	0	45	
	50	38	45	
CH_2cl_2 and	10	0	40	
EtOAc insoluble fr.	50	33	42	
Fraction enriched	0.01	30	34)	0.001ug/ml
with substance	0.1	40	34)	
same RF as histamine				

* In this publication the alga was identified as *Corallina sp.*

Table 1 shows the activity observed in the crude extract and fractions. It is evident from the table that the contractions produced by the substance on rat uterus are not concentration dependant, indicating that the oxytocic agent was histamine or histamine-like substance as this imidazole compound is known to produce concentration independent uterine contraction.[5,6]

As evident from the pharmacological experiments, a histamine like substance was suspected in the active water soluble fraction, which was subjected to extensive column chromatography over florisil using increasing concentrations of ammonia in ethanol as eluant. Fractions having the same TLC profile with Pauly's reagent[7] as the detecting agent were combined. This afforded a substance having a m.p. of 240°C, yield 23mg.

The ^{1}HNMR (D_2O,90MHz)(Fig.1) showed a complex multiplet at 3.54 corresponding to two $-(CH_2)$ groups of side chain and singlets at 7.55 and 8.53 corresponding to 1H each (imidazole ring protons). The mass spectrum (Fig.2) exhibited base peak at m/e 81 and M^+ at 183 besides major fragments at m/e 54, 80, 111, 122 and 149. Its picrate had a m.p. of 234-36° (lit. 238-242). On the basis of this spectral data and physical constant it was identified as histamine and its identity confirmed by determination of mixed m.p. with authentic sample.

It has been observed that the occurence of this biogenic amine in the alga is seasonal. *Amphiroa fragilissima* grows on *Gemmaria sp.*, a zoanthid, the water soluble fraction of which exhibited hypotensive activity.[8] Follow up investigations located the activity in the water soluble fraction and was found to be due to the presence of histamine. The zoanthid contains this amine when it is absent in the alga and is devoid of it when present in the seaweed. This observation indicated that there might exist some symbiotic relationship between these two marine organisms but it is not clear whether the histamine isolated is an algal metabolite

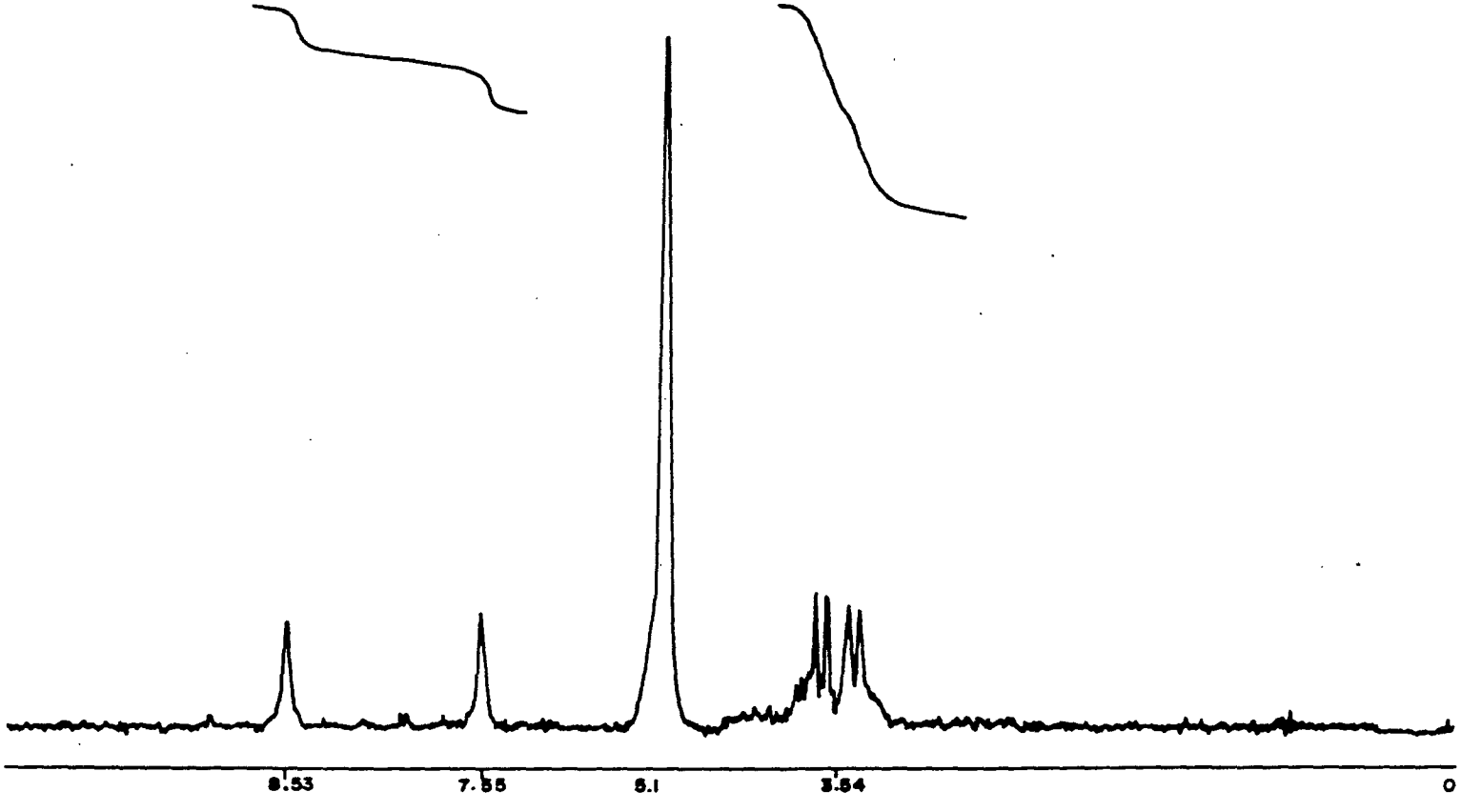

Fig. 1 ^{1}HNMR SPECTRUM OF HISTAMINE DIHYDROCHLORIDE

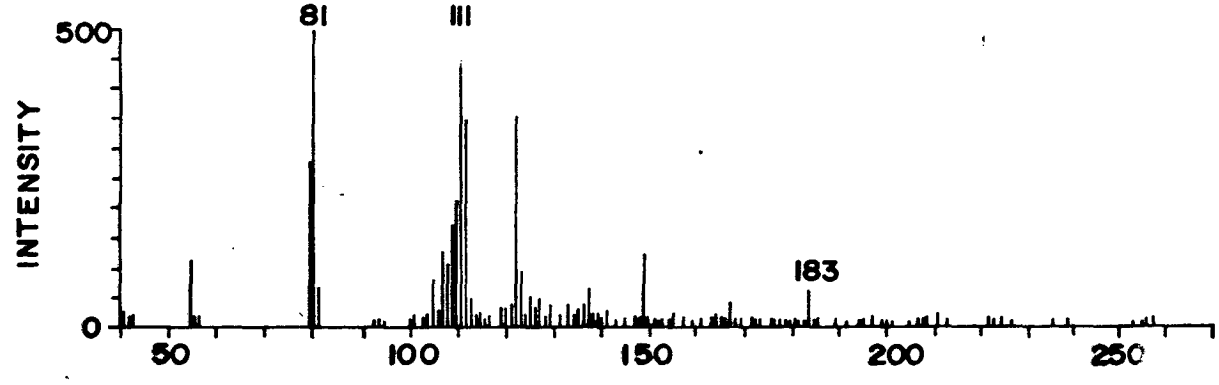

FIG.-2 MASS SPECTRUM OF HISTAMINE DIHYDROCHLORIDE FROM AMPHIROA FRAGILISSIMA

or has been originated from the zoanthid. Even though TLC of the acid mixture indicated the presence of four more compounds possessing a ß-imidazole nucleus, these metabolites could be transformation products of the histamine coming from the zoanthid. The presence of histamine in the red alga *Furcellaria lumbricallis* has also been reported recently by Barwell.[9]

EXPERIMENTAL

Collection and preparation of the extract

The fresh algal specimens (3Kg dry wt.) were hand picked from the intertidal zone of Anjuna beach (West Coast of India), washed, air dried and extracted thrice by immersion in absolute ethanol. After filtration the alcoholic extract was concentrated on a rotary evaporator at 50°C until all the solvent was removed. This crude extract obtained from it by sequential extraction with methylene chloride, ethyl acetate and water were tested for oxytocic activity.

The crude extract and its fractions were tested on isolated rat uterus using the method of Mehrotra *et al.*[5]

Testing for oxytocic activity

Female adult (140-200g) virgin rats maintained under uniform husbandary conditions (temp. 25 ± 1°C) and irrespective of physiological states of the reproductive cycle were pretreated 24 hrs. earlier with a 0.1mg diethyl stiboestrol intramuscularly. The animals were stunned by a blow on the head, laparotomized and the complete uterus was immediately removed. One of the uterine horns of freshly killed rat was suspended in an organ bath containing de Jalon's solution at 35°C aerated with carbogen (95% O_2 + 5% CO_2). Isotonic contractions were recorded through a frontal writing lever (1g tension, 5-10 fold magnification) on a moving Kymograph. Solutions of different concentrations of the testing crude extract and fractions were prepared in either distilled water or 50% ethanol depending on the solubility. Standard contractions were produced with oxytocin. Test substances were added to the bath in different doses and allowed to act for 5 minutes to produce contraction. The methylene chloride and ethyl acetate fractions were discarded as they did not show any activity.

Isolation of Active Principle

The TLC analysis of the active fraction in ammonia : acetonitrile(10:90) using Pauly's reagent[7] (diazo-benzo-sulfonic acid) as the detecting agent indicated the presence of compounds with a ß-imidazole nucleus one of which had the same RF value as histamine. This active fraction after concentration under reduced pressure on a rotary evaporator, was evaporated to dryness in a vaccum descicator over potassium hydroxide pellets and then chromatographed over florisil. The column was developed with increasing concentrations of ammonia in ethanol. Column effluence was monitored by TLC. Material eluted with 4% ammonia in ethanol contained, as a major constituent, the substance having the same RF value as histamine. It was tested again for the observed activity. Further purification of this histamine-like substance was carried out by repeated chromatography over florisil and then subjected to spectral analysis.

^{1}HNMR spectrum in D_2O, 90MHz (Fig.1)

3.53(4H,m); 7.55(1H,s); 8.53(1H,s)

Mass spectrum 70eV (Fig.2)

M^+ 183 and major fragments at m/e 149, 122, 111, 81(100%), 80 and 54.

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SUMMARY

SUMMARY

The thesis deals mainly with the chemistry of marine natural products isolated from the marine algae : *Acantophora spicifera, Stoechospermum marginatum, Amphiroa fragilissima* and the mangrove plant *Acanthus illicifolius*. These plants were selected on the basis of biological screening results of marine algae collected along the Indian coast. The biological evaluation of the marine flora has also been included in the thesis.

Besides a number of known compounds (acids, esters, steroids and oxazoles), some novel steroids, dipeptides and dimer have been identified from these marine sources.

A comprehensive survey of marine prostanoids has been presented, emphasis being laid on their isolation, chemistry and their probable biogenesis.