Isolation and Characterization of Petrosin from *Xestospongia sp.*, a sponge from the Read Sea.

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The School of Graduate Studies
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The Requirements for the Degree of
Master of Science in Chemistry

By
Paulos Barbe
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ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

The Isolation and Characterization of Petrosin From *Xestospongia* Species, a Sponge From The Red Sea

By

Paulos Barbe

Approved by:

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External Examiner

Dr. Berhanu Abegaz
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I would like to acknowledge Professor Kashman and his group, Tel Aviv University, Israel for generating spectral data. I am very greateful to Dr. Berhanu Abegaz for providing his invaluable advice during the preparation for the defense of thesis. I am also thankful to Ato Aron Uqbeab for collecting the sponge sample.

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Abstract

Isolation and Characterization

of

Petrosin from Xestospongia sp.,

a Sponge from the Red Sea

by Paulos Barbe

Advisor: Dr. Tarekegn Gebreyesus

The Sponge Xestospongia sp. from the Red Sea has been chemically investigated. The chloroform soluble portion of the ethanolic extract of the sponge yielded P-51, a minor metabolite which could not be identified, and P-46.

P-46 was obtained from the chloroform soluble portion of the ethanolic extract by repeated VLC and CC on silica gel; recrystallization yielded colorless rod-shaped crystals (0.002%)

P-46 was identified as petrosin on the basis of spectral studies including IR, EIMS, ^{13}C NMR, HMQC, one and 2D-^{1}H NMR as well as by comparison of its spectral and physical data with those reported for petrosin. The newly generated HMQC data confirmed the previously assigned structure of petrosin unequivocally.

Isolation and Characterization of Petrosin from Xestospongia sp., a sponge from the Red Sea.

I. Introduction

Sponges are primitive multicellular invertebrates belonging to the phylum porifera. They are mainly marine, but a few live in the fresh water. In marine habitats, sponges are among the most abundant invertebrates which make an important contribution to the biomass[1].

All Sponges are sessile and incapable of locomotion in the adult stage, living fastened to rocks, shells and other objects. The basic structure comprises a collection of cells which enclose a system of canals and chambers which open to the exterior by small pores[2].

Sponges were considered to be plants until about the mid 19th century when the last skeptics were finally convinced of the true animal nature of sponges[3]. Sponges, being sessile organisms, feed by extracting food from streams of water that they draw in through pores. They have world wide distribution and are found in a greatly diversity of habitats. The species vary grate in shape, size, structure and geographical distribution[2,4].

Classification of sponges presents great difficulties due to their highly variable characters and intermediate forms. The classification is based largely on skeletal structures. Sponges are usually divided into four classes[4].

1. Calcarea
2. Hyalospongea
3. Demospongea
4. Sclerospongea

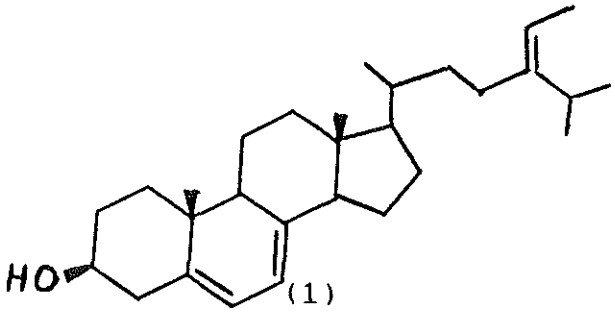
Over the last two decades, considerable chemical interest in these marine invertebrates has become evident. It has been shown that marine sponges are fertile sources of secondary metabolites with diverse and novel structures. Some of these compounds have shown to possess bioactivities, and can be a promising source of pharmaceuticals and agrochemicals for the future[5-7].

In the marine ecosystem, tolerance of sponges to the heavy pressure of predators is assumed to be not only due to the physical protection but to a well-developed chemical defense adaptation. It is believed that chemical defense should by far be the commonest mode of defense found among sponges. A great number of marine sponges produce bioactive secondary metabolites that serve to discourage attack of mobile predators, to kill potential fouling or contaminating organisms, or perhaps to prevent competitors from intruding into the living space of sponges[8-9].

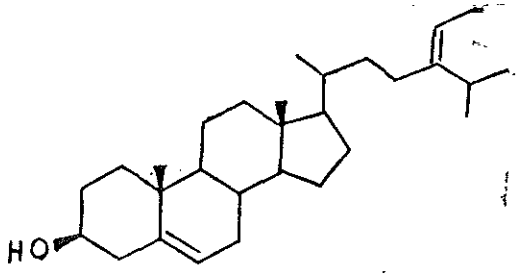
Despite the toxic chemicals sponges produce, a few highly specialized fishes and nudibranch mollusks select them as their main diet. The nudibranch mollusks feed on sponges, thereby storing sponge metabolites to use them for their own defense against predators. The presence of the same secondary metabolites in both nudibranchs and certain sponges is taken as evidence of a predator-prey relationship[9].

Not all secondary metabolites so far reported from sponges are believed to be true metabolites of sponges. Bacteria and other microorganisms present in marine sponges as endosymbionts are suspected to be the source of some of the metabolites isolated from the entire sponge assemblage. Therefore, some of the metabolites have been attributed to symbiotic organisms whereas the majority are considered to be true sponge metabolites[9-12].

One such example is the tracing of the true origin of(24Z) - Stigmata-5,7, 24(28)-trien-3β-01(1) isolated from Dysidea herbacea[12]. This sponge contains a large mass of blue green algae as endosymbionts which may be the source of this sterol (1). Nes et al[13] demonstrated that the cultured ciliated protozoan Tetrahymena pyriforms can biosynthesize 1 from isofucostrol (2) when 2 was added to the culture medium. On the other hand, sterols of the type Δ^{5}-, $\Delta^{5,7}$-, Δ^{7}-29- are common to blue green algae but are rare in sponges. Therefore, these results were taken as evidence that the true origin of 1 is largely the blue green algae, the endosymbiont of the sponge Dysidea herbacea.



(1)



(2)

The Red sea is a unique environment which is characterized by a highly diversified, rich and varying fauna and flora at the reef- covered coasts. Salinity and temperature of the water are higher than in other seas of the world. Sponges are among the few marine invertebrates that are largely distributed in the Red Sea and that contribute significantly to the biomass of the marine ecosystem[1,14].

Early investigations were limited to taxonomic classification of Red Sea invertebrates[1]. It is only recently that investigation of secondary metabolites of the Red Sea invertebrates has begun at the northern tip of the Red Sea by Professor Kashman's group[15-20]. A wide variety of invertebrates live in the Red Sea habitat. Some are endemic to the Red Sea. Because of the limited investigation of the marine life in the Red Sea as well as the uniqueness of that body of water a project to study the sponges of the Red Sea was initiated three years back.

In the course of this research work, the chemical investigation of a Red Sea sponge of the genus Xestospongia has been undertaken. The objective of the project was the isolation and identification of non-steroidal compounds. The Xestospongia sp has yielded several fractions. Further purification by chromatographic techniques led to the isolation of P-46 and P-51. P-46 has been characterized based on spectroscopic data. Work is in progress to identify P-51 which has been isolated in very minute quantities.

II. Literature background

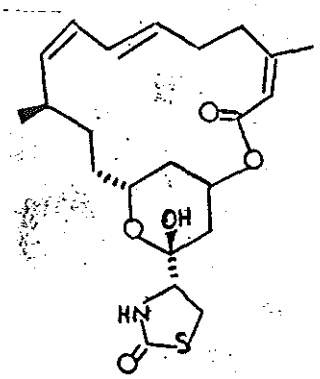
2. Marine sponge metabolites

2.1 Bioactive principles from marine sponges

Chemical defense is believed to be an efficient defensive mechanism of sponge, particularly in those sponges without spicules, from mobile predators and space competitors[8]. Thus sponges frequently produce large quantities of bioactive secondary metabolites that are thought to deter potential predators and inhibit the growth of fouling organisms. In recent years, several authors have reported the isolation of metabolites from sponges that are responsible for toxicity or anti-feeding activity to fish or act as inhibitors to other forms of marine life[21-22].

Latrunculin- A (3) and -B (4) are potent ichthyotoxic compounds isolated from Latrunculin magnifica[23]. Even when the sponge is squeezed into an aquarium, it causes poisoning and death of the fish within a short time.

Moreover, several sponges have been identified to yield ichthyotoxic secondary metabolites. Studies have shown that extracts of most of the tropical sponges so far tested are toxic to fish[24].



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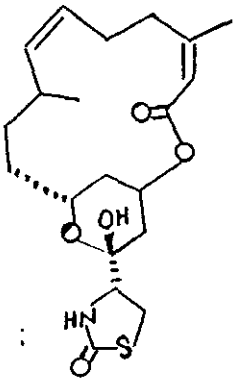
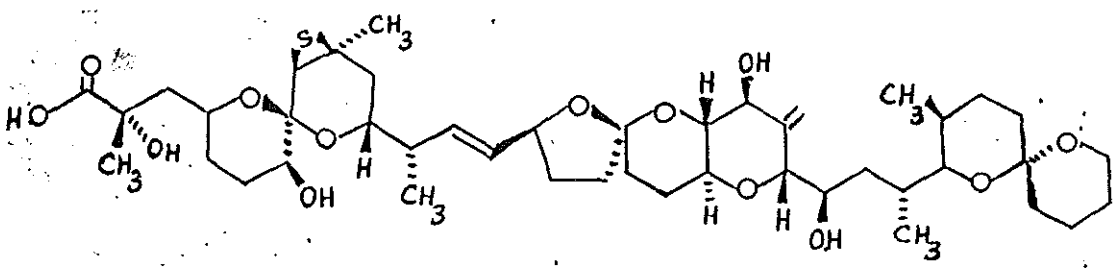


(4)

Extracts of many sponges show antibacterial and/or antifungal activities. As a consequence, a large number of novel antimicrobial metabolites have been isolated from marine sponges[21-22,25]. Schmitz et al have reported the isolation of a novel polyether antibiotic, acantifolicin (5), from the sponge Pandaros acanthifolium. Although acanthifolicin (5) is also a polyether, the structure differs from those of other known polyether antibiotics mainly by the incorporation of an episulfide group[26].



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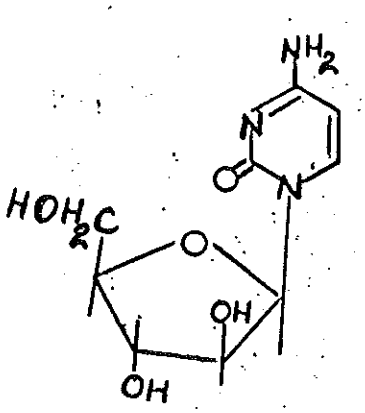
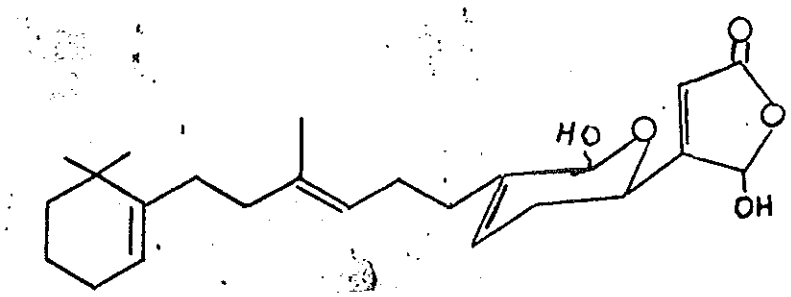


(8)

Studies of pharmacological and agronomical agents from marine organisms have become a major area of research endeavor in recent years. Indeed, many marine natural products are being screened for their pharmacological or agrochemical activities[29-31].

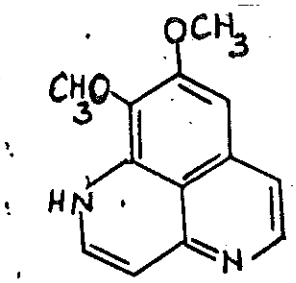
Jacobs et al[32] have identified novel biological activities for seventy compounds from a large pool of pure marine natural products. Studies have been conducted on these bioactive marine natural products in order to explore and extend knowledge on their pharmacology and mechanism of action. Manoalide (9) is one of three compounds whose study has reached an advanced stage among seventy selected compounds. Manoalide (9), a sesterterpenoid originally isolated from the sponge Laffariella variabilis[33], exhibited anti-inflammatory effect and inhibits irreversibly the enzyme phospholipase A_2 .



(9)

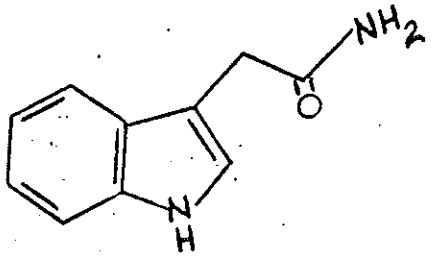
Another example of a pure marine natural product that has got interesting pharmacology is aaptamine (10), an alkaloid isolated from the sponge Aaptos aaptos. It has -adrenoceptor blocking activity. The pharmacology of

aaptamine (10) was investigated and revealed it to be an important agent both clinically and as a pharmacological probe[34].



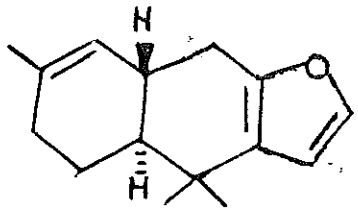
(10)

Sponges resisting overgrowth by space competitors are suspected to produce secondary metabolites with plant growth regulatory activity. Indeed, assays have shown that some sponges contain metabolites exhibiting plant growth regulatory activity. For example, indole-3-acetamide (11) is a well known growth regulator obtained from the sponge Dysidea etheria[31,35].



(11)

In addition, Insecti-cidal and insect repellent compounds have been reported from marine sponges[31,36-37]. The most abundant sesquiterpenes from Dysidea etheria[38] were employed in tests against grasshoppers. As a result, furodysinin (12) was proved to be a potent and fast acting toxin of all marine natural products tested so far on grasshoppers[31,39].



(12)

It is evident that marine Organism have been sources of a variety of bioactive compounds. As a consequence, research on marine natural products has grown fast in many fields such as ecology, pharmacology and agrochemistry. These multi-directional investigations would enable man to exploit marine natural products as a new source of economically invaluable compounds for the future[30-31,40-41].

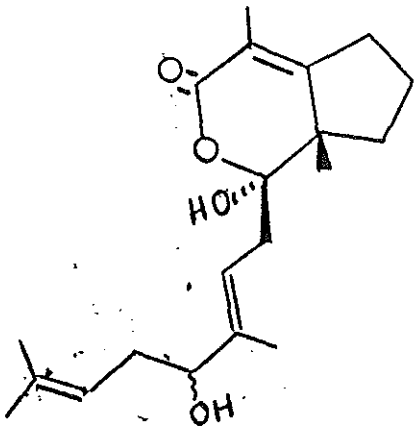
2.2 Secondary Metabolites of the genus Xestospongia

The sponges of the genus Xestospongia have been studied for the past several years and a number of interesting secondary metabolites have been reported. Many of the metabolites exhibit diverse biological activities including cytotoxicity, antibacterial, fungicidal, coronory vasodilative and cell division inhibition[42-45].

The sponges of the genus Xestospongia that have been studied so far include X.testudinaria[46], X.muta[47-48], X.exigua[44-45], X.caycedoi[37], X.sepra[49-50] X.vanilla[51-53], X.wiedenmayer[54], and eight unidentified ones[42-43,55-59]. The metabolites isolated include polyacetylenic compounds, pentacyclic polyketides, macrocyclic bis (1-oxaquinolizidines) and β-carboline alkaloids, heterocyclic peroxides, phospholipid fatty acids and sterols with unusual side-chain alkylation.

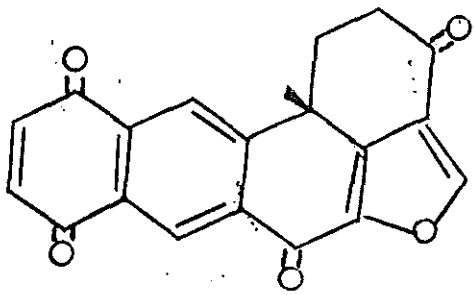
Polyacetylenes were known only as constituents of plants until the initiation of chemical investigations on marine organisms[17]. Since then, a large number of polyacetylenes were isolated from marine organisms, particularly, sponges. At present polyacetylenes are encountered commonly in the chemical investigations of sponges. A number of polyacetylenic compounds have been reported from the genus Xestospongia; many have shown antibacterial activities[43].

Although terpenes are the most abundant secondary metabolites of sponges, only a few compounds of this class were known to Xestospongia. For example, Xestolide (13) is a degraded triterpenoid isolated from the sponge Xestospongia vinilla[52].



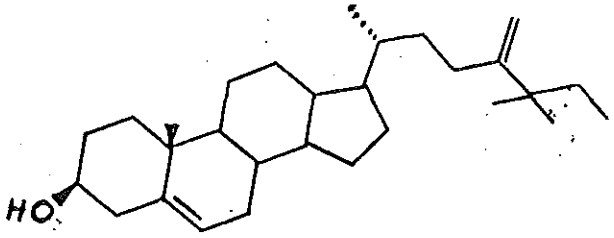
(13)

Xestospongia, however, is a source of very uncommon quinone and hydroquinone metabolites. Halenaquinone (14), isolated from Xestospongia exigua, not only is a rare polyketide secondary metabolite but also represents a new pentacyclic system. Halenaquinone (14) possesses in vivo antibiotic activity against Staphylococcus aureus and Bacillus subtilis[44].



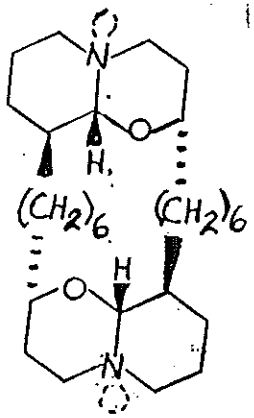
Sterols are the most frequently encountered natural products of marine sponges. They have been sources of numerous sterols possessing side chains with unusual alkylation patterns. Xestospongia was the first to yield sterols with quadruple methylated side chain. For

instance, Djerassi et al[48] isolated mutasterol (15) from Xestospongia muta. which was proved to be the first naturally occurring sterol with an acyclic quaternary carbon atom in the side chain.



(15)

The genus Xestospongia has also yielded macro cyclic and B- carboline alkaloids, and other nitrogen containing compounds[45,57-58]. These alkaloids and nitrogen containing compounds isolated from Xestospongia are indicated in Table 1. Xestospongin C (16) and its co-occurring seven quinolizidine alkaloids induce relaxation of blood vessels in vivo[45]. Whereas, petrosin (17), a related alkaloid isolated from the sponge Petrosia seriata[60] and more recently from a Xestospongia sp[61]., is a potent ichthyotoxin. A common biogenetic origin could link all these bis-quinolizidines and bis (1-oxaquinolizidine) alkaloids.



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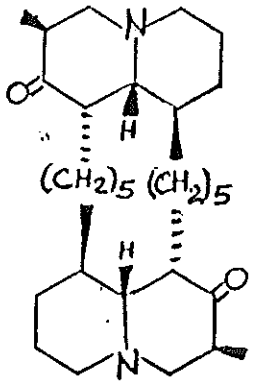


(17)

Table 1:

Metabolites from the Sponge Xestospongia and their biological activities

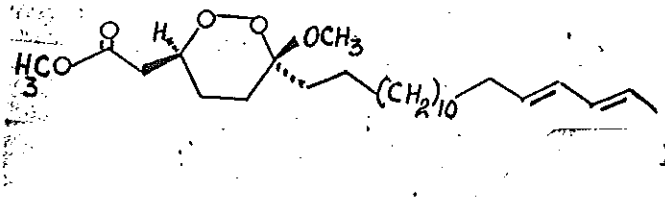
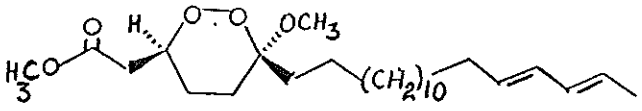
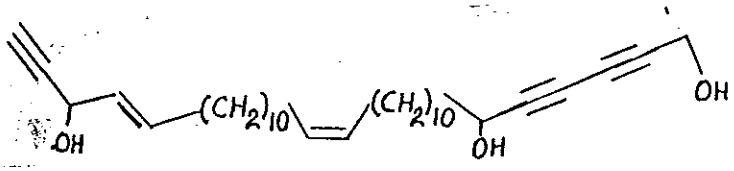
Metabolites	Source	References
	Xestospongiasp. (South pacific coral reef)	42
Xestin A (18) Potent cytotoxic and in vitro antitumor		
	Xestospongia sp. (South pacific coral reef)	42
Xestin B (19) Moderate activity (IC_{50}=3mg/ml) in vitor against P388 cells		
	Xestospongia sp. (Mele Bay, Vanvatus)	43
Melyne A (2o) Active against Giaridia		

Table 1:.....Continued

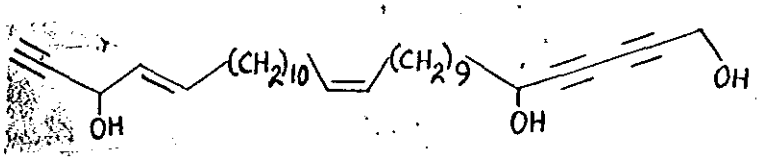
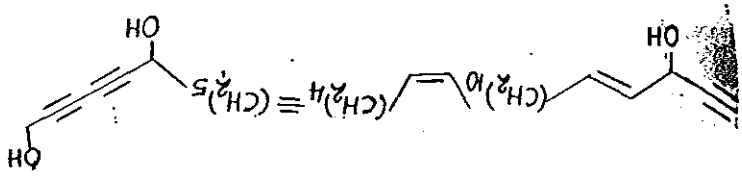
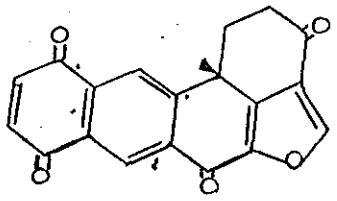
Metabolites	Source	References
 Melyne B (21) Active against bacteria	Xestospongia sp. (Melé Bay, Vanvatus)	43
 Melyne C (22)	Xestospongia sp. (Mele Bay, Vanvatus)	43
 Halenaquinone (14) in vivo antibiotic activities against Bacillus Subtilis and Staphylococcus avreus	Xestospongia exigua	44

Table 1:.....Continued

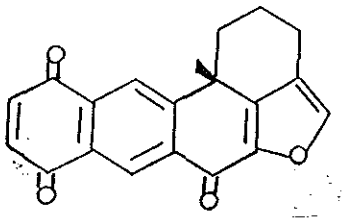
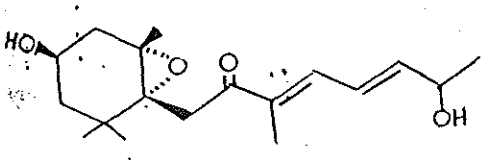
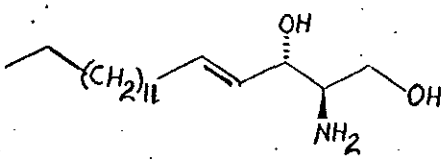
Metabolites	Source	References
 Xestoquinone (23) Cardiotonic	X.sepra	49
 (24) no reported bioactivity	X. vanilla	51
 (25) Exhibits activity against Candida albicans	Xestospongia sp. (Papua New Gunea)	57

Table1: .....Continued

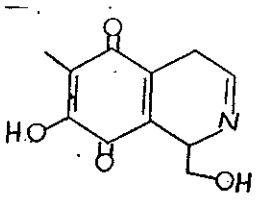
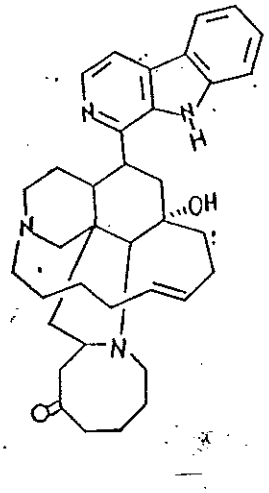
Metabolites	Source	References
	X.caycedoi	37
Renierol (26) Antibiotic activities against Staphylococcus aureus and mild cytotxicity against the L1210 cell lines		
	Xestospongia sp.	58
Manzamine E (27) Cytotoxic activity		

Table1:.....Continued

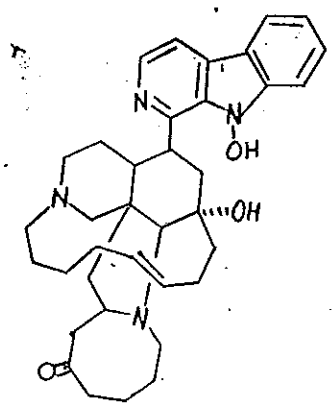
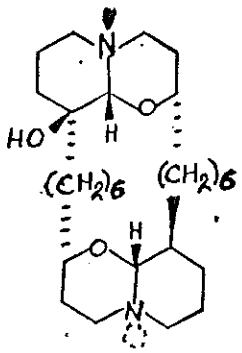
Metabolites	Source	References
 Manzamine F (28)	Xestospongia sp.	58
Cytotoxic activity		
 Xestospongin B (29) Vasodilative activity in vivo	X. exigua	45

Table 1:.....Continued

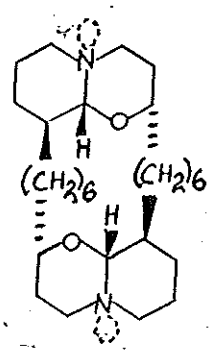
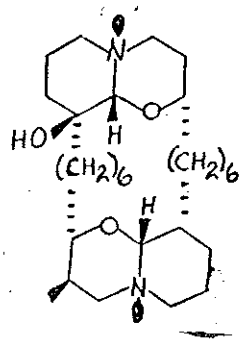
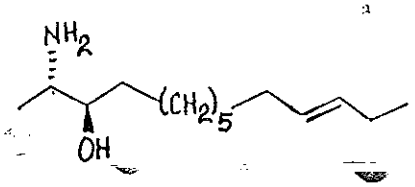
Metabolites	Source	References
 Xestospongin C (16) Vasodilative activity *in vivo*	*X. exigua*	45
 Xestospongin D (30) Vasodilative *in vivo*	*X. exigua*	45
 Xestoaminol A (31) Potent activities against parasites, microbes and, reverse transcriptase.	*Xestospongia sp.*	59

Table 1: .....Continued

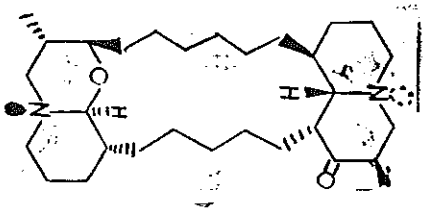
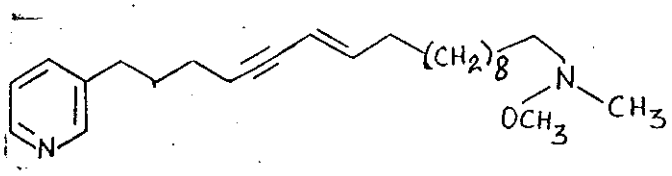
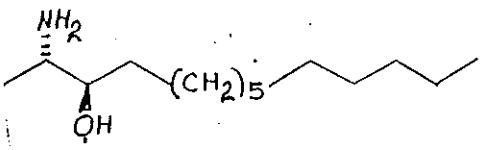
Metabolites	Source	Reference
 Aragupetorsin A (32) Possesses vasodilative activity <u>in vivo</u>	<u>Xestospongia sp.</u>	56
 Xestamine A (33)	<u>X. wiedeumayeri</u>	54
 Xestoaminol C (34) Active in assay against reverse transcriptase	<u>Xestospongia sp.</u>	59

Table 1: Continued

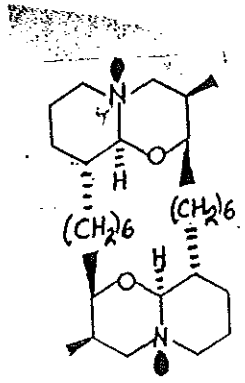
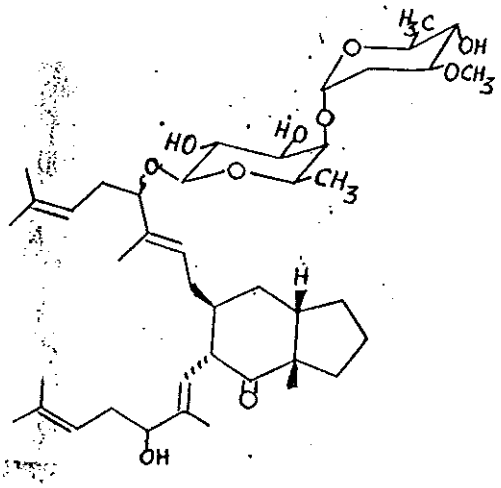
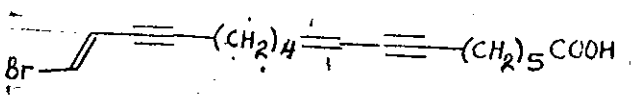
Metabolites	Source	Reference
	Xestospongia sp.	
Araguspongine -J (35) Vasodilative activity in vivo		
$BrCH=CH-CBr=CH-(CH_2)_4-CH=CH-C\equiv C(CH_2)_3CO_2H$ 36	Xestospongia muta	
Exhibits central nurvous system activity		

Table1: Continued

Metabolite	Source	Reference
	X.vanilla	53

Xestovinin A (37)

Exhibits antifungal activity against Pythium ultimum

	X.testudinaria	46

38

III. Results and Discussion

The sponge sample was collected in July 1989, from the Red Sea, around the Marine Biology Station of Asmara University at Massawa. The Sample was collected at a depth of 3-6 meters. A specimen was preserved in 10% formalin salt water solution. The sponge sample was identified as Xestospongia sp. by Mr. M. Ilan of Tel Aviv University, Israel. The sponge sample was sun-dried and stored in a deep freezer.

Extraction of the sponge (2kg) with 95% ethanol yielded a black gummy residue. The crude ethanol extract was partitioned between chloroform and water, and in turn the chloroform extract partitioned between pet. ether and 10% aqueous methanol. The 10% aqueous methanol extract was chromatographed repeatedly on VLC and CC to afford several fractions. Further purification led to the isolation of p-46 and p-51 as detailed in the experimental section.

Identification work of p-51 is in progress. p-46 was characterized by spectroscopic techniques.

Characterization of p-46

P-46 is quite soluble in moderately polar solvents such as chloroform and dichloromethane, but is sparingly soluble in acetone and methanol. P-46 was crystallized from acetone as colorless rod-shaped crystals, mp 221-222° C $[\alpha]_D^{20}=0$ (c= 0.015,$CHCl_3$). P-46 showed positive Dragendorff spray test. Rf 0.82 ($CHCl_3$/ethyl acetate/ methanol, 2:1:0.5).

The IR spectrum of P-46 (Fig.1) showed strong absorption band at 1710cm^{-1} due to a carbonyl group. The absence of absorptions above 3000 cm^{-1} suggests the absence of OH, NH and NH_2 groups in the molecule.

The Electron Ionization Mass Specturm (EIMS) of P-46 (Fig.2) showed a molecular ion peak at m/z 470. Two intense fragment ion peaks appear at m/z 455 (100%) and m/z 166.0 (40%). The relative intensities for the rest of the fragment ion peaks are very small (<10%). Based on intensities of isotopic peaks relative to the parent peak, about thirty carbon atoms may be deduced for P-46[62]. From the ^{13}C NHR spectra (both proton noise-decoupled, Fig. 4b and DEPT, Fig.5) the formula C_{15} H_{25} O could be deduced. If the number of carbons in $C_{15}H_{25}$ O is doubled, it will give the total number of carbons as thirty which was suggested based on intensities of the isotopic peaks. The displayed 15 signals for 30 carbons indicate the existence of an element of symmetry in P-46. This is in accordance with the observed optical activity.

NO. 007-1473

PERKIN-ELMER

CONCENTRATION Ca. = 50:1 mg

THICKNESS 0.1mm

PHASE KBr

REMARKS

Fig.1 IR Spectrum of P-46

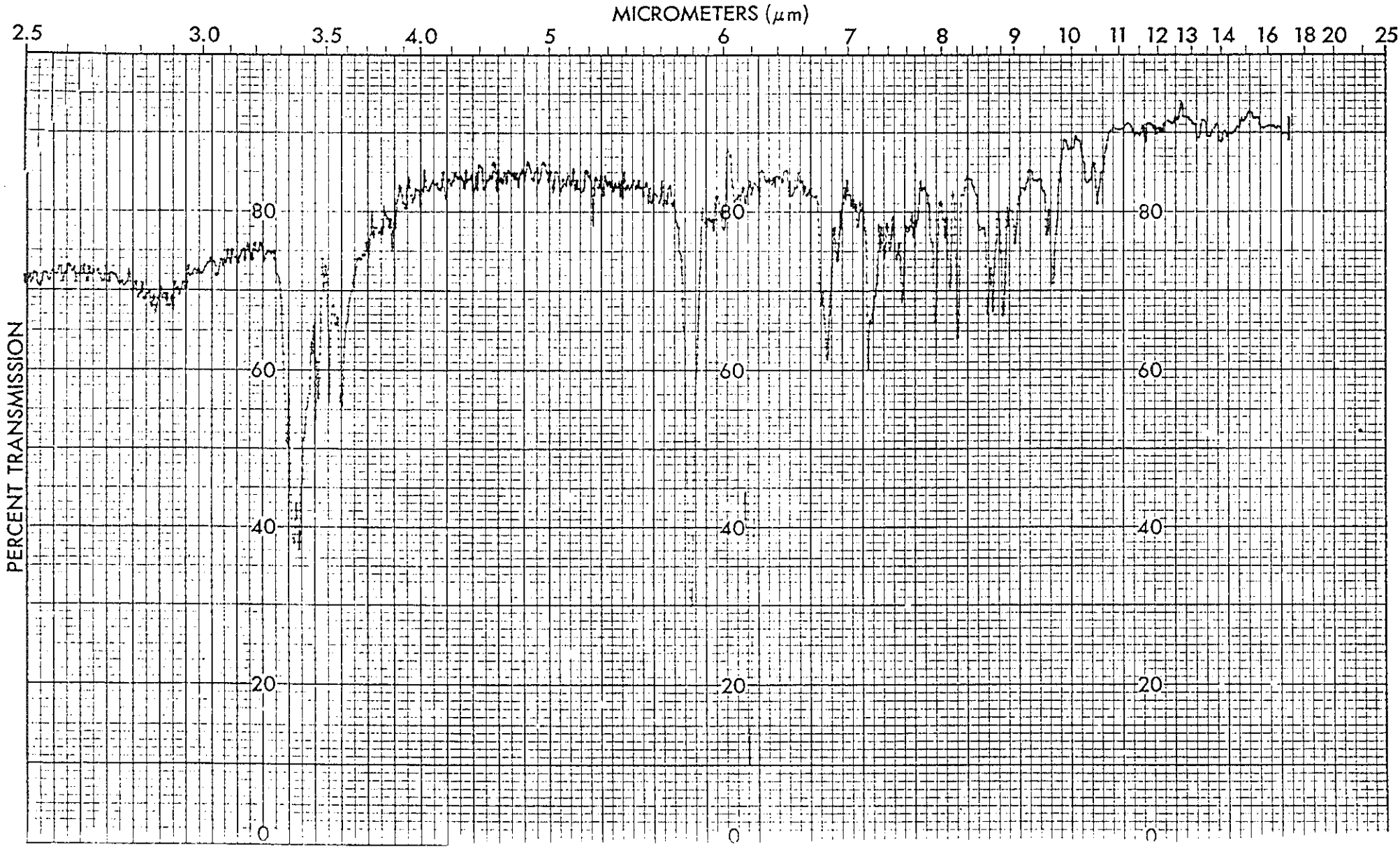
RESOLUTION

OPERATOR Gotschim A. DATE 9-03-91

SPECTRUM NO.

SAMPLE P-46

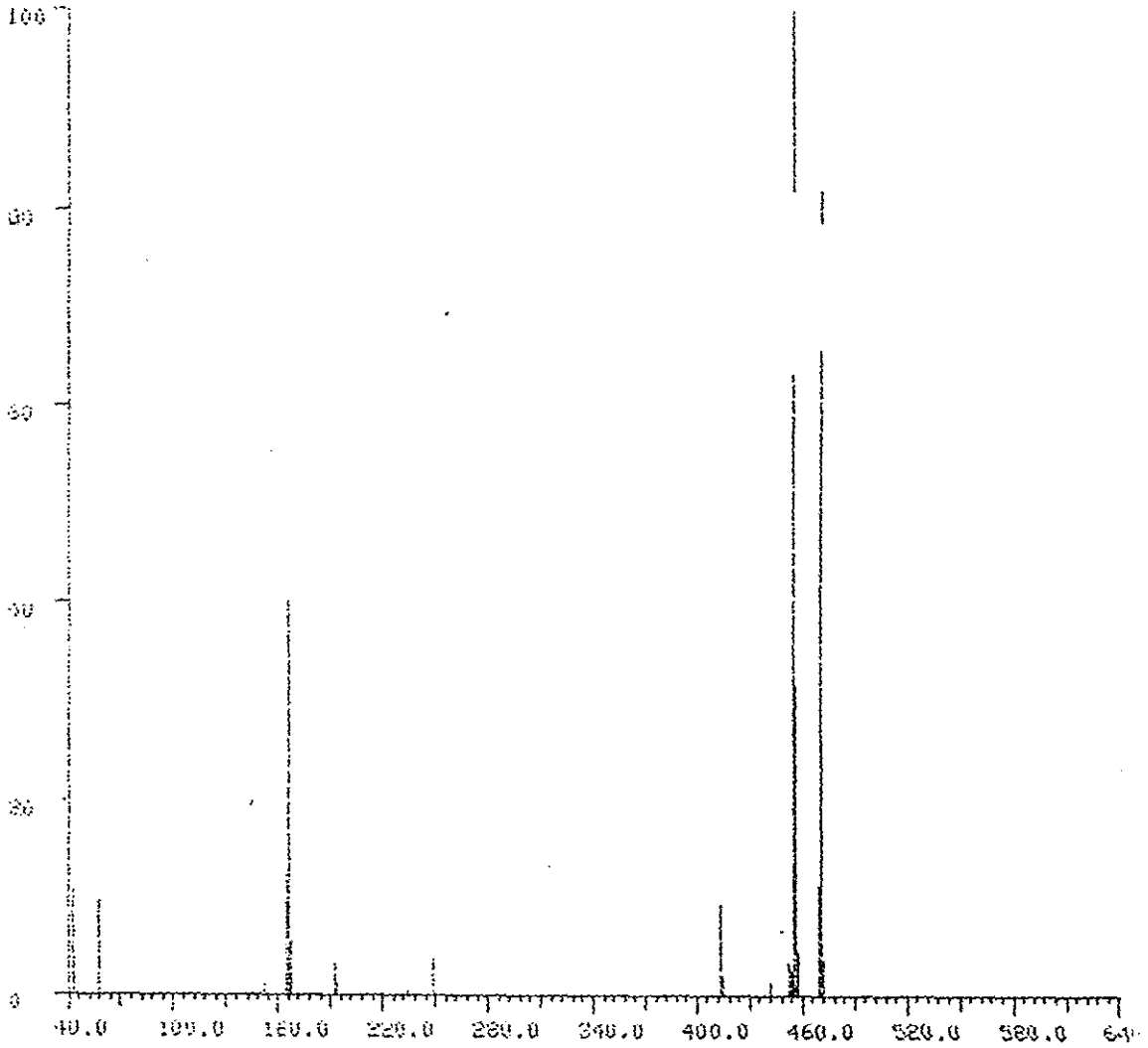
ORIGIN Xestolpongia sp.



21

5	0	471	12 3
6	0	1526	40 0
7	0	216	5 6
92	0	125	3 2
94	1	49	1.2
94.1		44	1.0
98.3		52	1 2
99	1	150	3 8
413	4	357	9.3
414	5	85	2.1
442.2		59	1 4
452	3	138	3 3
453	3	102	2 6
455	5	3806	100 0
456.3		1187	31 1
457.4		168	4.3
469	3	425	11 0
470.3		3108	81 6
471.3		1008	26 4
472.3		143	3 6

Fig. 2 Mass Spectrum of P – 46



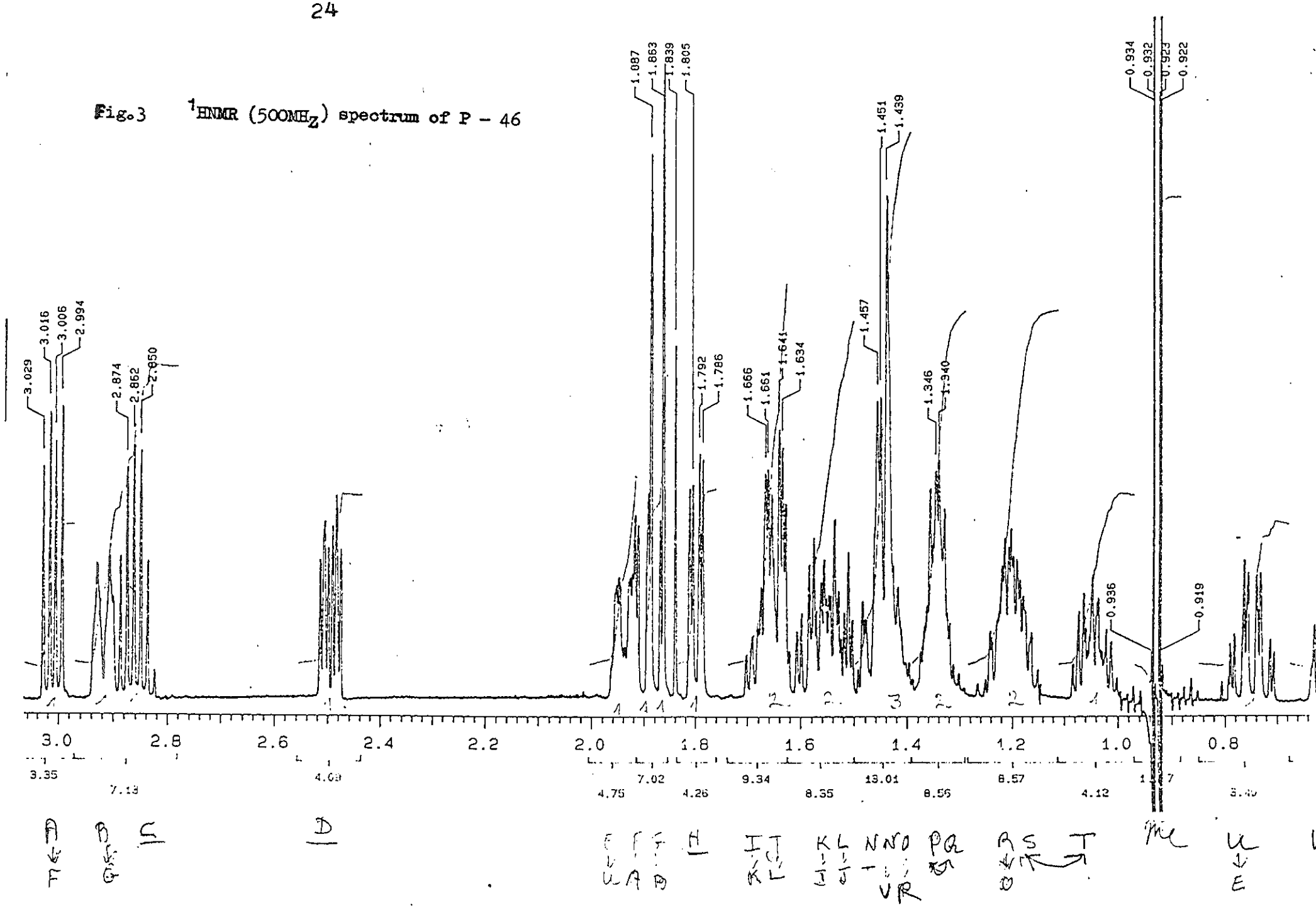
Therefore, it requires doubling of the number of C, H and O in $C_{15}H_{25}O$. This would give $C_{30}H_{50}$ O with 442 atomic mass units.

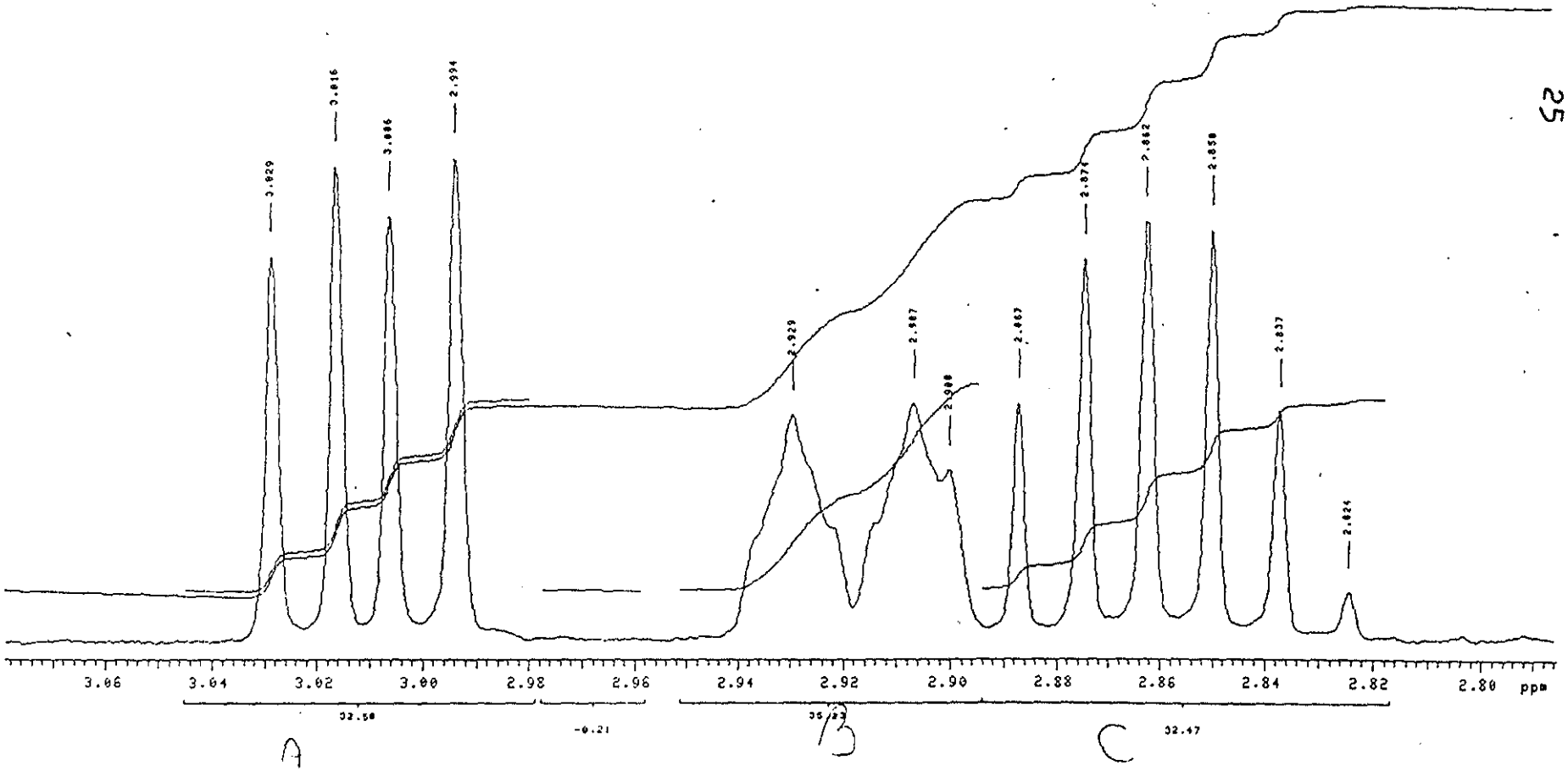
The molecular ion peak is an even mass number. In accordance with the nitrogen rule, P-46 may contain none or an even number of nitrogens[62]. But the presence was indicated by the Dragendorff positive test. The above deduced $C_{30}H_{50}$ O formula differs by 28 atomic mass units from the molecular ion mass, 470. This substantiates the existence of two nitrogen atoms. The molecular formula, therefore, may be written as $C_{30}H_{50}N_2O_2$ for P-46.

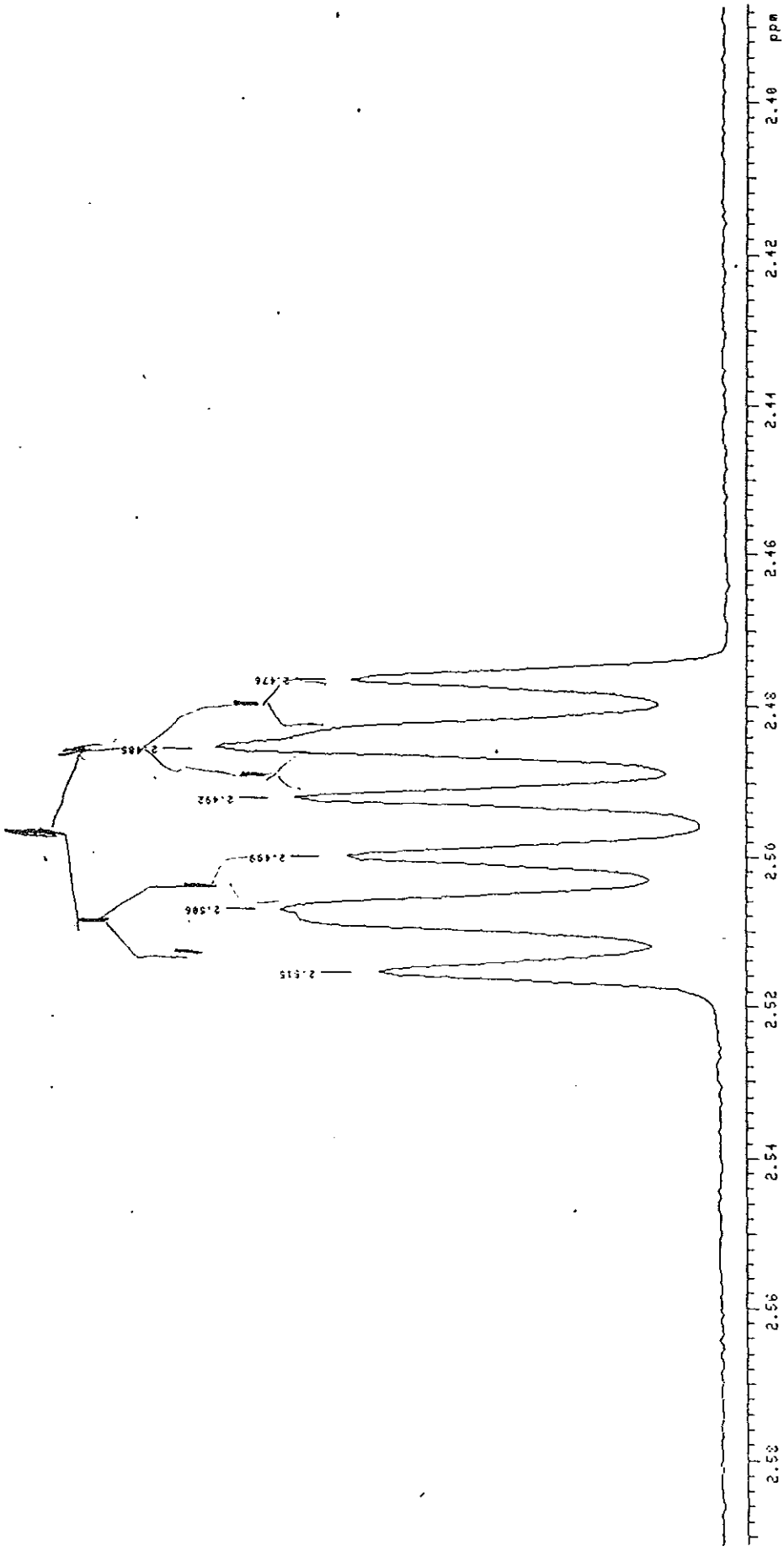
The 500 MHz ^1H NMR Spectrum (Fig. 3) of P-46 depicts all proton signals in the upfield region (δ 3.04-0.57). These proton signals may be attributed to methyl, methylene or methine protons . The signals integrate for 25 protons. Table 2 displays the ^{1}HNMR data of P-46.

The proton noise-decoupled ^{13}C NMR spectra (fig. 4a and Fig. 4b) of P-46 taken in deuterochloroform displayed only 15 signals. The DEPT spectrum (Fig. 5) exhibited the presence of one methyl, nine methylene, four methine groups. The remaining one quaternary carbon appearing at 214.5 ppm in the ^{13}C NHR (125 MHz) is assignable to a carbonyl carbon. This carbonyl carbo sipnal appears at 202ppm in ^{13}C NMR (90MHz) spectrum. But a carbonly carbon chemical shift should have been relatively down field at about 210-215 ppm than in ^{13}C NMR (90 MHZ). Other signals in ^{13}C NMR (125MHz) spectrum (Fig.4b) of P-46 are upfield shifted by 2.8 ppm compared to that recorded on 90 MHz instrument (Fig.4a). The tertiary nature of the nitrogen atom follows from the lack of absorption at about 3500 cm^{-1} in the IR spectrum. Furthermore, in ^1H NMR spectrum (Fig. 3) the signal for N-H is absent at about 7.40 ppm. Down field signals at 70.3 (d), 64.7 (t) and 55.9 (t) can be assigned to carbon atoms attached to a nitrogen. Table 3 displays the ^{13}C NMR data of P-46.

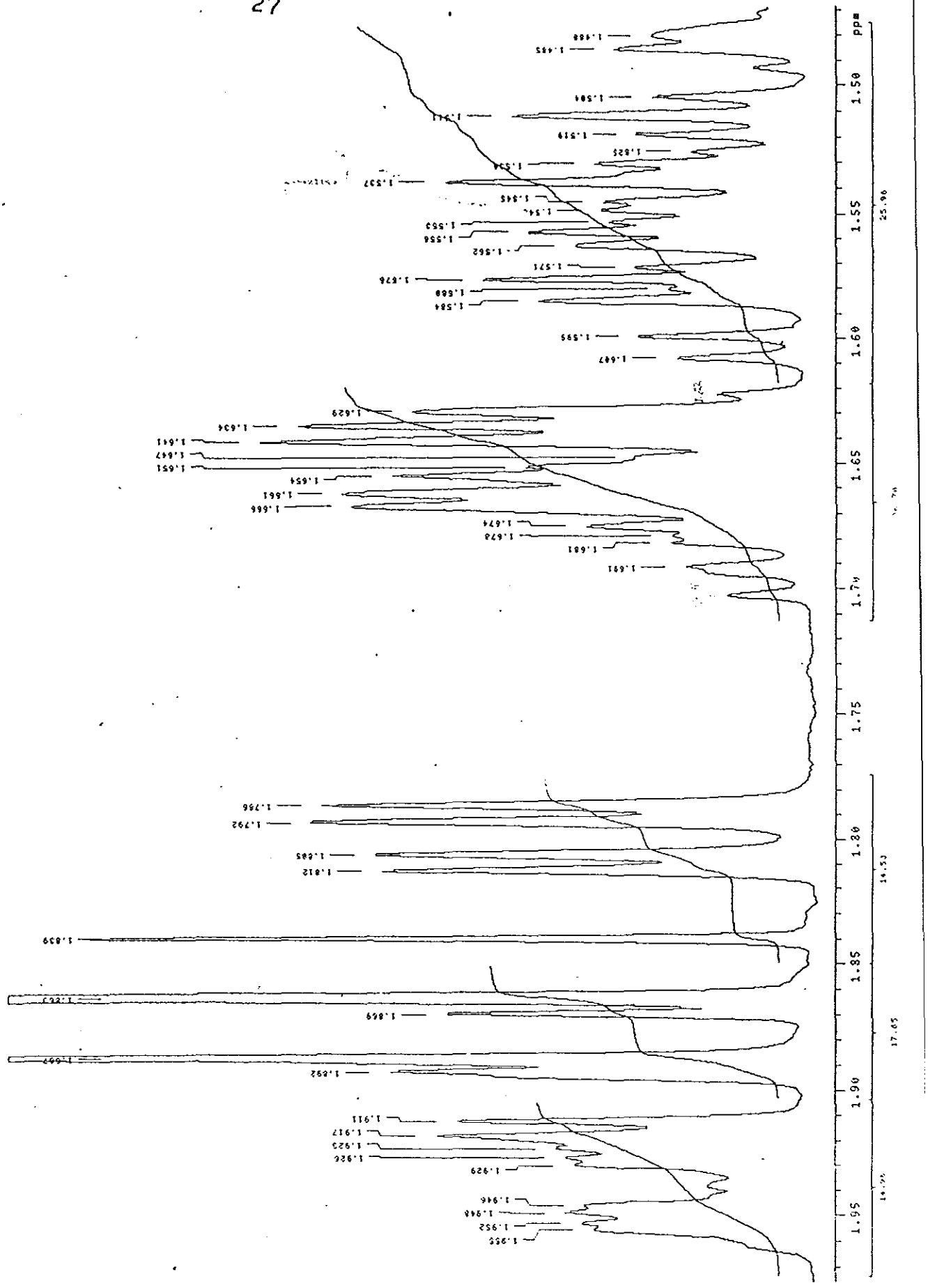
Fig.3 ^{1}HNMR (500MH$_{Z}$) spectrum of P – 46

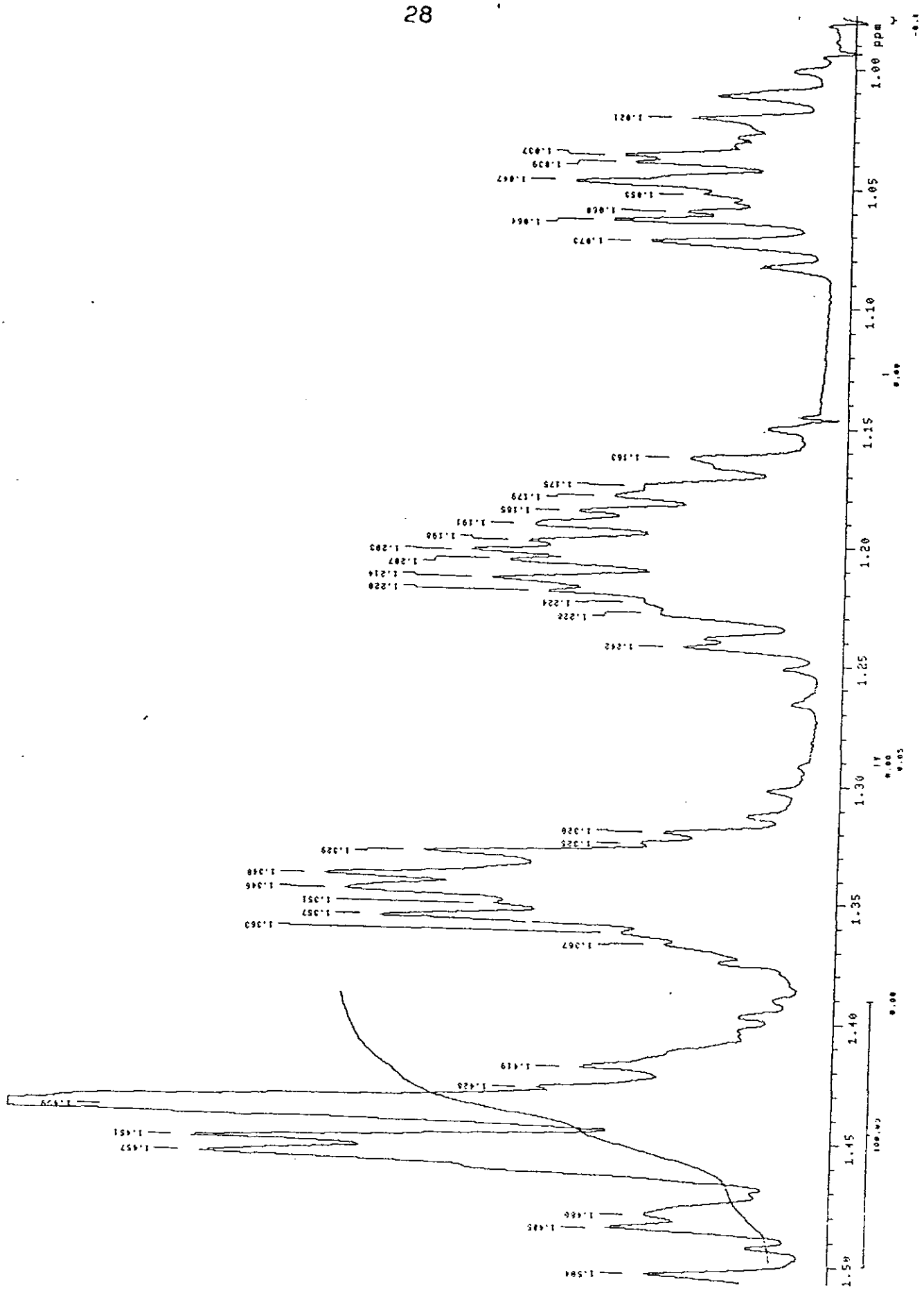



3.029
3.016
3.006
2.994
2.929
2.907
2.888
2.863
2.857
2.852
2.838
2.837
2.824
3.06
3.04
3.02
3.00
2.98
2.96
2.94
2.92
2.90
2.88
2.86
2.84
2.82
2.80
ppm
32.58
-0.21
35.23
32.47
A
B
C


2.476
2.485
2.492
2.499
2.506
2.515
2.40
2.42
2.44
2.46
2.48
2.50
2.52
2.54
2.56
2.58
ppm

D


1.488
1.495
1.504
1.511
1.519
1.525
1.534
1.537
1.545
1.547
1.553
1.556
1.562
1.571
1.576
1.584
1.588
1.595
1.607
1.629
1.634
1.641
1.647
1.651
1.654
1.661
1.666
1.674
1.678
1.681
1.691
1.756
1.792
1.805
1.812
1.839
1.863
1.869
1.897
1.902
1.911
1.917
1.923
1.926
1.929
1.946
1.948
1.952
1.955
ppm
1.50
1.55
1.60
1.65
1.70
1.75
1.80
1.85
1.90
1.95


1.00 ppm
1.05
1.10
1.15
1.20
1.25
1.30
1.35
1.40
1.45
1.50
1.021
1.037
1.039
1.047
1.053
1.060
1.064
1.073
1.163
1.175
1.179
1.185
1.193
1.196
1.203
1.207
1.214
1.220
1.224
1.226
1.242
1.326
1.325
1.329
1.348
1.346
1.351
1.357
1.363
1.367
1.419
1.426
1.457
1.461
1.466
1.485
1.504

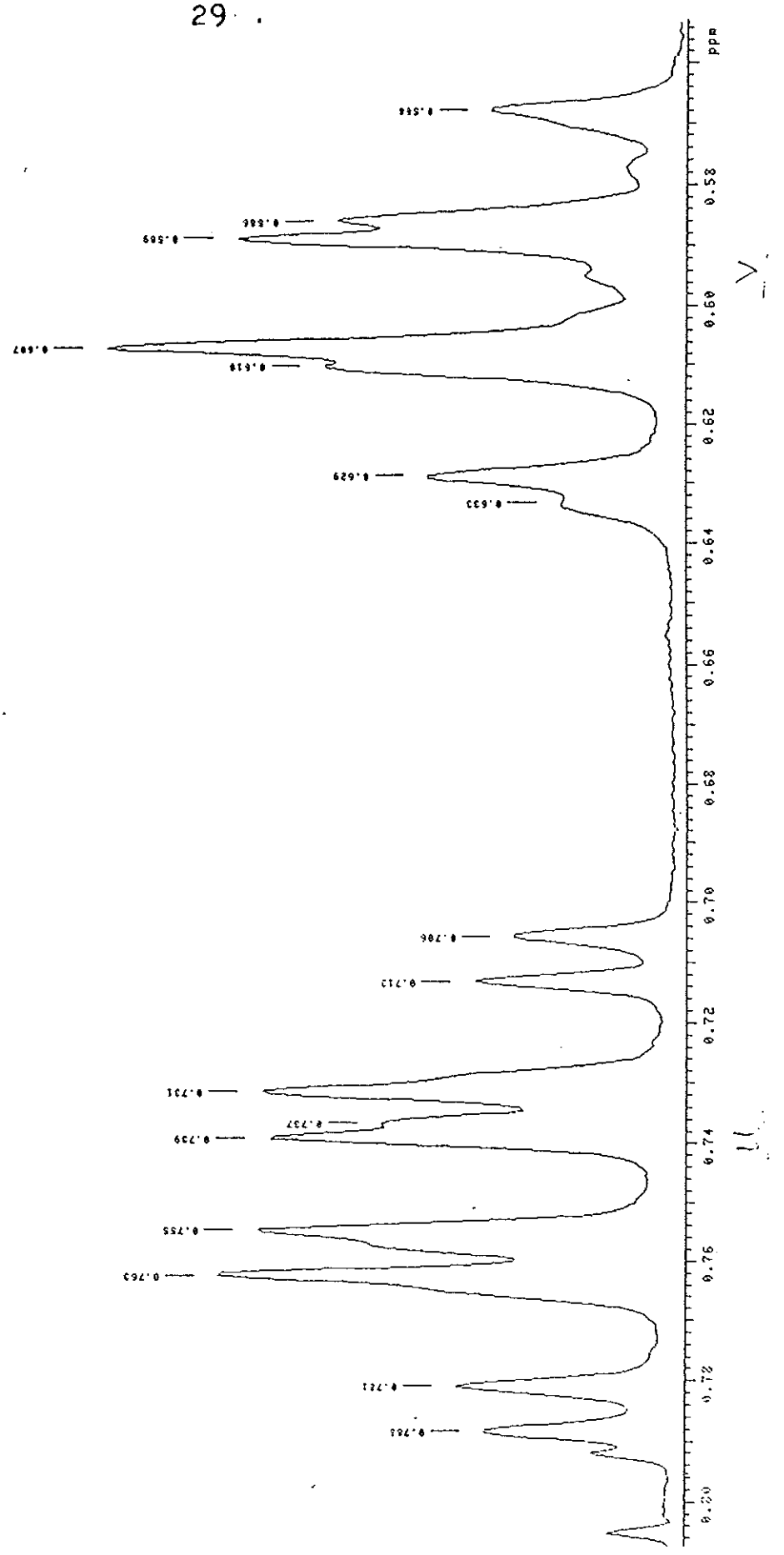

ppm
0.58
0.60
0.62
0.64
0.66
0.68
0.70
0.72
0.74
0.76
0.72
0.566
0.586
0.589
0.607
0.618
0.629
0.633
0.706
0.713
0.731
0.737
0.739
0.755
0.763
0.721
0.761

Table 2: ^1H NMR data of P-46 (500MHz)

Proton assignment	δH&M	J (Hz)	Proton assignment	δH&M	J(Hz)
H_A	3.04 dd	11.0.6.5	H_M	1.43 m	
H_B	2.94 brd	11.0	H_N	1.43 m	
H_C	2.86 ddd	13.0,6.5,6.5	H_O	1.43 m	
H_D	2.51 ddd	11.5,4.5,3.5	H_P	1.31 m	
H_E	1.93 m		H_Q	1.31 m	
H_F	1.88 dd	11.0,10.5	H_R	1.18 m	
H_G	1.86 dd	13.0,11.0	H_S	1.18 m	
H_H	1.79 dd	10.0,3.5	H_T	1.02 m	
H_I	1.66 m		H_U	0.74 dd	24,12.5,3.5
H_J	1.63 m		H_V	0.57 dd	21,11,1.5
H_K	1.55 m		H_{M-}	0.92 d	6.5
H_L	1.48 m				

δH = Chemical Shift of protons
J = Coupling Constant in HZ
M = Multiplicity

Fig. 5 DEPT Spectrum of P-46

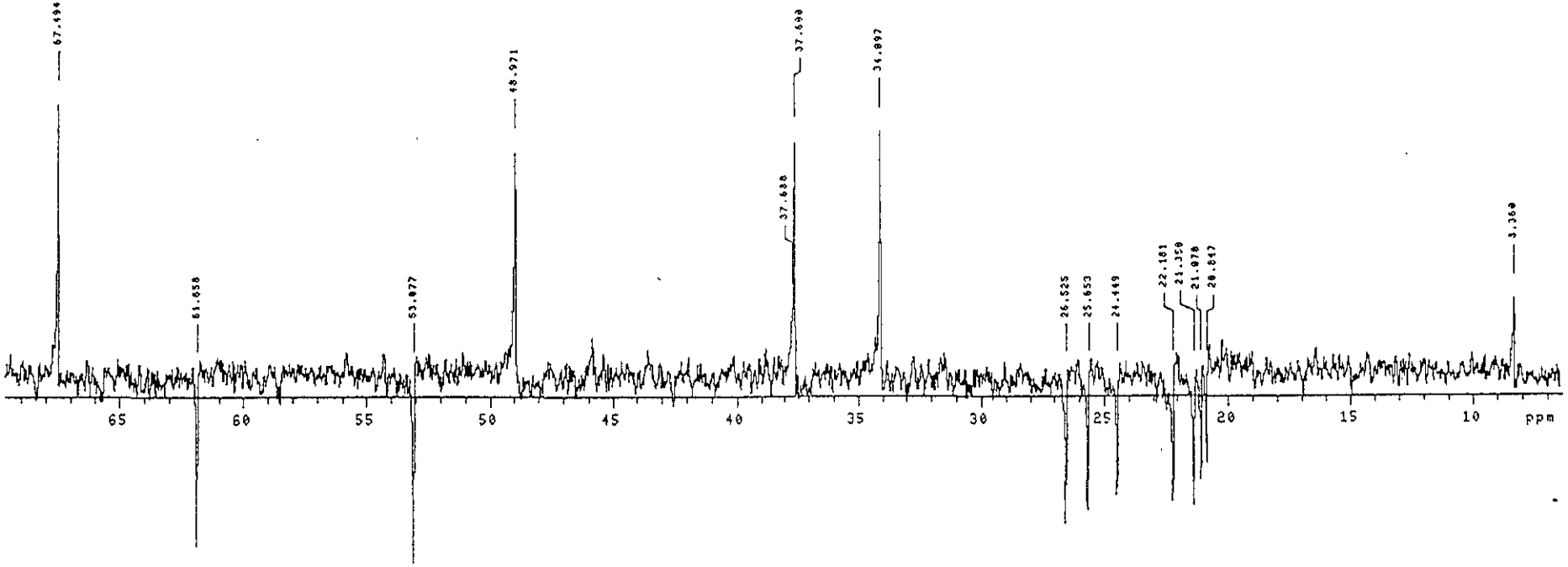


Fig.4b ^{13}CNMR (125 MHz) spectrum of P-46

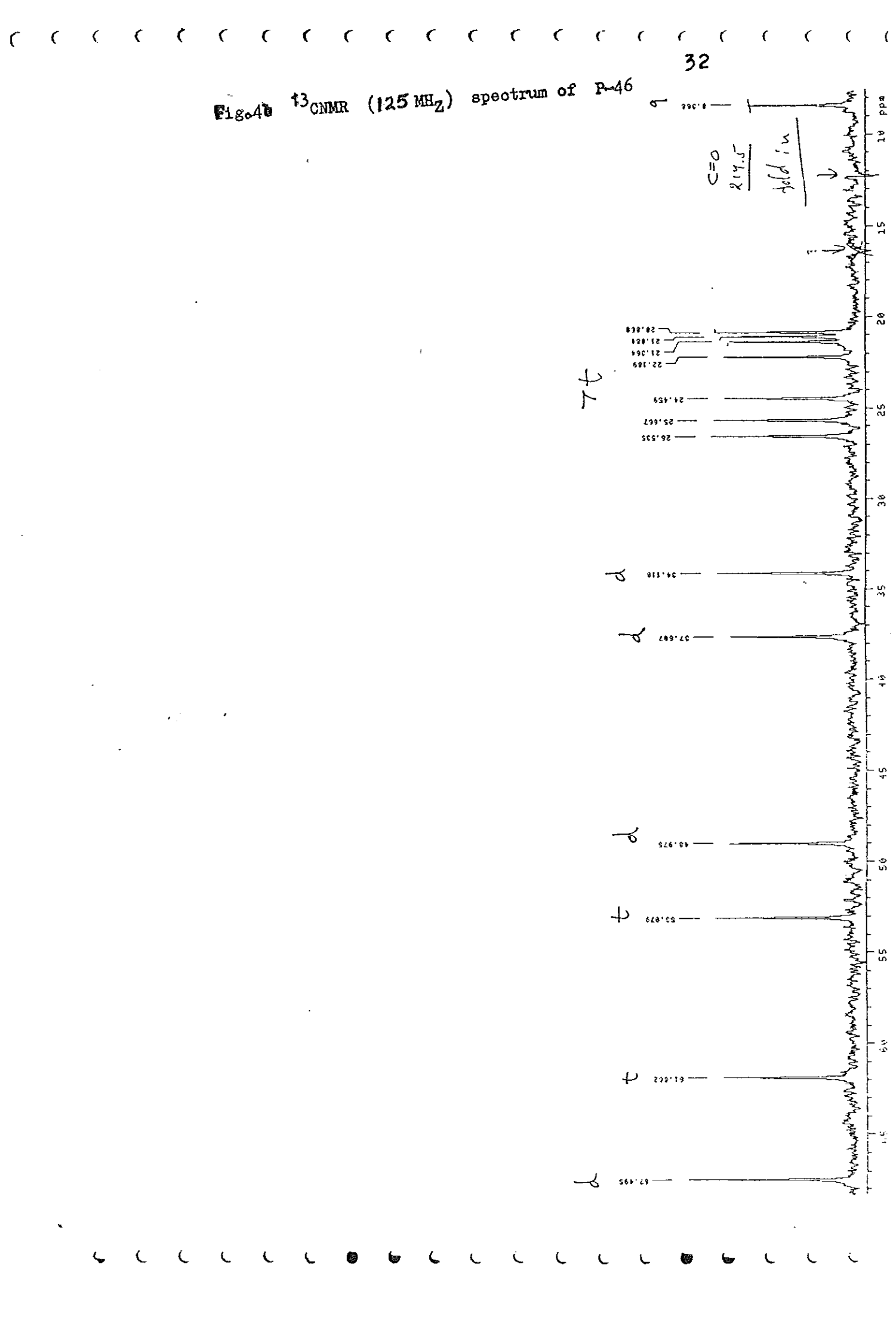


Fig.4a ^{13}CNMR (90 MHz) spectrum of P-46

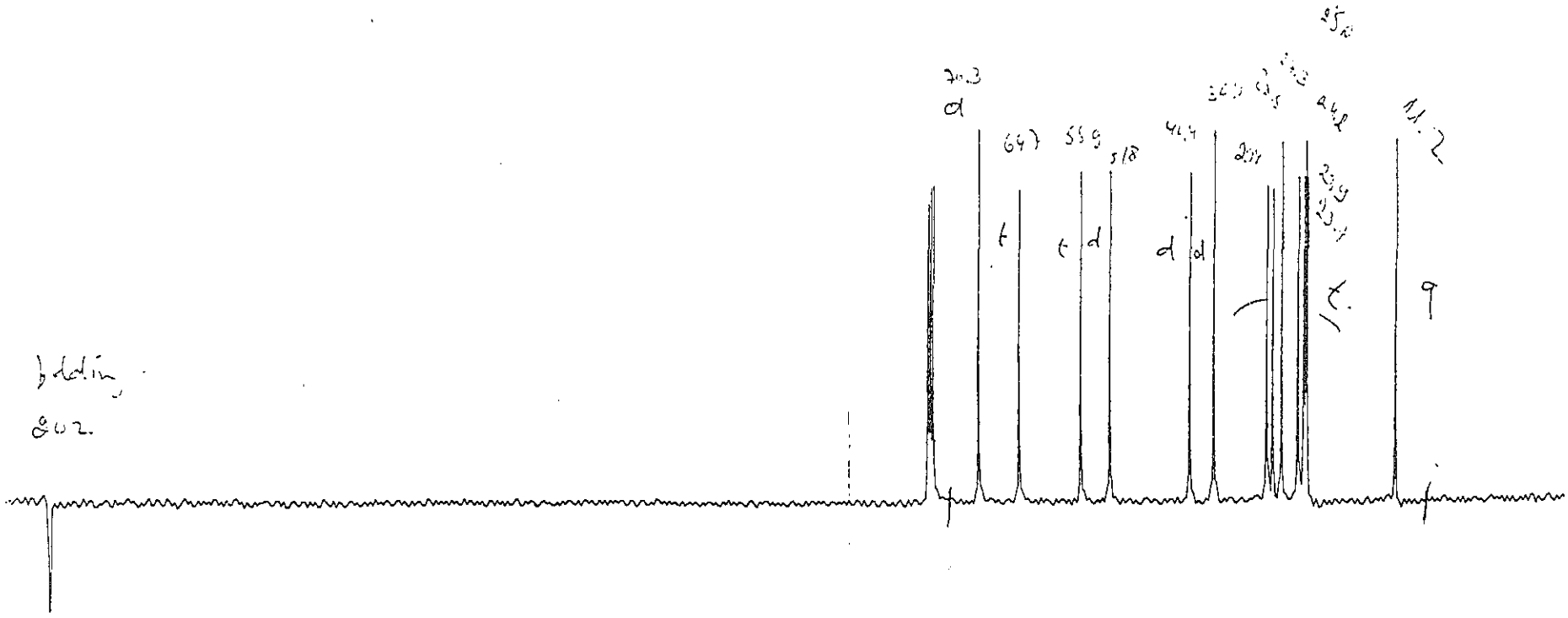


Table 3 : ^{13}C NMR (90 MHz & 125 MHz) data of P-46

Carbon assignment	δC&M (90MHz)	δC&M (125MHz)	Carbon assignment	δC&M (90 MHz)	δC&M (125 MHz)
C-a	202 S	214.50 S	C-i	28.5t	25.667t
C-b	70.3 d	67.495 d	C-j	27.3t	24.459t
C-c	64.7 t	61.862 t	C-k	25.0t	22.189t
C-d	55.9 t	53.079 t	C-l	24.2t	21.364t
C-e	51.8 d	48.975 d	C-m	23.9t	21.084t
C-f	40.4 d	37.607 d	C-n	23.7t	20.860t
C-g	36.9 d	34.110 d	C-o	11.2q	8.368q
C-h	29.4 t	26.535 t			

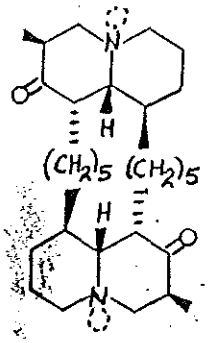
δC = Chemical Shift of Carbons
M = Multiplicity

The double bond equivalent of the molecular formula $C_{30}H_{50}O_2N_2$ is equal to seven. Two of

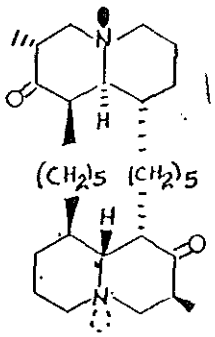
$$r + db = 7$$

the seven are attributed to two carbonyl groups in the molecule. Because of the absence of olefinic carbons in the ^{13}C NMR Spectrum, the remaining five stand for the number of rings in the structure of P-46. P-46 with molecular formula $C_{30}H_{50}N_2O_2$, therefore, is a pentacyclic alkaloidal compound.

In recent years, macrocyclic alkaloidal compounds constituting five rings in their structures were reported from the sponge Petrosia seriata[60,63] and more recently from Xestospongia sp[61]. The spectral data of P-46 was compared with the reported spectral data of macrocyclic bis-(1-oxaquinolizidine) and bis-(quinolizidine) alkaloids. Two macrocyclic alkaloids, petrosin (17) and petrosin - A (39), from Petrosia seriata[63] were found to exhibit similar spectral properties as P-46.



Petrosin (17)

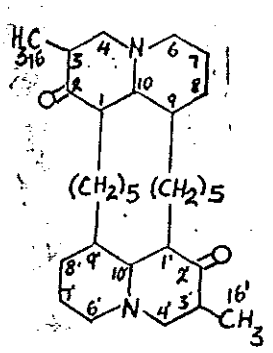


Petrosin-A (39)

Petrosin (17) and petrosin-A(39) are reported to be diastereomeric compounds. The molecular formula $C_{30}H_{50}N_2O^2$ reported for these compounds is identical with the one deduced for P-46. All exhibited molecular ion peaks at m/z 470. Petrosin and petrosin-A show only 15 signals for thirty carbons like P-46 on proton noise-decoupled ^{13}C NMR spectrum. Petrosin and petosin-A are reported to possess two fold axis (C_2) and a center of symmetry (S_2), respectively. P-46 is also a symmetrical molecule. All do not exhibit optical activity. Hence, P-46 may be a diastereomer of petrosin

(or petrosin-A) or it may be identical with one of the two.

As shown in Table 4, the chemical shifts of carbons atoms C1,1 to C10,10 of petrosin and petrosin-A are nearly identical with that of P-46. The methylene carbons, however, show slight variation in their chemical shifts. The chemical shift data of P-46 and petrosin (17) are in good agreement for carbons C11,11 to C15,15 . Based on the similarity in the ^{13}C NMR chemical shift, the common frame- work of 17 and 39 is suggested as well for P-46.



Frame-work of P-46

The assignment of the carbon signals is based on chemical shift comparison with the reported data of 17 and 39. The most upfield signal at 11.2 ppm is assignable to a methyl group (C16,16'). The most down field shifted signal at 214.5 ppm is assigned to carbonyl carbon (C2 ,2'). The signals at δ 70.3, 55.9 and 64.7 are assignable to carbon atoms that are attached directly to the nitrogen. Accordingly they are assigned to C10(10'), C6(6') and C4(4'), respectively. The doublets at δ 40.4, 51.8 and 36.9 are assigned to C3(3'), C1(1') and C9(9'), respectively, in P-46. Two triplet carbons at δ 23.7 and 28.5 are assignable to C7 (7') and C8(8'). The remaining five triplets with close chemical shifts are due to methylene carbon atoms linking the two quinolizidine moities. The signals at δC= 25.0, 24.2,27.3,23.9 and 29.4 are assigned to C11(11'), C12 (12'), C13(13'), C14(14'), and C15(15'), respectively, in P-46.

Table 4 : ^{13}C NMR data of P-46, petrosin and petrosin-A

Assignment	Petrosin δ C&M	P-46 (90MHz) δ C&M	P-46 (125MHz) δ C&M	Petrosin-A δ C&M
C1 ,1'	51.9 d	51.8 d	48.975 d	51.6 d
C2 ,2'	213.8 s	202. s	214.50 s	213.9 s
C3 ,3'	40.5 d	40.4 d	37.607 d	40.3 d
C4 ,4'	64.7 t	64.7 t	61.862 t	64.8 t
C6 ,6'	56.0 t	55.9 t	53.079 t	56.0 t
C7 ,7'	25.1 t	23.7 t	20.860 t	25.0 t
C8 ,8'	29.5 t	28.5 t	25.667 t	30.0 t
C9 ,9'	37.0 d	36.9 d	34.110 d	36.0 d
C10,10'	70.4 d	70.3 d	67.495 d	70.8 d
C11,11'	24.4 t	25.0 t	22.189 t	26.3 t
C12,12'	24.1 t	24.2 t	21.364 t	26.1 t
C13,13'	27.4 t	27.3 t	24.459 t	29.1 t
C14,14'	23.9 t	23.9 t	21.084 t	25.6 t
C15,15'	28.8 t	29.4 t	26.535 t	32.0 t
C16,16'	11.2 q	11.2 q	8.368 q	11.3 q

δ C = Chemical Shift of Carbons
M = Multiplicity

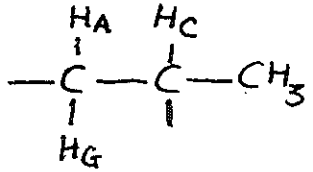
The inverse-detected Heteronuclear Multiple Quantum Coherence {HMQC} Spectrum (Fig.6) provided important information on the carbon-hydrogen connectivity. Using HMQC assignment can be made unambiguously for directly linked H and C in p-46 (see Table 5).

Table 5: HMQC direct C-H Correlation data

Assignment of Carbon	Direct Correlated Proton & δ_H	Assignment of Carbon	Direct Correlated Proton & δ_H
C1,1'	H_D (2.5)	C10 ,10'	H_H (1.79)
C2,2'	-	C11 ,11'	H_J (1.63), H_L (1.48)
C3,3'	H_C (2.86)	C12 ,12'	H_K (1.55), H_J (1.66)
C4,4'	H_A (3.04), H_G (1.86)	C13 ,13'	H_P (1.31), H_Q (1.31)
C6,6'	H_F (1.88), H_B (2.94)	C14 ,14'	H_T (1.02), H_R (1.18)
C7,7'	H_N (1.43), H_W (1.18)	C15 ,15'	H_E (1.93), H_U (0.74)
C8,8'	H_O (1.43), H_V (0.57)	C16 ,16'	H_{MM} (0,92)
C9,9'	H_M (1.43)		

δ H = Chemical shift of protons (ppm)

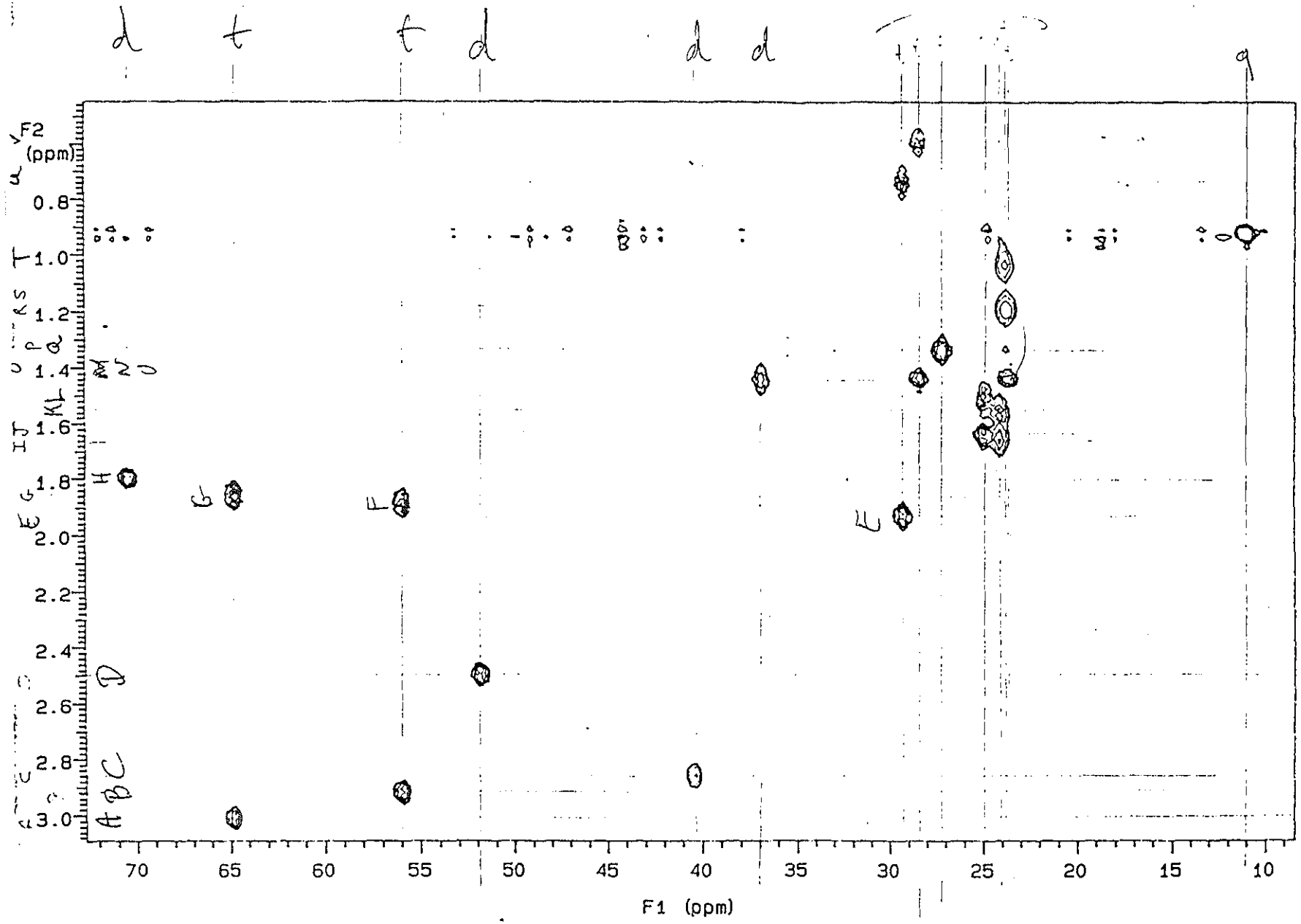
The 500 MHz ^1H-^1H COSY NMR spectrum (Fig.7) depicts coupling of Hc with H_{MM}, H_A and H_G (J= 6.5, 6.5,13Hz). Linking carbon atoms to which these protons are attached provides fragment 1.



fragment 1

None of the protons in fragment 1 exhibit a coupling with other protons as shown in the 500MHZ H-H COSY NMR spectrum. Therefore, they represent an isolated spin system. Hence, it requires attachment of nitrogen to the down field shifted methylene carbon and the carbonyl carbon to the methine carbon in fragment 1. This leads to an extended fragment 2.

Fig. 6 HMQC ^{13}C-^{1}H Correlation Spectrum of P-46



PLOTS OF INDIVIDUAL F1 TRACES

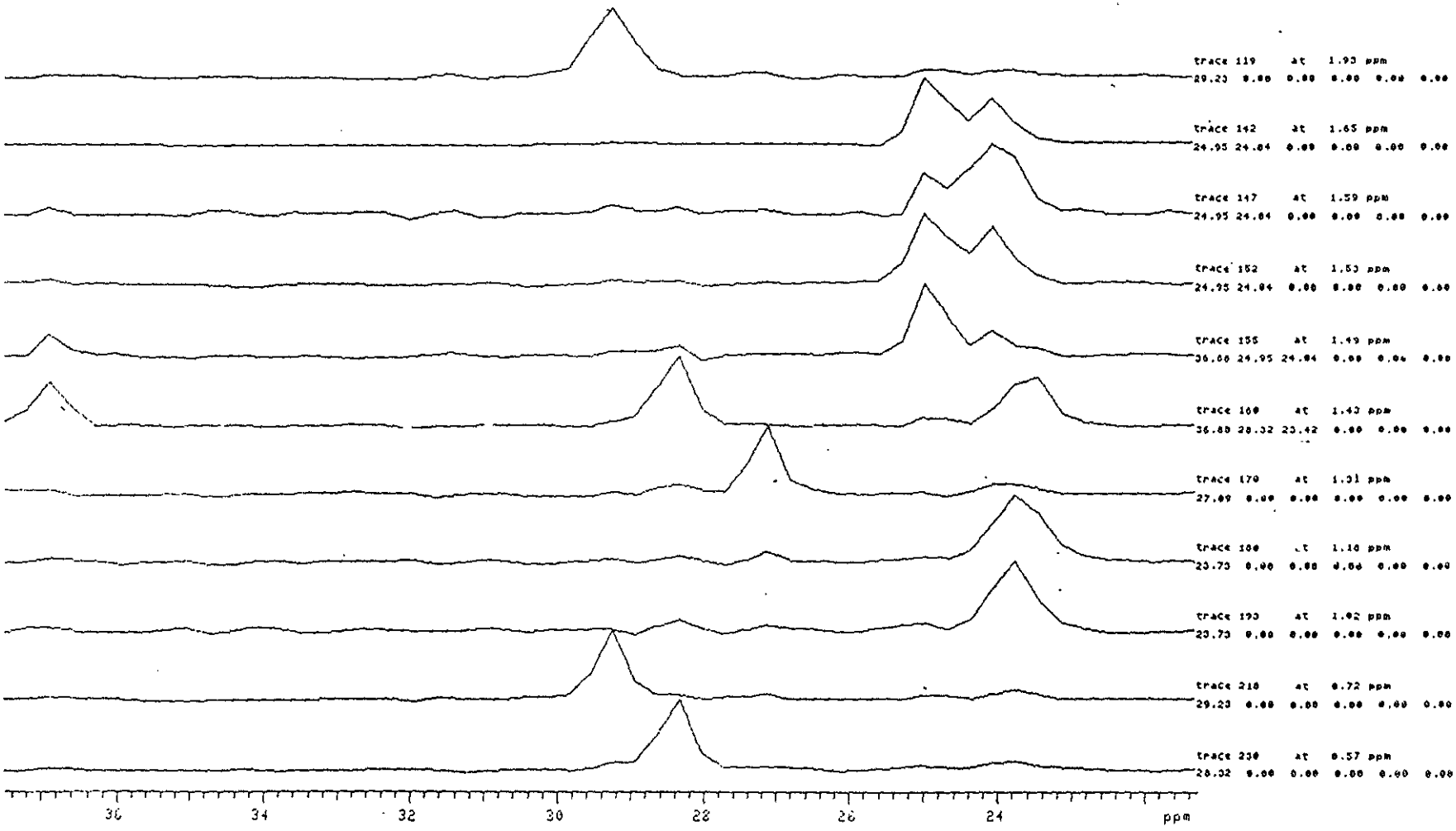
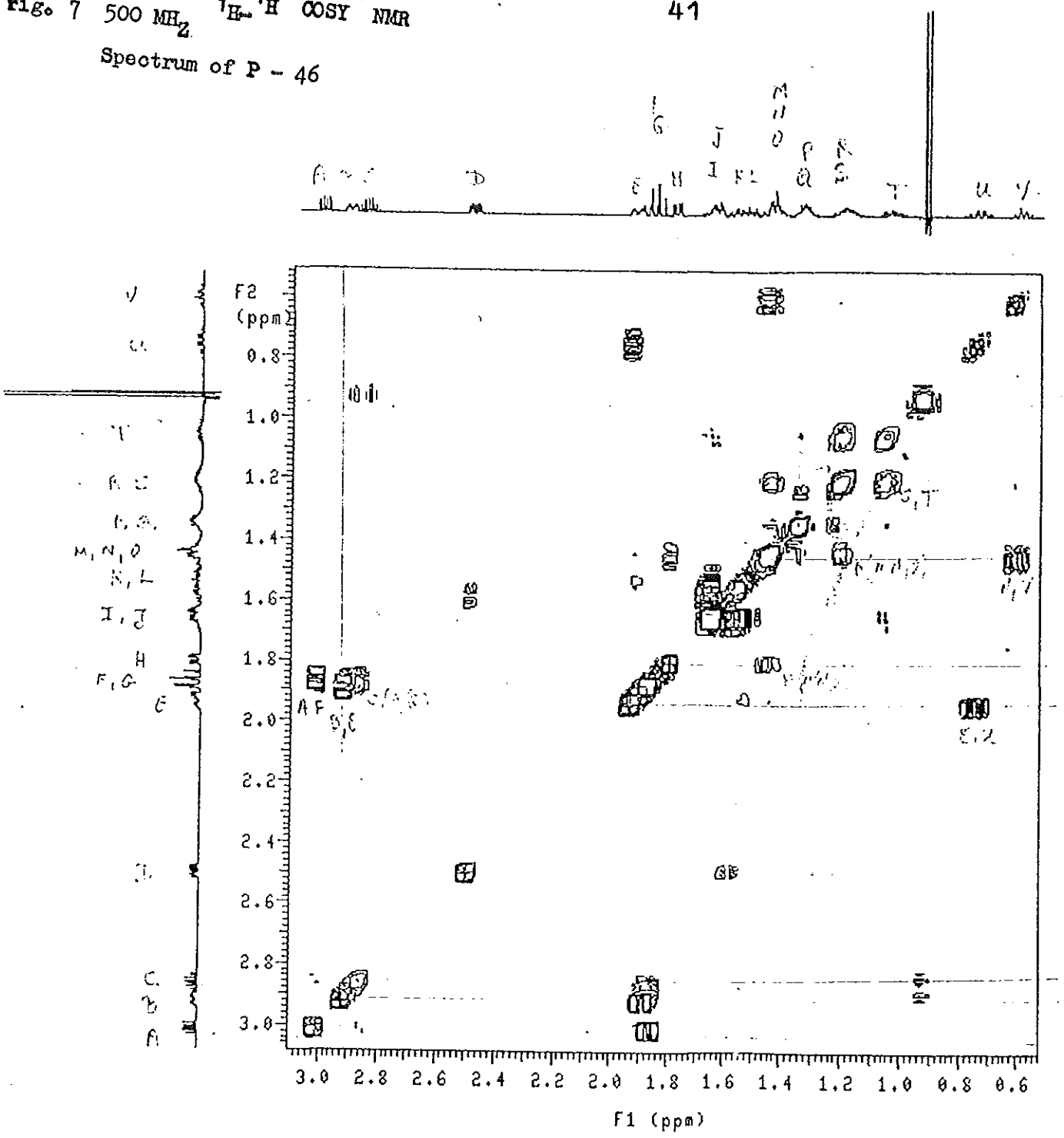
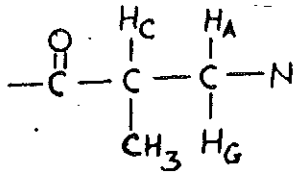


Fig. 7 500 MH_Z ^1H-^1H COSY NMR

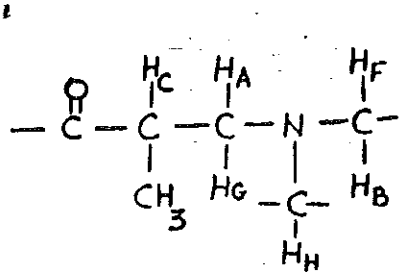
Spectrum of P - 46





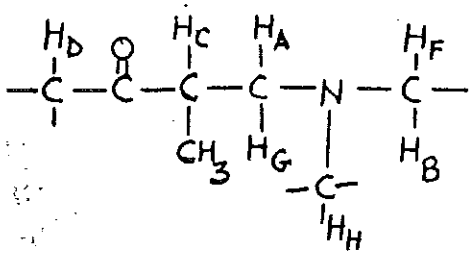
fragment 2

In quinolizidine rings, carbon atoms directly attached to nitrogen exhibit down field signals due to the dish ielding effect by the electrongative nitrogen atom. Hence, signals at δ 70.2(d) and 55.9(t) may be assigned to carbons attached to nitrogen. As a result, fragment 3 is obtained.



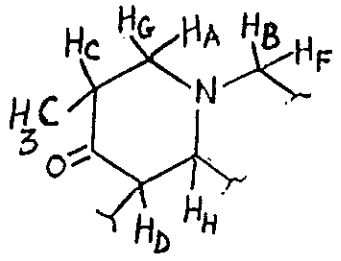
fragment 3

The α- carbon to the carbonyl carbon is strongly deshielded ($\sim$+31 ppm)[64]. Hence, the most down field shifted carbon atom (51.8) among the remaining carbons not in fragment 3 could be assigned to the carbon next to the carbonyl carbon in fragment 3. One, therefore, may write fragment 4.



fragment 4

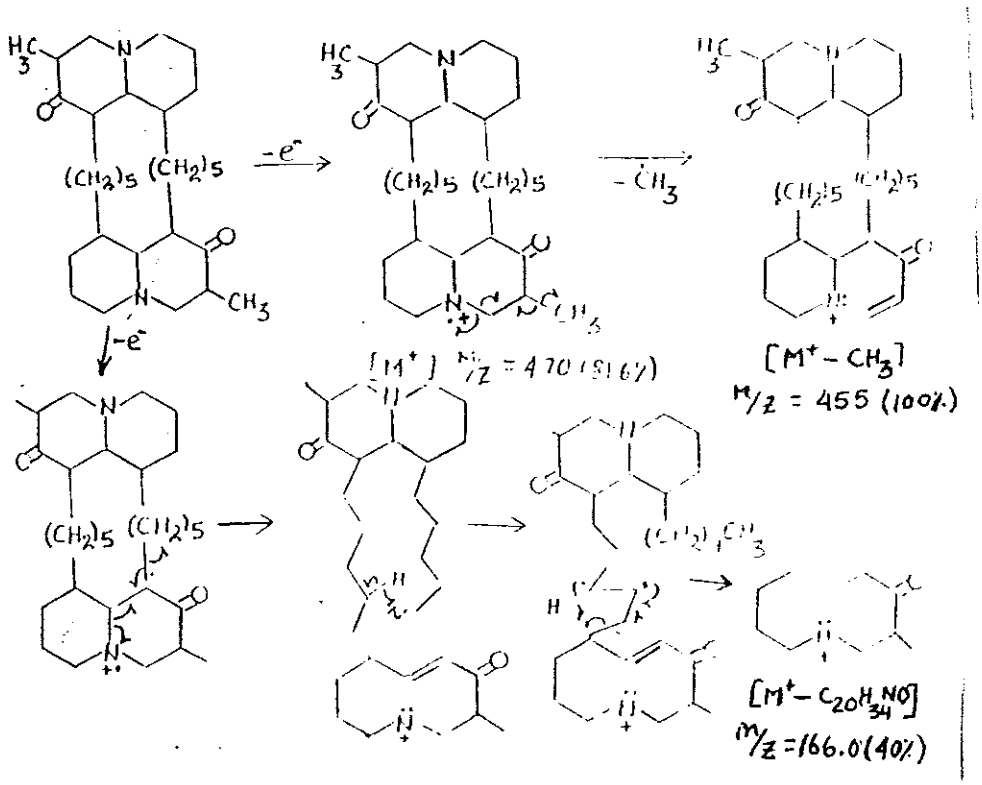
The presence of a coupling between H_D and H_H (J=3.5 Hz) supports the direct linking of the carbons bearing these protons. This leads to fragment 5.



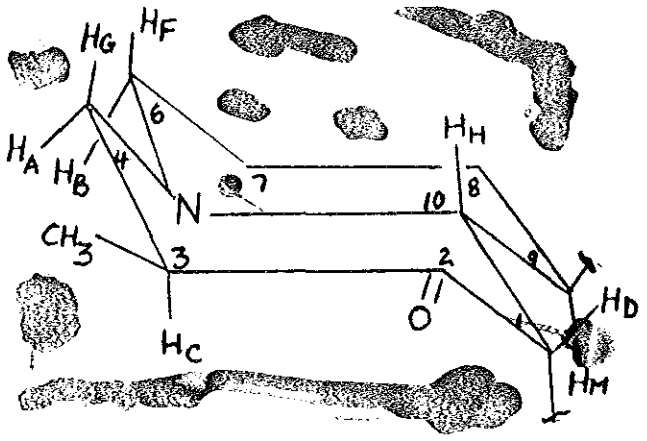
fragment 5

Fragment 5 obtained based on the H NMR spectra as well as ^{13}C NMR Chemical shift presents strong support to the suggested frame-work of P-46.

Further support may be obtained from the EIMS (Fig.2). The base peak is at m/z 455 which is due to loss of a methyl group. The following Scheme show the EIMS fragmentation patterns for the intense peaks in the mass spectrum of P-46.



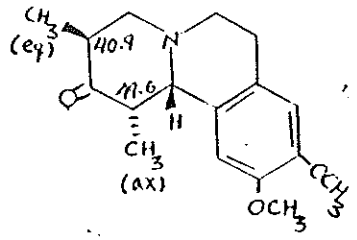
The stereochemistry of P-46 has been established based on ^{13}C NMR one-and 2D H NMR data. A trans conformation was assigned to the quinolizdine ring on the basis of the upfield-shifted H NMR value of H_H (1.79ppm). If it were cis, H_H would appear below about 3.5ppm[65]. Also the IR spectrum of P-46 (Fig.1) exhibits "Bohlmann bands" at 2850 cm^{-1} 2830cm^{-1} and 2780cm^{-1} suggesting the trans conformation [65].



40

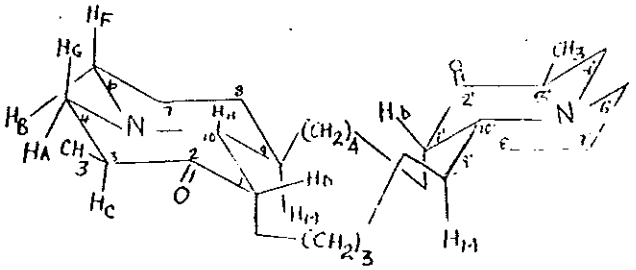
Trans quinolizidine moiety in petrosin or petrosin-A

The stereochemistry of the two adjacent chiral centers to H_H (CH-10,10') could be deduced based on the shape of signals, the doublet of doublets, of H_H. H_H showed a doublet of doublets appearing roughly as a 1:1:1:1 quartet (J=10Hz ,3.5Hz) at 1.79ppm. This indicates that H_H has axial-axial ($J=10H_z$) and axial-equatorial ($J=3.5H_z$) relationship with adjacent protons, H_D (CH-1,1) and H_M (CH-9,9'), in P-46. The adjacent H_D to H_H displays a ddd (J=11.5Hz, 4.5Hz and 3.5Hz) signal centered at 2.49 in the H NMR spectrum. The common coupling constant 3.5 Hz for H_D and H_H requires equatorial-axial relationship. These facts indirectly suggest that H_H and H_M to be axial-axial (J=10Hz) oriented. As a result, the bridging alkyl chains take equatorial position at C9(9') and axial position at C1(1). Further evidence is obtained from comparison of the chemical shift of C3(3') with model compound 41[66]. The chemical shift of C3(3') (40.4ppm) requires the axial orientation at C1(1') for the alkyl chain in P-46. This presents a confirming evidence to equatorial orientation of H_D, and in turn of the axial-axial relationship of H_H and H_M. Again this requires the equatorial orientation at C9(9') for the alkyl chain in P-46.

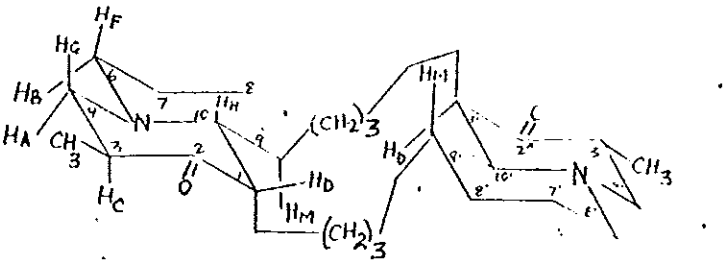


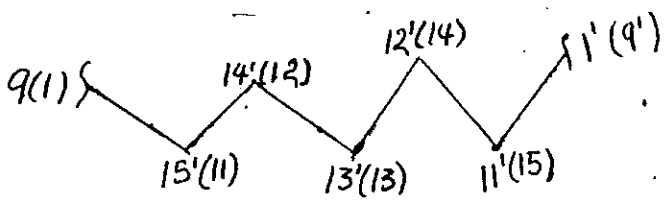
41

The longitudinal magnetic susceptibility of the lone electron pair on nitrogen shields axial protons [H_G (1.86) and H_F (1.88)] whereas it deshields equatorial protons [H_A (3.04) and H_B (2.94)]. The 500MHz H-H COSY NMR spectrum showed a coupling between H_G and H_C (JH_G-H_C=13Hz), a large coupling constant suggesting axial-axial relationship. Thus, it is logical to assume equatorial position for the methyl group. Taking into account all of the above arguments, one may suggest two possible structures, 17 and 39, of petrosin and petrosin-A, respectively, for P-46.



17





bridging alkyl chain in 17 and 39

As mentioned above, H_B proton H_C, H_F, H_H and H_M are axial protons whereas H_B, H_D and H_A are equatorial in the quinolizidine ring. The ^1H NMR data of P-46 are not as in good agreement as observed in the ^{13}C NMR data with petrosin (17) and pertosin-A (39) (see table 6)

Table 6: ^1H NMR data of P-46, Petrosin and petrosin-A

Assignment	Petrosin δH(ppm)	P-46 δH(ppm)	Petrosin-A δH(ppm)
CH-1,1'	2.52	2.51	2.51
CH-3,3'	2.89	2.86	2.92
CH-4.4' (e)	3.05	3.04	3.03
CH-4,4' (a)	1.90	1.86	1.83
CH-6,6' (e)	2.96	2.94	2.92
CH-6,6' (a)	1.92	1.88	1.84
CH-7,7'	1.68,1.56	1.18,1.43	1.55
CH-8,8'	1.97,0.78	1.43,0.57	1.82,0.80
CH-9,9'	1.47	1.43	1.58
CH-10,10'	1,74	1,79	1.77
CH-11,11'	1.68,1.63	1.63,1.49	1.76,1.71
CH-12,12'	1.25,1.08	1.55,1.66	1.30,1.12
CH-13,13'	1.38,1.20	1.31	1.28
CH-14,14'	1.48,1.35	1.02,1.18	1.20,1.58
CH-15,15'	1.48,0.63	1.93,0.74	1.28,0.74
CH-16,16'	0.96	0.92	0.96

Two alternative structures suggested above for P-46 differ only in the absolute configuration of the quinolizidine moieties. Their relative configurations are the same.

As reported by Braekman et al[60,63], petrosin crystallized readily from acetone/hexane solvent system, mp 215-216°C. Whereas petrosin-A could not be induced to crystallize. On the other hand , P-46 crystallized readily from acetone as a colorless rod shaped crystals, mp 221-222°c. The ^{13}C NMR data of P-46, petrosin (17) and petrosin-A (39) are nearly identical for C1(1') to C10(10') of the quinolizidine moities. But the ^{13}C NMR data of P-46 for the alkyl chain methylene carbons (C11 (11') to C15(15') are nearly identical with that reported for petrosin compared to petrosin-A. Therefore, the alternative structure of petrosin-A supported for P-46 was ruled out on the basis of above mentioned evidences. As a result, P-46 was identified as petrosin (17).

The structure of petrosin was established by Braekman et al[60] using X-ray diffraction analysis. The same group analyzed the structure of petrosin based on the spectroscopic data. P-46, possesses two fold pseudo-axis passing through the middle of the 16-membered ring and perpendicular to the mean plane of the same ring. This explains the optical inactivity and only half of the signals displayed for the total number of protons and carbons in P-46 in the ^{1}H NMR and ^{13}C NMR spectra.

Conclusion

A Chemical investigation of the chloroform soluble portion of the ethanolic extract of Xestospongia sp. (collected near Massawa, Read Sea) has been undertaken. In the process two compounds. P-46 (petrosin) and P-51 were isolated. P-46, petrosin, was fully characterized. Further purification and identification work of P-51 is being carried out by professor Kasahun's group at Tel Aviv University in Israel.

The Structure of petrosin was established based on spectral studies including IR, EIMS, ^{13}C NMR , HMQC, one and 2D ^{1}HNMR as well as by comparison of spectra and physical data with those reported for the same Compound by Braekman's group[63]. In petrosin the carbon-hydrogen connectivities established by Braekman's group[63] were confirmed unambiguously using HMQC data.

Petrosin, which was also found in this investigation was the first representative of a bis-quinolizidine alkaloid in nature. It was isolated earlier from Petrosia Seriata[60,63] and more recently from Xestospongia sp.[61]

V. Experimenta

General : Melting point was measured by using a Boetius hot-stage apparatus and is uncorrected. IR (KBr) spectrum was recorded on a Perkin Elemer 727 B spectrometer. Optical rotations were measured in chloroform on a Perkin Elemer 241 polarimeter. H NMR and ^{13}C NMR spectra measurement were performed in deuterochloroform on Bruker MA 360 MH_z and Bruker MA 500 MH_z spectrometers. The chemical shifts were referenced to the solvent signals. Mass spectrum was taken with DuPont 21-491 B instrument. All solvents used were either freshly distilled or analytical reagent grade.

Collection of the Sponge Sample

The sponge sample was collected in July 1989, from the Read Sea, around the Marine Biology Station of Asmara Universitiy at Massawa. The sample was collected at a depth of 3-6 meters. A specimen was preserved in 10% formaline salt water solution. The sponge sample was identified as Xestospongia sp. by Mr.M.Ilan of Tel Aviv University, Israel. The collected sponge sample was sun-dried and stored in a deep freezer.

Extraction and fractionation

The extraction of 2kg of the dried sponge was carried out in four batches of 500g each. 500g of the dried sponge was cut in small pieces and socked in 3.5L of 95% ethanol three times for 48 hrs at room temperature. The combined extract were filtered and concentrated to give a black gummy residue (600g). This was partitioned between chloroform (3x400ml) and water (400ml). The water extract was kept aside. The combined chloroform extract was concentrated under reduced pressure to yield a residue (179g). This residue was partitioned between 10% aqueous methanol (400ml) and pet. ether (4x400ml). The combined pet. ether extract was evaporated in vacuo to give 83g residue. This non-polar fraction was not further investigated. The removal of solvent from the aqueous methanol extract gave 96g residue.

The residue (96g) from the aqueous methanol extract was chromatographed in portions on VLC (Vacuum Liquid Chromatoprapy). It was eluted with solvent mixtures of pet.ether-ethylacetate and ethyl acetate-methanol. Fractions eluted with 80% ethyl actate-pet. ether were

combined and concentrated in vacuo to yield 18.60g crude extract. This crude was subjected in portions, 3g at each run, to column chromatography over silica gel 60H (120g). The column was eluted with pet. ether-chloroform mixtures with increasing proportion of chloroform and then ethyl acetate in chloroform. Fractions of 100ml were taken and combined based on TLC monitoring. Fractions eluted with 10% ethyl acetate in chloroform yielded P-46. P-51 was eluted from the column with 40% ethyl acetate in chloroform. Further purification and identification work of P-51 is in progress.

P-46: The combined fractions eluted with 10% ethyl acetate in chloroform were concentrated to give an oily residue. P-46 was crystallized as colorless rod shaped crystals by treating the oily residue with acetone. It was further purified by repeated recrystallization from acetone (40mg). MP 221-222° c. $[\alpha]_D^{20}=0$ (C=0.05, $CHCl_3$). P-46 showed positive Dragendorff spray test. Rf=0.82 ($CHCl_3$ /ethyl acetate/methanol, 2:1:0.5).

Mass Spectrum - $^{M/z}$ (rel.int.) :470.3 (M.81.6%) 455.5 (M $-CH_3$,100%), 166.0 (M-$C_{20}H_{34}NO$ 40%), 58(9.5%) 43(11%), 192.0 (3.2%),234 (1.0%), 249.1 (3.8%), 414.5 (2.1%), 442.2 (1.4%).

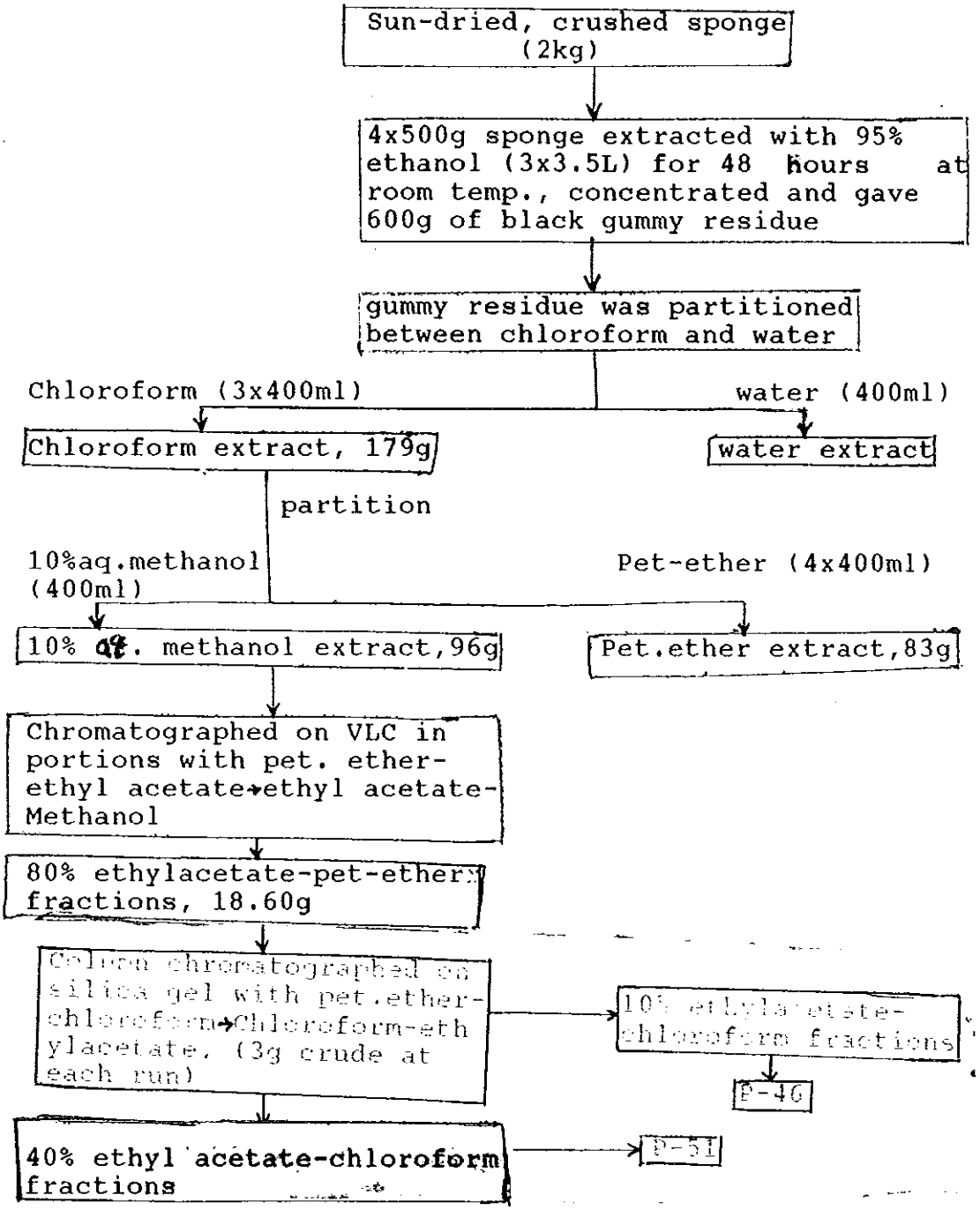
Proton-nuclear magnetic resonance :- (Bruker MA 500 MH_z, ($DCCl_3$) : See Table 2.

Infrared spectrum ν max (KBr) : 1710cm^{-1} ,2960 cm^{-1} 2980cm^{-1}, 2850 cm^{-1}, 2830 cm^{-1}, 2780cm^{-1}.

^{13}C NMR Spectrum :- (Bruker MA 360)

δC: 51.8 (d), 202 (s), 40.4(d), 64.7 (t), 55.9 (t), 23.7 (t), 28.5 (t), 36.9 (d), 70.3 (d), 25.0 (t), 24.2 (t), 27.3 (t), 23.9 (t). 29.4(t), 11.2 (q)

Table 7: Flow chart for extraction and fraction of the sponge Xestospongia sp.



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DECLARATION

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

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