Secondary metabolites from a few marine organisms and its applications

Ph.D Thesis

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2005

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Thesis submitted to Goa University
for the degree of
Doctor of Philosophy
in
Chemistry

By
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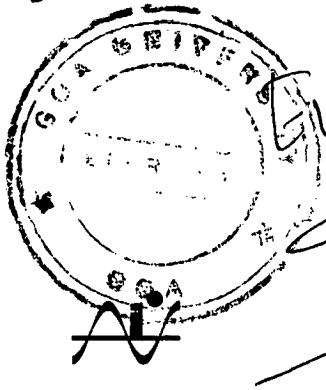

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For the Lord giveth wisdom:
Out of his mouth cometh
Knowledge and understanding.

(Proverbs 2:6)

Dedicated to my parents

Declaration

As required under the University ordinance 0.19.8 (vi), I state that the present thesis entitled "Secondary metabolites from a few marine organisms and its applications" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

26/4/05

Dr. (Mrs) Solimabi Wahidulla
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26/04/05

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Certificate

This is to certify that the thesis entitled "Secondary metabolites from a few marine organisms and its applications", submitted by Ms. Rani Rajamanickam for the award of the degree of Doctor of Philosophy in Chemistry is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any Universities or Institutions.

28/4/05

Dr. Solimabi Wahidulla
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Acknowledgement

A dream begins with a teacher who believes in you, who tugs and pushes and leads you to the next plateau, who suggests rather than dogmatizes and inspires his listener with the wish to teach himself. I have always looked back with appreciation to all the brilliant teachers in my life, but am filled with a sense of gratitude to one who touched my human feelings-my teacher, my guide- ***Dr. Solimabi Wahidullah,*** *Scientist, NIO. This thesis is an outcome of her altruistic and steady support, advice and teaching, in the face of all her trials and tribulations. My association with her has not only enriched my scientific knowledge and understanding, but has also taught me to be simple in thoughts and actions, patient with friends and colleagues and benign towards all around me.*

I owe my sincere gratitude to ***Dr. Shailaja Anand,*** *Scientist, NIO. It was my privilege to be associated with her. I had the opportunity to carry a part of my thesis under her support and guidance. The practical knowledge I received during my association with her was really inspiring. It has been a learning experience for me wherein I learned to work meticulously and thoroughly equip myself in the practical knowledge of marine pollution. I enjoyed working in the laboratory with her where I could put my ideas to work and realize my abilities as a chemist.*

I am proud and happy that I have been associated with one of the best scientific teams (Bio Organic chemistry group) in NIO and this association has inculcated in me a lure for scientific endeavors. I am highly indebted to my project leader ***Dr. C. G. Naik,*** *Scientist, NIO, for giving me an opportunity to work in the Bio Organic chemistry laboratory under the project "Development of potential drugs from the Indian ocean". Working under this project has developed in me the skills in scientific methods and has also exposed me to the use and operation of different modern instruments making me a 'hands-on' kind of person, which I believe is the biggest asset to me.*

My sincere thanks to ***Dr. Lisette D'Souza,*** *Scientist, NIO, not only for her help and support but also for her kind, cheerful ways that created a lovely working ambience. I thank* ***Dr. Parameswaran,*** *Scientist, NIO, for his timely help and support throughout the course. I am also grateful to* ***Dr. S. W. A. Naqvi,*** *Scientist, NIO, for his kindness.*

I am grateful to ***Dr. K. S. Rane*** *(Head, Chemistry Department, Goa University) and* ***Dr. S. P. Kamat*** *(Co-guide),* ***Dr. V. S. Nadkarni*** *(subject expert), Goa University, for their constant help and guidance.*

I thank to ***Dr. Suryanarayana,*** *Scientist, NIO for all his encouragement and* ***Dr. Raghukumar*** *for his help.*

I acknowledge CSIR for the award of Senior Research Fellowship (CSIR-SRF).

I am grateful to ***Dr. Satish Shetye,*** *Director, NIO, for providing the necessary infra structure to carry out this research.*

I am grateful to ***Dr. Madhusudanan,*** *Scientist, CDRI, Lucknow for some of the spectral data.*

My sincere thanks to ***Mr. Bhobe, Mr. Uday Mandrekar*** *and* ***Mr. E. Sasi*** *for all the help they rendered, both in the laboratory and during field work.*

I also thank ***Mr. Mahale, Mr. Shyam*** *and* ***Mr. Uchil*** *for scanning and skillfully tracing all the figures.*

When we honestly ask ourselves which people in our lives mean the most to us, we often find that it is those who, instead of giving advice, solutions or cures, have chosen to share our pain and touch our wounds with a warm and tender heart. I wish to express my immense love and gratitude to my friends ***Supriya Tilvi, Divya Shridhar, Celina Rodrigues*** *and*

Tonima Kamat for just being there with me and for me whenever I needed them the most. I am also grateful to my friends Ammar, Varsha, Rakhee, Prabha Devi, Dnyaneshwar, Trupti, Rajesh, Mahesh, Vaibhavi, Yogita, Reena, Ely, Shirish, Marylene, Sushant, Ankush, Gulshan, Tilvi, Nilesh, Sunil and Srikant and for their unconditional help, kindness, care and concern.

I am grateful to Mr. Paneer Selvam, Technical officer, NIO, for his unwavering support in some of the most imperative moments!

The happiest moments of my life are the ones, which I have passed at home in the bosom of my family. Good, honest, hardheaded character is a function of home. If the proper seed is sown there and properly nourished for few years, it will not be easy for that plant to be uprooted. All that I am today is what my parents have given me. I have no adequate words to express my love and gratitude to my parents; therefore I dedicate this thesis to my dear parents as an expression of my gratitude, love and care for them.

I owe my sincere gratitude to my brothers and all my other family members & friends for their love and moral support.

(Rani Rajamanickam)

GENERAL REMARKS

Compounds used are commercially available. All the solvents used were dried and freshly distilled. All melting points were measured on a digital melting point apparatus (Electothermal 9100) and were uncorrected.

Silica gel 60 F_{254} plates (Merck, 0.2 mm) TLC plates (aluminium sheets) were used. Silica gel (Merck, 60-120 mesh, 200-400 mesh) was used for column chromatography.

Infra red spectra were taken on Shimadzu FTIR spectrophotometer while ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 300 MHz spectrometer in $CDCl_3$ using TMS as internal standard unless otherwise stated. ESI-MS and FABMS were recorded on QSTAR XL (Applied Biosystems, Canada) mass spectrometer. The chemical shifts are expressed in downfield to TMS, which is used as internal standard.

All figures, tables, structure numbers and references in a section refer to that particular section only.

ABBREVIATIONS

AcOH	Acetic acid
AHH	Aryl hydrocarbons hydroxylase
amu	atomic mass unit
[α]	Specific rotatory power
α	Alpha
BaP	Benzo(a)pyrene
BDH	Bombay drug house
BHT	Butyl hydroxy toluene
BOA	Benzoxazoline-2-one
bs	Broad singlet
BuOH	Butanol
β	Beta
CI-MS	Chemical Ionisation Mass spectra
$CHCl_3$	Chloroform
^{13}C	Carbon 13
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
CID	Collisionally induced dissociation
DEPT	Distortionless Enhancement by Polarisation transfer
DMSO	Dimethyl sulphoxide
d	Doublet
dd	Double doublet
D_2O /HOD	Deuterated water
δ	NMR chemical shift [ppm]
°C	Degree celsius
Da	Dalton
DMF	Dimethylformamide
DNA	Deoxy ribonucleic acid
Δ	Position of double bond
E	Entegenen
EH	Epoxide hydrolase
Et_2O	Diethyl ether
eV	Electron volts
ECOD	7-ethoxy coumarin-O-deethylase
EROD	7-ethoxyresorufin-O-deethylase
ESI-MS	Electrospray ionization-Mass spectrometry
EP	Ergosterol peroxide
EDTA	Ethylene diamine tetra acetic acid
e.g.	Example
FTIR	Fourier transform infra red spectroscopy
FABMs	Fast atom bombardment
$FeCl_3.6H_2O$	Ferric chloride

fmol	Femto moles
g	Grams
gL^{-1}	Grams per litre
gL^{-2}	Grams per two litres
γ	Gamma
HBSS	Hank's balance salt solution
^1H	Proton NMR
^1H-H^1 COSY	Correlated Spectroscopy
HMQC	Heteronuclear Multiple Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
Hz	Hertz
H_2O	water
HOCs	Hydrophobic organic compounds
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPLC-F	High performance liquid chromatography-Fluorescence
i.p.	Intra peritoneal
IR	Infra red
J	Spin-spin coupling constant [Hz]
KBr	Potassium bromide
Kg	Kilogram
KH_2PO4	Potassium dihydrogen phosphate
KCl	Potassium chloride
L	Litre
LC_{50}	Lethal concentration
LC/MS/SIM	Liquid chromatography/mass spectroscopy/ Selected ion monitoring
LMPA	Low melting point agarose
LSI	Liver somatic index
m/z	Mass to charge ratio
M^+	Molecular ion
MHz	Megahertz
MeOH	Methanol
m. p.	Melting point
mm	Millimeter
mg	Milligram
$MgSO_4.7H_2O$	Hydrated magnesium sulphate
MS/MS	Tandem mass spectroscopy
MFO	Monooxygenase
μM	Micromolar
M	Molar
$[M+H]^+$	Protonated molecular ion
$[M+Na]^+$	Sodium adduct of molecular ion
$[M+K]^+$	Potassium adduct of molecular ion
NMR	Nuclear Magnetic Resonance

NaOH	Sodium hydroxide
NaOAc	Sodium acetate
NaCl	Sodium Chloride
Na_2CO_3	Sodium carbonate
N_2	Nitrogen
Na_2HPO_4	Disodium hydrogen phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	10^{-9} metre
$NaNO_2$	Sodium nitrite
nmol	Nanomoles
NMPA	Normal melting point agarose
PAHs	Poly aromatic hydrocarbons
PAEs	Phathalic acid esters
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PDB	Potato dextrose broth
PVC	Poly vinyl chloride
ppm	Parts per million
ppb	Parts per billion
%	Percent
pH	-log10 $[H^+]$
QM^+	Quasi molecular ion
q	quartet
RNA	Ribonucleic acid
Rf	Retention factor
Rt	Retention time
Sp.	Species
S	Second
TLC	Thin Layer Chromatography
TMS	Tetra methyl silane
TOF	Time of flight
t	Triplet
TAG	Triacylglycerols
UV	Ultra violet
µg	Micrograms
µL	Microlitres
V	Volts
v/v	Volume/volume
Wt.	Weight
Z	Zusamemen

Introduction

If examined at molecular level, the basic process of life occurs in much the same way with all living organisms. This is particularly apparent if we consider the animals and plants on the earth's crust, including freshwater. All higher animals have analogous respiratory pigments and all higher plants use the same chlorophyll in photosynthesis. This leads to the same sugars and amino acids from all plants and the same proteins from all animals being formed. This is called primary metabolism and primary metabolites are the components that are involved in these processes. However, it is also true that some plants can be safely eaten while others are poisonous and the poison differs from species to species. Such toxic compounds stem from processes that start with primary metabolites. Because of this and as a consequence of the unfortunate choice of the term primary metabolism, these processes and the compounds resulting from them have been called secondary metabolism and secondary metabolites respectively. Despite their names, secondary metabolites are by no means of secondary importance to the life of the organism. What is true is that secondary metabolites are in general restricted to certain organisms where they fulfil special roles, thus contributing to the uniqueness of these organisms as well as being essential to their survival.

To avoid difficulties the alternate term **'Natural Products'** was invented for the secondary metabolites. However, secondary

metabolites are not the only ones to have a natural origin; primary metabolites have a natural origin as well.

The ocean provides a huge resource bank to the discovery of novel compounds (Cragg *et al.*, 1997). Many compounds, which are diverse, novel and bioactive, have been isolated from marine organisms. The natural compounds with existing drug-like properties (Harvey, 1999) have the potential to become new therapeutic agents for various diseases.

Besides considering how man has used marine natural products to his advantage, we also have to examine in what way marine natural products are involved in regulating marine life and thus contribute to the sharp differentiation between the marine and terrestrial ecosystems. These ecological aspects are much less understood than nutritional and pharmacological ones.

Secondary metabolites have different roles to play within and outside the organisms. Thus, they may provide defense against potential predators not only to marine invertebrates as in molluscs but also form part of the plant defense system with the production of antifeedants and phytoalexins (Dixon, 2001). They may also act as antibacterial, antifouling as exemplified by the alga belonging to family Caulerpaceae and Udotaceae (Paul & Fenical, 1987). Marine metabolites like asterosaponins from the starfish and holothurins from

sea cucumber are known to inhibit fertilization of sea urchin eggs (Ruggieri & Nigrelli, 1960; Faulkner, 1984, 1986). These are the type of molecules a chemist is interested as they are expected to be of biomedical importance.

In order to evaluate the biomedical potential of any plant or animal one must consider both the chemical ecology of the organism and its evolutionary history. It is interesting to note that the majority of marine natural products currently in clinical trials or under preclinical evaluation are produced by invertebrates such as sponges, tunicates, molluscs or bryozoans. The wealth of bioactive metabolites isolated from these soft-bodied, sessile or slow-moving invertebrates that usually lack morphological defense structures such as spines or a protective shell is no coincidence but reflects the ecological importance of these constituents for the respective invertebrates. It has been repeatedly shown that chemical defense through accumulation of toxic or distasteful natural products is an effective strategy to fight off potential predators (e.g. fishes) or to force back neighbours competing for space (Proksch & Ebel, 1998; Proksch 1999; Mc Clintock & Baker, 2001).

Recent studies (Faulkner, 1994) have shown that unique secondary metabolites are produced not only by the organisms such as sponges, soft corals, algae, ascidians, bryozoans and molluscs (Michl *et al.*,

1993, Attaway & Zaborsky, 1993) but also by microorganisms associated with these invertebrates (Davidson, 1995).

Most, if not all, marine invertebrates harbour microorganisms that include bacteria, cyanobacteria and fungi within their tissues where they reside in the extra and intra cellular space (Vacelet & Donadey, 1977; Wilkinson, 1992). In some cases these associated microorganisms may constitute up to 40% of the biomass as in Mediterranean sponge *Aplysina aerophoba* (Vacelet, 1975; Friedrich *et al.*, 1999).

On the other hand many invertebrates are filter feeders and consume microorganisms from the inhaled seawater by phagocytosis. The relationships of marine invertebrates and marine microorganisms that may serve as food or that live either permanently or temporarily inside of marine macro organisms are highly complex and far from being understood (Wilkinson, 1992; Steinert *et al.*, 2000). It is believed that microorganisms might perhaps also be involved in the biosynthesis of natural products that are recovered for example from sponges. Okadaic acid first isolated from the sponges of the genus *Halichondria* such as *H. okadai* or *H. melanodocia* with habitats in two oceans was subsequently recognized as the metabolite of a marine microalgae, the dinoflagellate, *Prorocentrum lima* (Shimizu, 2000). Some marine bio-products originally isolated from macro organisms, such as sponges, have been subsequently discovered to be localized in

microbial associates (Bewley *et al*, 1996). If these symbiotic microorganisms can be isolated and cultured, optimization of production in marine microbial bioreactors may lead to an industrially feasible supply option. If the source of the compound is the macro organism itself, development of *in vitro* production methods could provide bulk supply of the compound (El Sayed, 2000).

Development in these fields can be understood from the recent reviews wherein marine microorganisms have been referred to as a new biomedical resource (Fenical & Jenson, 1993). The same authors have commented on both the chemical and ecological perspectives of pursuing marine bacteria as a source of new secondary metabolites (Jenson & Fenical, 1994; Fenical, 1993). A significant number of marine bio-products with pharmaceutical potential have been identified from heterotrophic marine microorganisms isolated from coastal sediments (Fenical, 1993; Davidson, 1995; Kobayashi and Ishibashi, 1993).

Fungi are well represented within the marine environment (Kohlmeyer & Kohlmeyer, 1979), especially in association with wood; nevertheless, marine fungi have received very little attention from natural products chemists. As interests have turned to marine microorganisms, the fungi have begun to be recognized as a likely source of potentially useful natural products (Davidson, 1995).

All of the fungi studied have been found to be associated with solid substrates. The shell of the crab *Chinoecetes opilio* was the source of a fungus of *Phoma sp.* producing diterpenoids phomactins A, B, B1, B2 and D (Sugano *et al.,* 1991, 1994). The surfaces of marine algae are a good nutrient-rich source of microorganisms. For example, the surface of *Enteromorpha intestinalis* was the source of a strain of *Penicillium sp.* that produces the novel polycyclic alkaloids communescins A and B (Numata, 1993). Although these two molecules differ only in the structure of the acyl group, they exhibit roughly a 10-fold variance in their cytotoxicity against P388 lymphocytic leukemia (Davidson, 1995). Two classes of metabolites have been recently isolated from unidentified fungi obtained from the marine sponge *Jaspis johnstoni*, known as the source of the cyclic peptide jaspamide (also termed jasplakinolide) (Zabriskie *et al.*, 1986; Crews *et al.*, 1986).

Marine fungi are adapted to a very distinct set of environmental pressures and there is increasing evidence that these adaptations include the production of unique secondary metabolites (Jensen, 2002).

The development of seawater-based isolation and fermentation techniques have recently enabled the discovery of exciting marine

fungal metabolites such as the neomangicols, structurally unprecedented halogenated sesterterpenes that possess cytotoxic and antibacterial properties (Renner, 1998).

Clearly, marine fungi represent a frontier resource for the discovery of structurally unique secondary metabolites with anticipated biomedical potential (David *et al.*, 2003).

Filamentous fungi are good candidates for industrial production of heterologous proteins (van den Hondel *et al.*, 1991), organic acids (Kubicek & Rohr, 1986), and pigments (Hajjaj *et al.*, 1997; Theobald *et al.*, 1993). They are ubiquitous inhabitants of soil. Many of their metabolites are contaminants of feed and foodstuff. Mycotoxins are toxic metabolites produced by fungi, which when contacted by skin or ingested or inhaled can cause very serious diseases in human beings (Hsieh, 1987). They are capable of antibiotic activity, which represents a mechanism of challenge with other microorganisms. Among toxic fungi, a very important genus is *Aspergillus.* In particular, the species *A. flavus, A. fumigatus* and *A. parasiticus* are producers of very important toxic metabolites called aflatoxins. Aflatoxin B1 (AFB1) is by far the most toxic aflatoxins (Coulombe, 1993). It is potent carcinogen (Robens & Richard, 1992) and induces renal or hepatic lesions in many animal species, including human beings (Mollenhauer *et al.*, 1989; Newberne & Butler, 1969).

The sea also has been a dumping ground for thousands of years, offloading rubbish, sewage and more recently industrial waste. Marine pollution frequently originates on land, entering the sea via rivers and pipelines. This means that coastal waters are much more polluted than the open seas, especially estuaries and harbours being badly affected. Additional pollution is actually created at sea by activities such as dredging, drilling for oil and minerals and shipping. By the latter half of the 1990s, it has become clear that oceans, routinely treated as limitless sources and sinks for human consumption and waste, were changing in response to intense fishing, pollution and climate change (NRC, 1995).

Most of the coastal areas of the world have been reported to be damaged from pollution, significantly affecting commercial coastal and marine fisheries. Therefore, control of aquatic pollution has been identified as an immediate need for sustained management and conservation of the existing fisheries and aquatic resources (Islam & Tanaka, 2004).

Many synthetic organic chemicals (e.g. organochlorines, organophosphates, polycyclic aromatic hydrocarbons and organometals) are of growing environmental concern, because of their toxicity and high persistence in the environment and in biological systems. Furthermore, the high lipophilicity of many of these

xenobiotics greatly enhances their bioconcentration/ biomagnification, thereby posing potential health hazards on predators at higher trophic levels (including human beings). Nowadays, persistent xenobiotic compounds have been found in every part of the ocean: from Arctic to Antarctic and from intertidal to abyssal (Islam & Tanaka, 2004).

Most xenobiotic compounds occur only at very low concentrations in the environment, and their threats to marine life and public health are still not well understood. However, sub-lethal effects of these compounds over long-term exposure may cause significant damage to marine population, particularly considering that some of these compounds may impair reproduction functions of animals while others may be carcinogenic, mutagenic or teratogenic (Islam & Tanaka, 2004). The biomarker approach is suggested as a suitable strategy for studying sublethal effects of pollutants, providing an early indication of possible adverse effects in the organisms (Hugget *et al.*, 1992).

The work reported in this thesis deals mainly with (1) fungal metabolites of industrial importance, (2) fish biliary metabolites that are used as biomarkers of polycyclic aromatic hydrocarbon (PAH) pollution and bioremediation of PAHs (3) anti-protozoal and anti-microbial activity of some synthetic analogs of benzoxazoline-2-one a constituent of mangrove *Acanthus ilicifolius*.

It has been divided into three chapters:

Chapter 1: is a presentation of the study carried out on the secondary metabolites produced by marine fungus *Aspergillus sulphureus* isolated from marine grass environment. It has been sub divided into the following sections.

Section I: reviews the literature on the genus *Aspergillus.*

Section II: describes the isolation and structural elucidation of secondary metabolites produced by *Aspergillus sulphureus* using NMR and ESI-MS techniques. These include:

(i) Kojic acid

(ii) Major lipids with special reference to triglycerides, sterols and phthalates.

Section III: deals with the attempts made to enhance the production of Kojic acid, an industrially important compound by using alginate elicitor and different synthetic and commercial culture media.

Section IV: In this section, the isolation and structural elucidation of acetyl cyclopiazonic acid a novel antifungal metabolite that was obtained from the culture medium of *Aspergillus sulphureus* during the

course of investigations on the enhancement of kojic acid production has been dealt with.

Chapter 2: studies the effect of Polycyclic Aromatic Hydrocarbon (PAH) pollutants on the estuarine fish *Oreochromis mossambicus* in the presence of nitrite. Bioremediation of these pollutants using biosurfactants (saponins) has also been evaluated. It has been divided into the following sections.

Section I: gives a broad description of polycyclic aromatic hydrocarbons in the marine environment.

Section II: The effect of nitrite on the metabolism and impact of phenanthrene in the estuarine fish, *Oreochromis mossambicus* has been discussed in this section. The metabolites and DNA adducts formed in the above study have been characterised by ESI-MS.

Section III: The studies made on the emulsifying and surfactant properties of saponins with respect to bioavailability and bioelimination of PAH has been reported in this section. The novel saponin was isolated from a mangrove plant *Lumnitzera racemosa* and identified on the basis of spectral data.

Chapter 3: Miscellaneous studies

In this chapter antimicrobial and leishmanicidal activities of Benzoxazoline-2-one and its derivatives have been reported.

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Chapter 1

Study of Secondary Metabolites of Marine Fungus Aspergillus sulphureus

Section I

Genus Aspergillus: Literature Review

INTRODUCTION

For more than four decades, there has been an ongoing quest to discover new drugs from the sea. Most efforts have been directed towards chemical studies of marine invertebrates. Although, these studies have proved that marine invertebrates are an important source of new biomedical leads, a fact well demonstrated by the number of compounds currently in clinical trials, it has proven notoriously difficult to obtain adequate, reliable, and most importantly, renewable supplies of these compounds from nature. Compounding the supply problem is the inherent structural complexity of many marine natural products, which most often eliminates the possibility of commercially viable synthesis. Because of these problems, and the fact that more than 40 years of extensive research has shown that it is difficult to isolate novel metabolites from marine invertebrates, a new avenue of study focusing on marine microorganisms has been garnering considerable attention.

As evident from the literature, marine microorganisms including bacteria, fungi and micro algae have received increasing attention over the past two decades. In recent reviews, marine microorganisms have been introduced as a new biomedical resource.

From the discovery of penicillin more than 60 years ago for the treatment of microbial infection, to the more recent anticancer chemotherapeutic agent adriamycin and the important immuno-suppressant drug cyclosporin A, microorganisms have yielded over 120 of today's most important medicines. In an effort to improve the rates of discovery of biologically active novel secondary metabolites, marine microbial resources are being sought. Bacteria and fungi isolated from marine environments, namely seawater, sediments and marine organisms often produce metabolites identical or similar to those from terrestrial species.

Although, new biomedically active compounds are being isolated from marine microorganisms, the potential for any one of these metabolites to reach the clinic is very much dependent upon the aggressiveness with which they are tested in diverse disease areas. The biomedically relevant secondary metabolites reported to date from marine microorganisms represent diverse structure classes that include terpenes, peptides, polyketides and compounds of mixed biosynthetic origin. The producing strains range from obligate marine bacteria such as *Flavobacterium ulginosum* to ubiquitous fungal genera such as *Penicillium.*

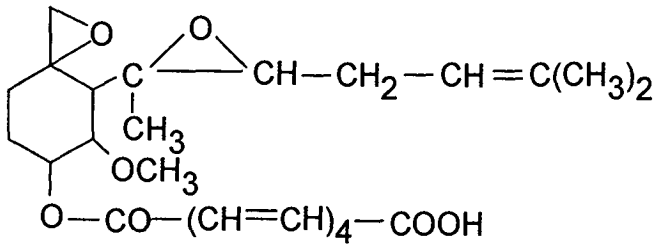
In the recent years, chemical studies of culturable marine microorganisms have led to the discovery of numerous, structurally

novel, biologically active secondary metabolites (Fenical, 1993; Davidson, 1995; Kobayashi & Ishibashi, 1993; Okami, 1993). Although most of these compounds were isolated from marine bacteria, a growing number of new structures have been reported from filamentous marine fungi. The fungi producing these novel metabolites have been collected from diverse marine sources including fish, algae, invertebrates and sediments. Marine strains may produce unusual secondary metabolites as an environmental adaptation.

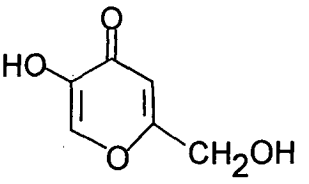
LITERATURE REVIEW

Aspergillus species are well known common inhabitants of our environment recognized as organisms of economic, public health and general biomedical importance. Certain species are useful and at the same time harmful, e.g. *A. fumigatus*, which produces fumagillin (Eble & Hanson, 1951) an antibiotic once used as an amoebicide, may also cause aspergillosis in man and animals (Wilson, 1971).

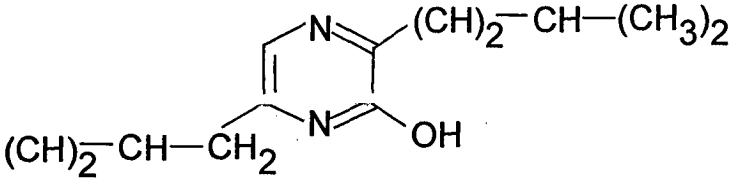
Kojic acid a metabolite of *A. flavus* and *A. oryzae* (Ogawa *et al.*, 1995) is useful as a starting material for the synthesis of flavour enhancing food additive. *A. oryzae* is also known to be a source of flavacol besides aspergillic acid and its related compounds (Wilson, 1971). Flavacol is also reported to be a metabolic product of *Aspergillus sclerotiorum*.



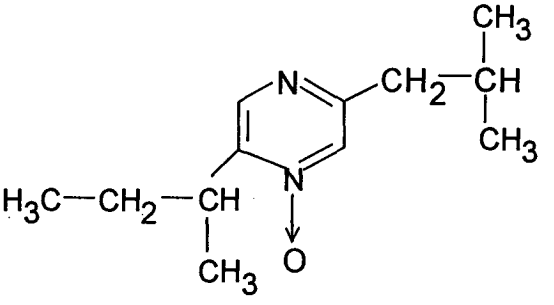
FUMAGILLIN



KOJIC ACID



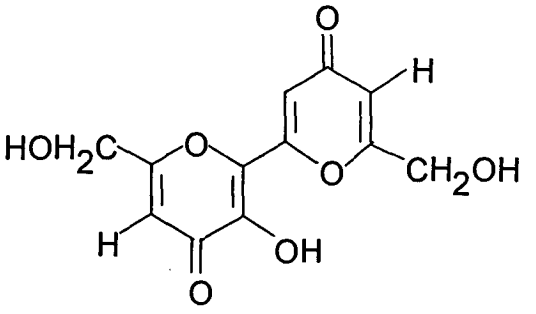
FLAVACOL



ASPERGILLIC ACID

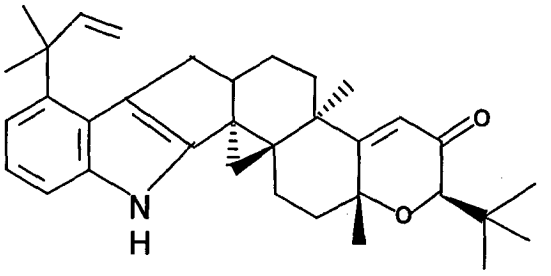
Invasion of developing cottonseed *Gossypium hirsutum* by aflatoxigenic *Aspergillus sp.*, *A. flavus* and *A. parasiticus* is reported to

yield a bright greenish yellow fluorescent compound known as BGY-F as a result of reaction of host plant peroxidase with the fungal metabolite, kojic acid (Hampden *et al.*, 1999). The following structure has been assigned to it.



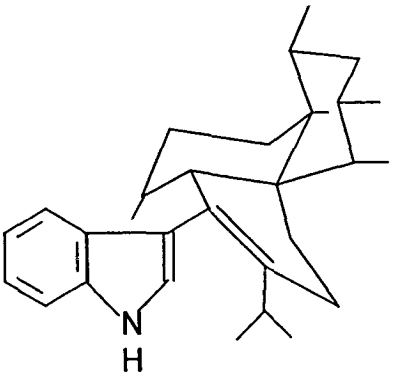
BGY-F

Several strains of *Aspergillus flavus* grown on various food materials (Rex *et al.*, 1980b; Wilson & Wilson, 1964) are reported to produce aflatrem (Rex *et al.*, 1980a), a toxin, which causes trembling and convulsion in mice and if the dose is high enough, death (Wilson, 1977; Wilson & Wilson, 1964).



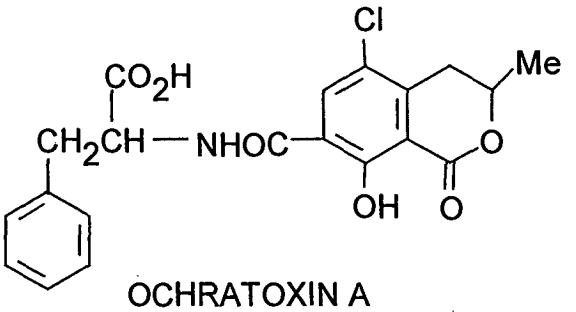
AFLATREM

Aflavinine is yet another indole metabolite reported from the tremogen producing strains of *A. flavus* species.

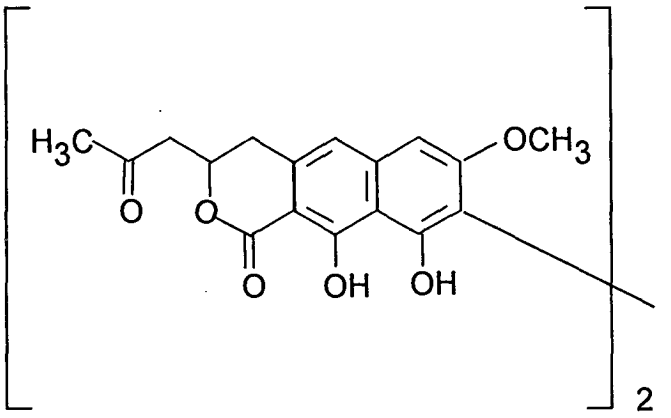


AFLAVININE

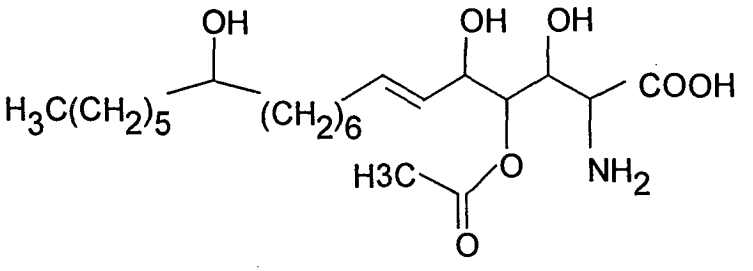
A. ochraceus is also known to be a source of cyclodepsipeptides destruxins A and B. Ochratoxin A, a mycotoxin discovered in 1965 as a secondary metabolite of *A. ochraceus* strain (Sco, 1965; Van der Merwe *et al.*, 1965), was subsequently found to be present in many other species of the genus *Aspergillus* and *Penicillium* including *A. alliaceus, A. ostianus, A. sclerotiorum, A. sulphureus, A. melleus* and *A. petrakii*. An antibacterial antibiotic SC-28762/viriditoxin (Jiu & Mizuba, 1974), and an antifungal antibiotic fumifungin (Mukhopadhyay *et al.*, 1987) are reported to be metabolites of *A. fumigatus* Fresenius, isolated from Himalayan soil samples.



OCHRATOXIN A



VIRIDITOXIN



FUMIFUNGIN

Certain fungi produce specialized resting bodies known as sclerotia as a means for surviving adverse environmental conditions, which other fungal bodies cannot tolerate such as harsh climate, nutrient deficiency and desiccation. Generally sclerotia remain viable in soil for several years, and provide primary inoculums from the producing species where conditions again become favourable for fungal growth. Sclerotia are found under natural conditions or in solid substrate fermentations, but are not commonly produced in the liquid fermentation cultures generally employed in studies of microbial

metabolites. Accordingly, many novel metabolites of common fungi such as *Aspergillus* have not been characterized.

Sclerotia have been recently recognized as potential source of natural insecticide. Many sclerotia, which are subjected to predation by fungivorous insects and anthropods during their period of dormancy in soil, have been shown to contain metabolites that exert adverse physiological effects on insects. Gloer *et al* (1988) and Wicklow *et al* (1988) have reported the isolation of four anti-insectant aflavinine derivatives from the sclerotia of *A. flavus* used in controlling the dried fruit beetle *Carpophilus hemipterus* (Nitidulidae: Coleoptera). From the same source Gloer *et al* (1989) and TePaske *et al* (1990) have reported a related metabolite, aflavazole. An insecticidal indole diterpene, nominine useful in controlling the corn earworm *Helicopverva zea* (Lepidoptera) has also been identified by Gloer *et al* (1989). It is evident from the literature, as mentioned above, that genus *Aspergillus* produces insecticidal compounds.

Our interest in biologically active compounds from marine source under the project, "Development of Potential Drugs from the Indian Ocean", led us to examine *Aspergillus sulphureus*, a fungus isolated from the sediment of sea grass, for its secondary metabolites.

Aspergillus sulphureus (Fresenius) Thom and Church belongs to order Ascomycetes, subclass Euascomycetes, group Plectomycetes and family Aspergillaceae. It is a facultative marine fungus, i.e. those from fresh water or terrestrial origin able to grow and sporulate in the marine environment.

A. sulphureus has been extensively studied by different groups of workers. Many studies have evaluated the toxicity of mycotoxins to mammals, but there is little information on their action against fungal cells, even though mycotoxins are frequently acting against fungi in nature. A crude extract of *A. sulphureus* is reported to have dose dependent growth inhibiting effect on *Cryptococcus neoformans*. Out of eleven *Aspergillus* species, extracts of *A. sulphureus*, *A. ochraceus* and *A. konagawaensis* were effective against two mosquito species causing 80% mortality in second stage larvae of *Aedes fluvitilis* and *Culex quinquefasciatus* (De Moraes *et al.*, 2001). Pigments, xanthomegnin, viomellin, rubrosulphin and viopurpurin, are known to be metabolites of *A. sulphureus* and *A. melleus* (Durley *et al.*, 1975; Simpson, 1977). Indole compounds like radarins, sulpinines A, B and C, secopenitrem A and B and 10-oxo-11, 33-dihydropenitrem B, from *A. sulphureus* are effective in controlling coleopteran and lepidopteran insects (Laakso *et al.*, U.S.P. 1992 (a, b), 1993, 1994).

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Section II

Secondary Metabolites Produced by Aspergillus sulphureus

INTRODUCTION

In the last few years marine microorganisms have emerged as a new source for the discovery of novel biologically active compounds (Fenical, 1993; Kohlmeyer and Kohlmeyer, 1979; Fenical and Jensen, 1993). These microorganisms can originate and be isolated from sediments, and also from open oceans or marine surfaces including marine living organisms.

In a program searching for bioactive compounds biosynthesized by marine fungi we studied a fungal strain, *Aspergillus sulphureus* (Fresenius) Thom & Church, isolated from sea grass sediment. *Aspergilli* are industrially important microrganisms due to their production of organic acids, statins and various extracellular enzymes. There are reports on it being a producer of ribonuclease (Gomes *et al.*, 1998). Recently, Nemec *et al.*, (1997) analysed free and hydrolysed fatty acids and sterols of five different *Aspergillus sp.* and two *A. niger* strains as chemotaxonomic markers for their characterization. The present work is concerned with the isolation and structural characterization of 2-hydroxymethyl-5-hydroxy-γ-pyrone (Kojic Acid) **(1)** and major lipids produced by this fungal strain. These include triacyl glycerols: 1, 3-distearoyl-2-palmitoleoyl glycerol **(2)**, 1, 3-distearoyl–2-oleoyl glycerol **(3)** and 1, 3-dipalmitoyl-2-oleoyl glycerol **(4)**, 1, 3-dioleoyl-2-palmitoleyl glycerol **(5)**, tristearoyl glycerol **(6)**,

phthalate esters: o-phthalic acid bis- (n-octadecyl 9Z, 12Z-diene) ester **(7)** and bis-(n-pentadecyl) phthalate **(8)**. Besides, the extract contained other triglycerides belonging to C_{46} and C_{48} groups but in lesser concentrations. So far, no literature is available on the lipid components including triacylglycerols of the fungus studied in the present investigation. Triacylglycerols as source of these lipids particularly those containing high proportions of polyunsaturated fatty acids of nutritional value are now in commercial production. Phthalates degradation by microorganisms is known but their biosynthesis by fungus is being reported here for the first time. Similarly, *Aspergillus sulphureus* has also been found to be a new fungal source of kojic acid reported herein for the first time.

This section deals mainly with the identification of above mentioned metabolites of *A. sulphureus* produced when mycological broth was used as the fermentation medium. Filtration of crude n-butanol extract through Sephadex LH-20 followed by repeated chromatography over silica gel and gradient elution with methanol:chloroform yielded light yellow crystals of compound **1**, m.p. 154°C. ESI-MS **(Fig.1)** in negative mode exhibited intense $[M-H]^-$ ion at m/z 141 and in positive mode it showed peaks at m/z 143 $[M+H]^+$, 165 $[M+Na]^+$ and $[M+K]^+$ at m/z 181. ESI-MS in positive mode also displayed additional peaks at m/z 307 $[2M+Na]^+$ and m/z 449 $[3M+Na]^+$. This suggested that the molecular weight of the compound **1** is 142 amu.

Its IR spectrum (KBr pellet) **(Fig.2)** showed absorption at 3176 cm^{-1} and 1660 cm^{-1} due to hydroxyl (-OH) and α,β-unsaturated carbonyl group respectively. The ^1H NMR spectrum (300 MHz, DMSO) **(Fig.3, Table 1)** indicated two singlets, each for one olefenic proton, at δ 6.33 and 8.02.

Table 1: ^1H and ^{13}C NMR data of compound 1

Carbon No.	δ_H (DMSO)	δ_H (D_2O exchange)	δ_C (DMSO)	δ_C (CD_3OD)
2			167.5 (s)	170.4 (s)
3	6.33(s, 1H)	6.33 (s)	109.3 (d)	110.8 (d)
4			173.4 (s)	176.8 (s)
5			145.1 (s)	147.4 (s)
6	8.02 (s, 1H)	8.02 (s)	138.7 (d)	141.0 (d)
7	4.28 (d, 2H, J=4.5Hz)	4.28 (s)	58.9 (t)	61.2 (t)
5-OH	9.05 (s, 1H)			
7-OH	5.68 (t, 1H)			

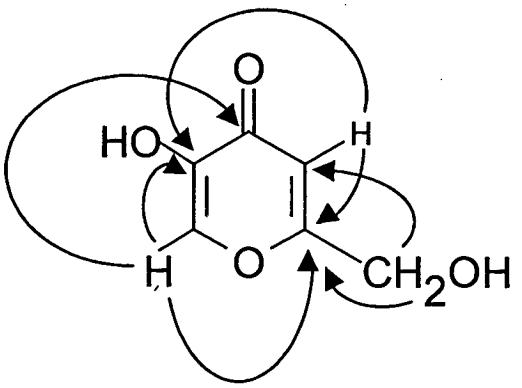
A doublet, integrated for two protons was evident at δ 4.28 (J=4.5Hz) and indicated presence of a methylene group adjacent to an oxygen function. Two D_2O exchangeable protons at δ 5.68 (triplet) and broad singlet at δ 9.05 were assigned to –OH groups, one next to methylene (methylene doublet at δ 4.28 is reduced to a singlet on D_2O exchange) and the other –OH on a quarternary carbon. The signal at δ 9.05 was

assigned to –OH and not –NH on the basis of even molecular weight. A less intense signal at δ 2.5 and an intense broad singlet at δ 3.3 were assigned to the methyl groups of DMSO-d_6 and the HOD (in DMSO). The ^{13}C NMR spectrum (300 MHz, DMSO & CD_3OD) **(Fig.4, Table 1)** of compound **1** showed signals which on the basis of DEPT experiments **(Fig.5)** were inferred as three quarternary carbons at δ 170,176 and 145, two methines at δ 110 and 138 and one methylene carbon at δ 61.2.

Proton carbon chemical shift correlation for all the carbons directly bonded to the protons was established by HMQC experiment **(Fig.6)**. The cross peaks from the HMQC spectrum **(Table 2)** identified the sets of directly bonded carbon and hydrogens.

Table 2: HMQC & HMBC data of (1)

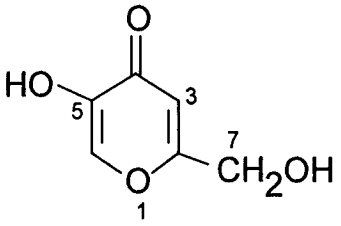
HMQC		HMBC	
δ_H	δ_C	δ_H	δ_C
4.28	58.9 (CH_2)	8.02	145, 170, 176
6.33	109.4 (CH)	6.33	145, 170
8.00	138.7 (CH)	4.28	110, 170



Scheme 1: illustrates the use of HMBC to build structural segments.

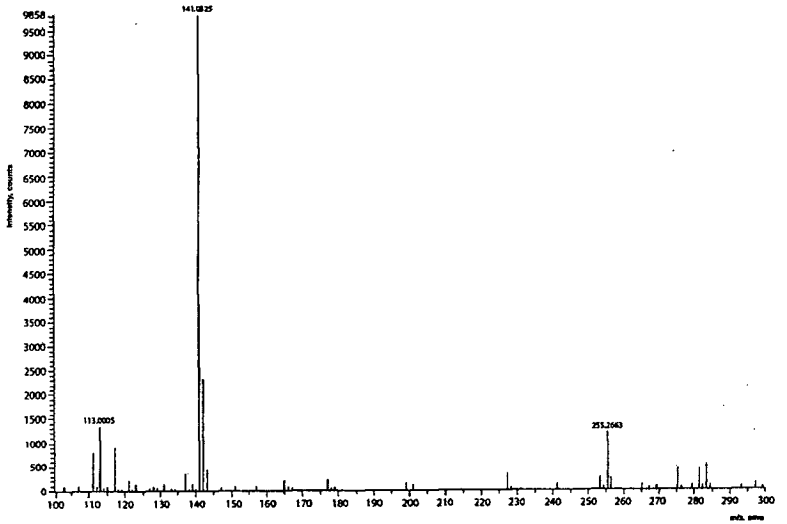
HMBC experiment **(Fig.7, Scheme 1)** established the long range proton carbon chemical shift correlation .The spectrum showed correlation of H-6 proton with C-5, C-2 and C-4; the olefenic H-3 proton showed correlation with C-5 and C-2. Similarly methylene protons H-7 correlated with C-2 and C-3. Thus, compound **1** was identified as 2-hydroxymethy-5-hydroxy-γ-pyrone, which is identical to kojic acid.

On the basis of above spectral data, the compound was identified as 2-hydroxymethyl-5-hydroxy-γ- pyrone (Kojic acid). The data is well in agreement with the values reported in the literature (Wilson, 1971).



1

Kojic acid is an organic acid produced by various fungal or bacterial strains, such as *Aspergillus oryzae, Penicillium* or *Acetobacter* species (Gerhard *et al.*, 2004) during fermentation of various carbon containing substrates (Chen *et al.*, 1991a). Kojic acid continues to draw attention due to its varied application in the field of medicine (anti-inflammatory and painkiller) (Chen *et al.*, 1991a), food science (precursor for flavor enhancers (Le Blanc *et al.*, 1989) and anti-browning agent) (Chen *et al.*, 1991b) and cosmetic industry (as whitening agent) (Ohyama & Mishima, 1990). Kojic acid has many biological functions such as insecticide and antibiotic. In addition it may have other uses in the future for the production of novel biodegradable plastics from kojic acid, if it can be produced industrially at reasonable cost (Futamura *et al.*, 2001). Based on the literature survey, it was found that *Aspergillus sulphureus* is a new source of kojic acid.



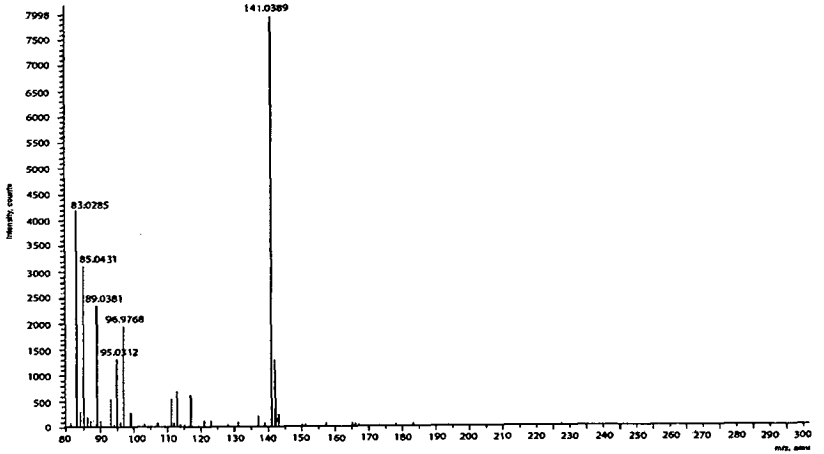
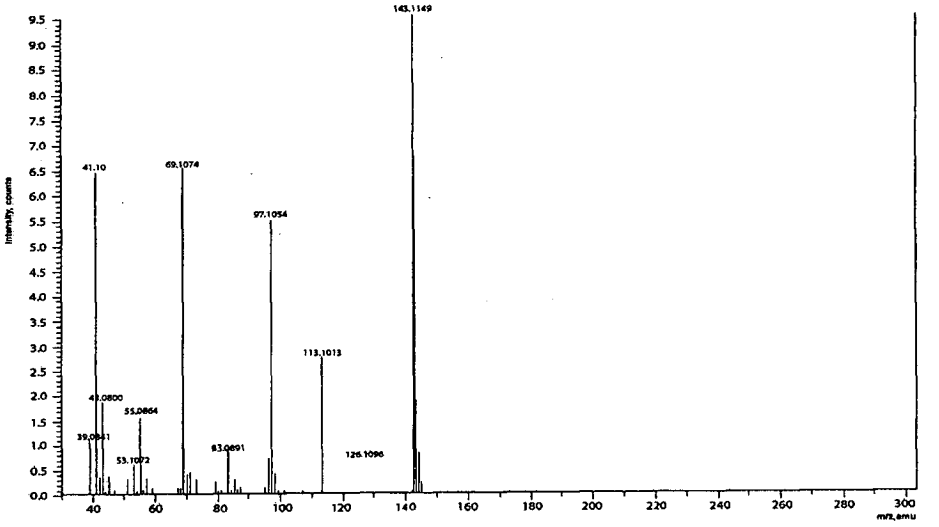
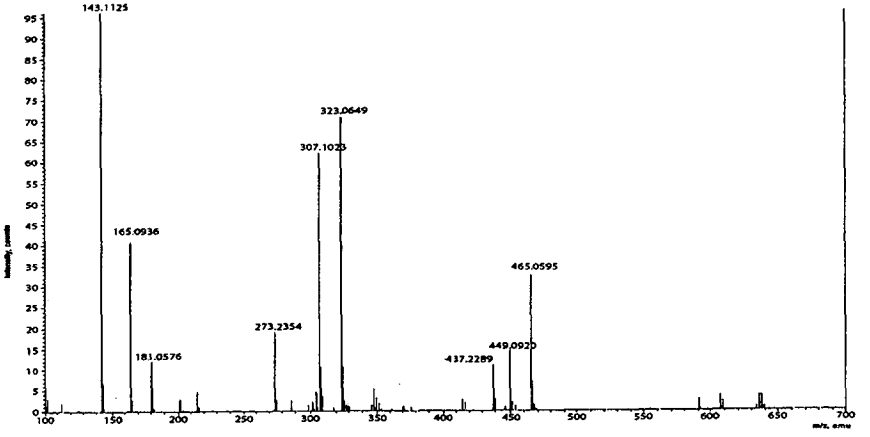


Fig.1: ESI-MS of kojic acid in the negative ion mode





ESI-MS of kojic acid in the positive ion mode

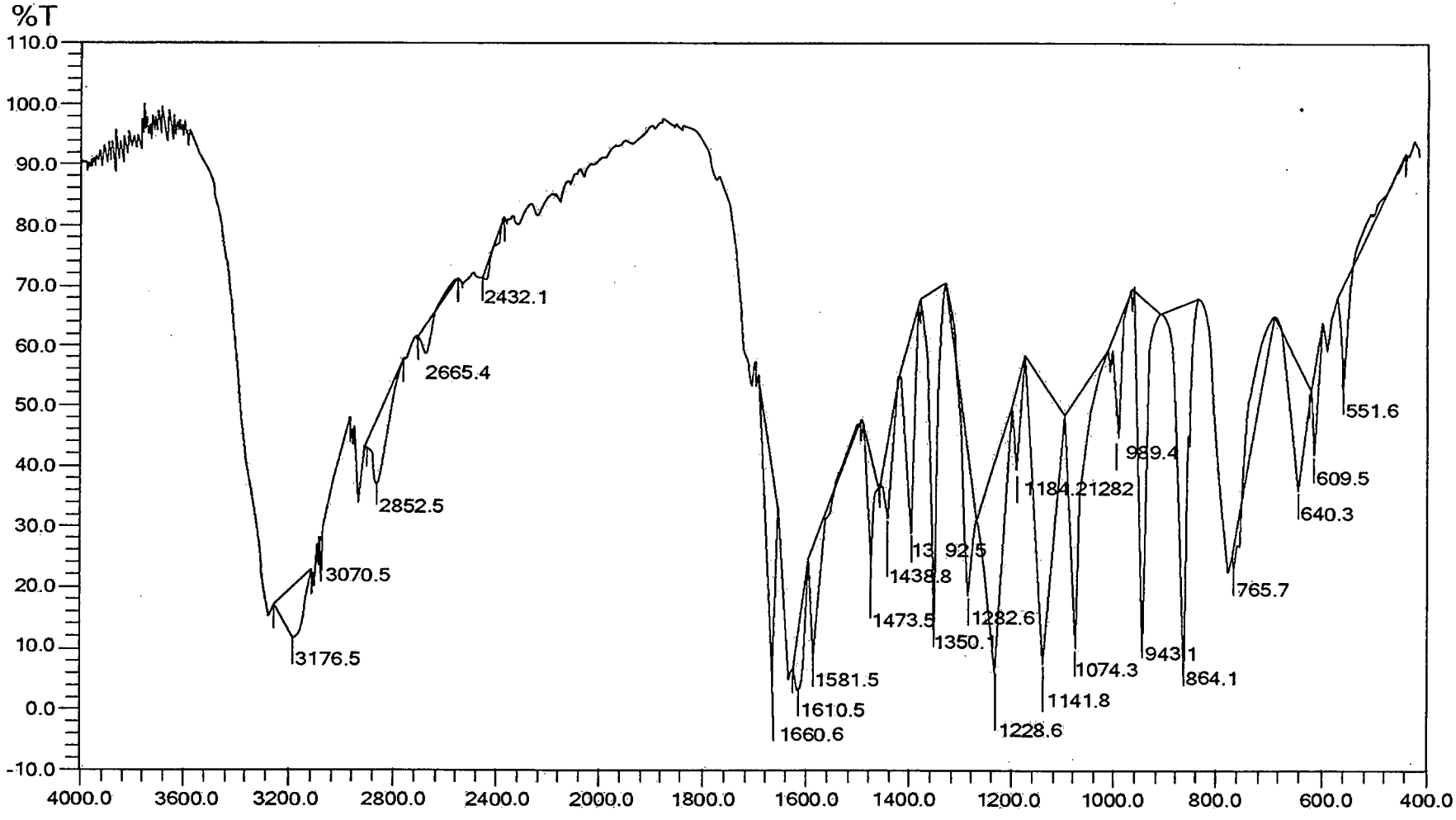


Fig.2: IR spectra of kojic acid

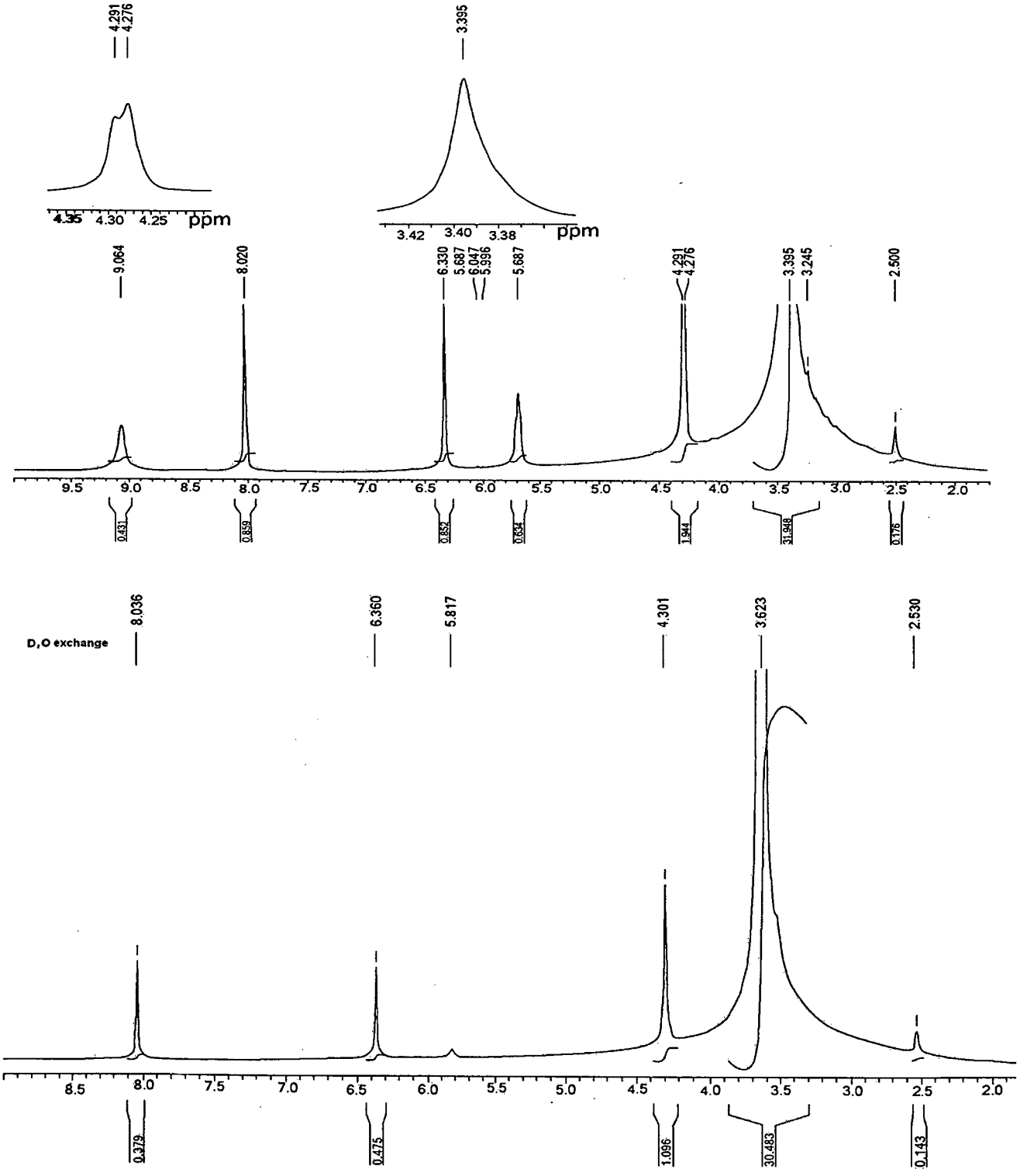


Fig.3: ^{1}H NMR spectra of kojic acid

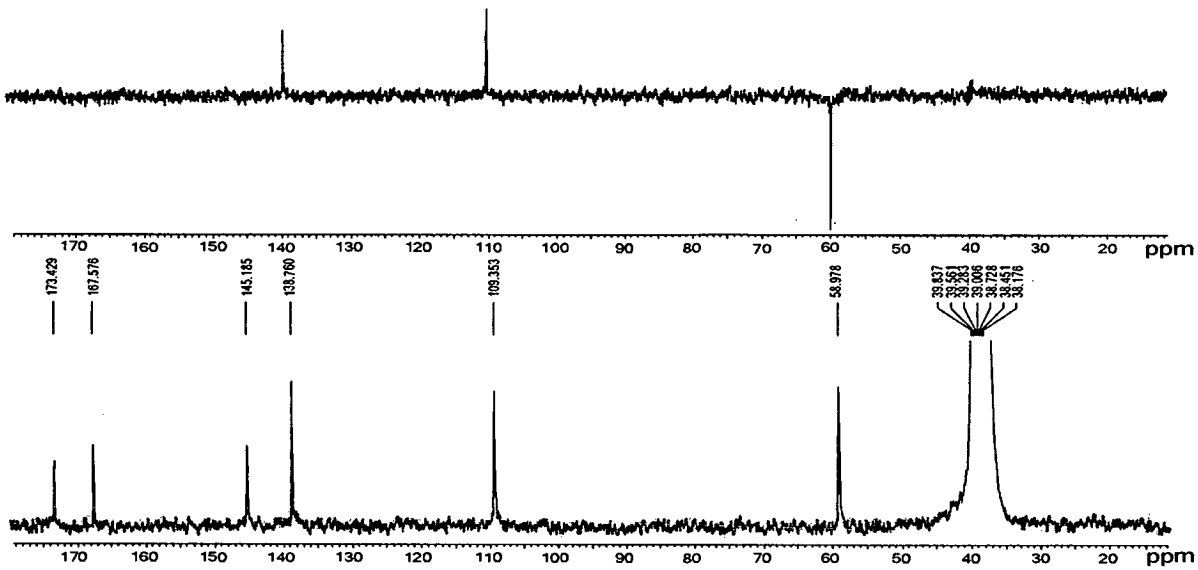


Fig.5: ^{13}C NMR & DEPT spectra of kojic acid (DMSO)

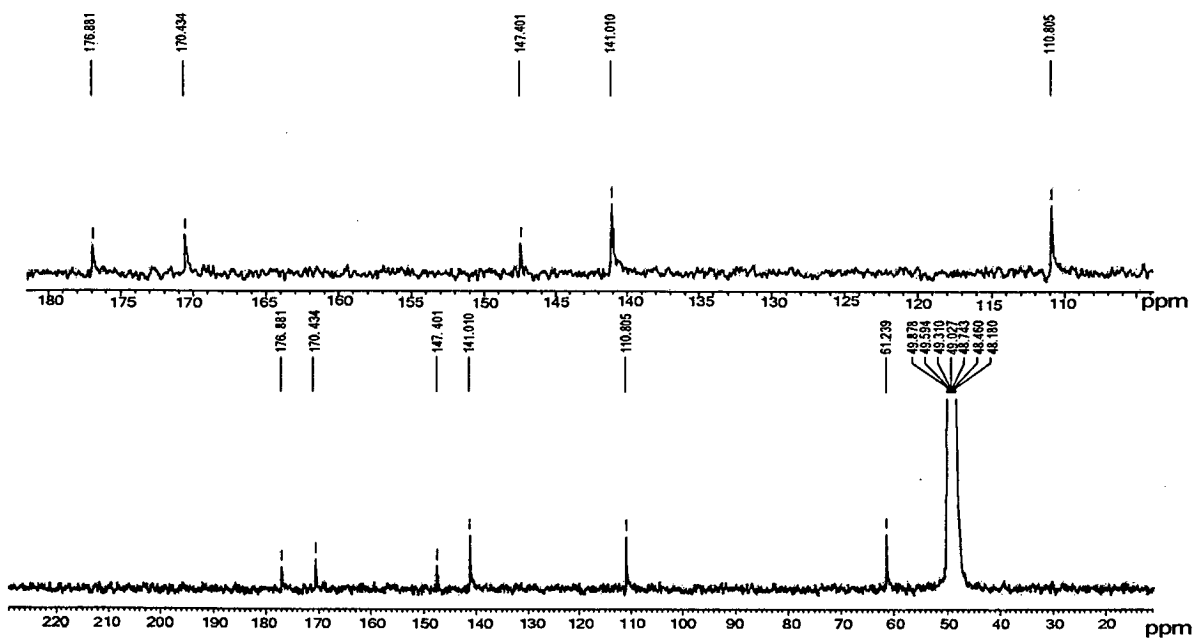


Fig.4: ^{13}C NMR & DEPT spectra of kojic acid (in methanol)

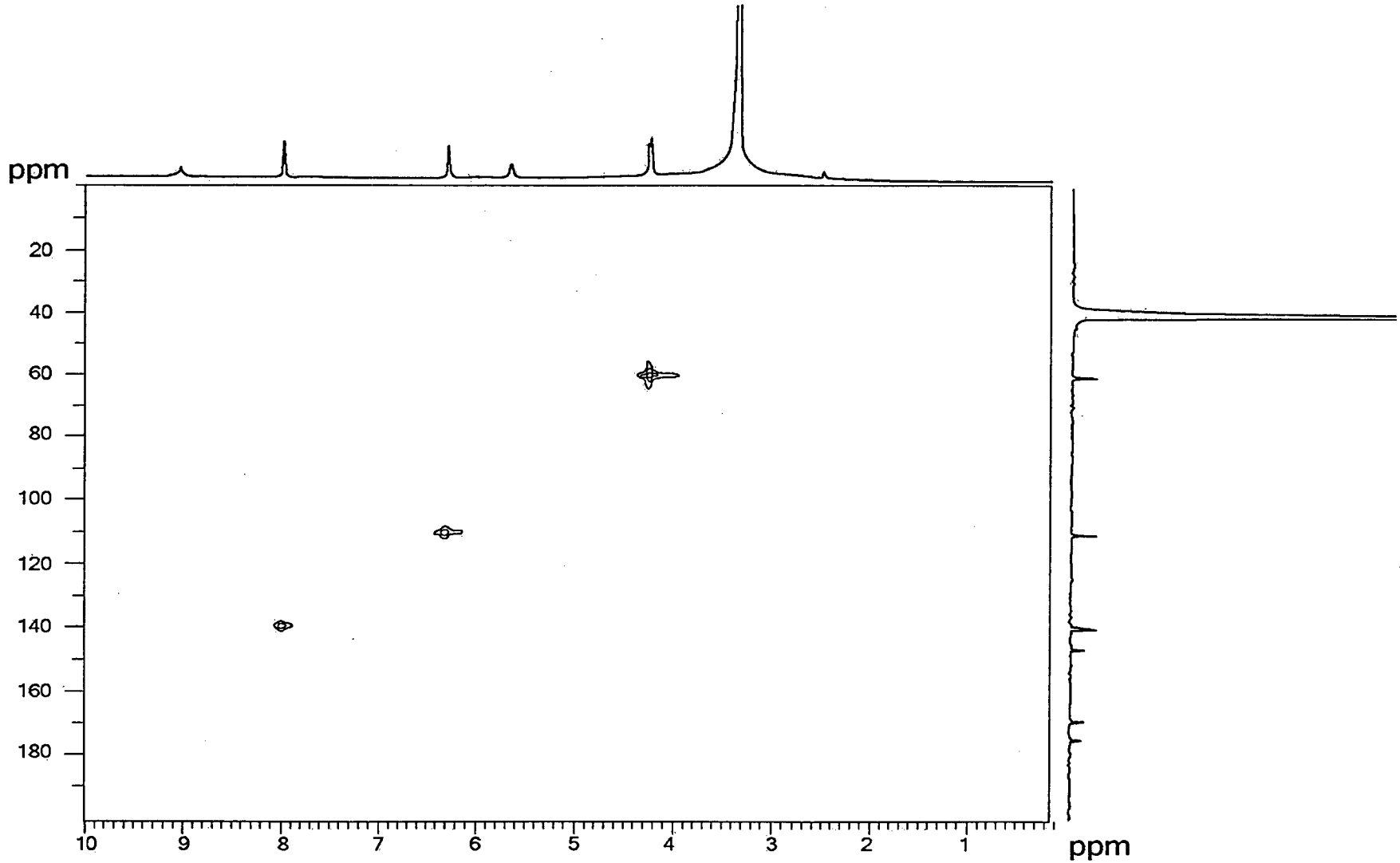


Fig.6: HMQC spectra of kojic acid

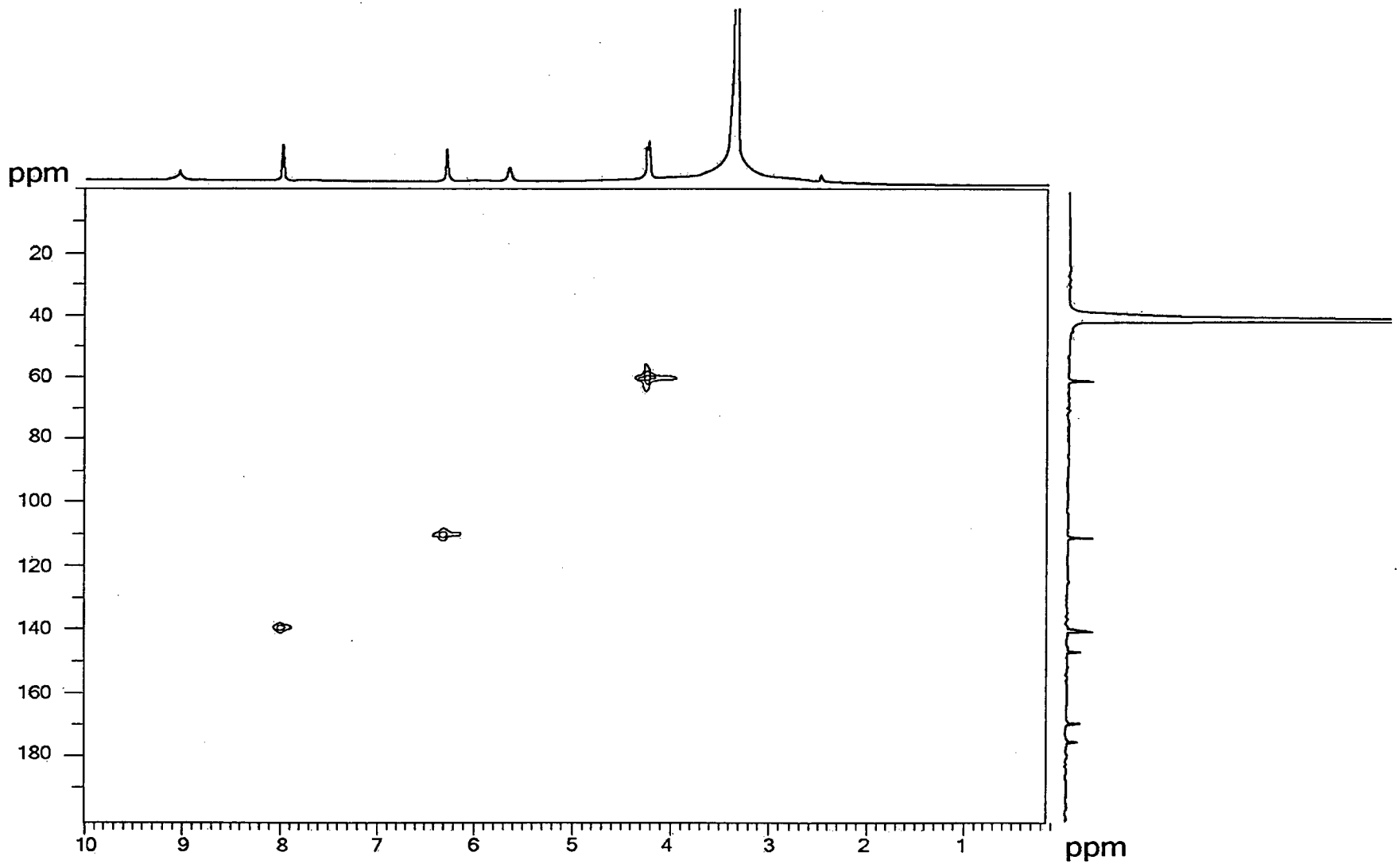


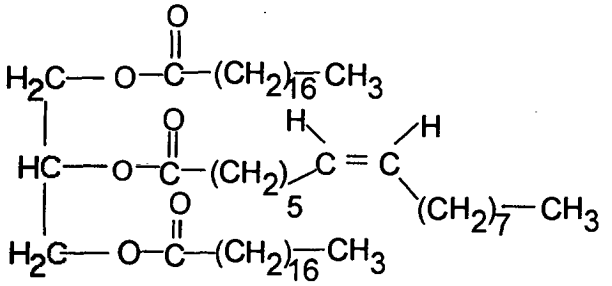
Fig.6: HMQC spectra of kojic acid

Mass spectral and NMR spectroscopic analysis of lipids:

Chloroform soluble lipid fraction chromatographed over silica gel column (60-120 mesh) and gradient eluted with increasing concentrations of ethyl acetate in petroleum ether yielded four major compounds A, B, C, and D in order of their increasing polarity and in a chromatographically pure state evidenced by homogeneity on TLC plates.

On the basis of spectral data compound A was identified as a triglyceride (TAG) 1,3-distearoyl-2-palmitoleyl glycerol (18^0:16^1:18^0), as the major component. In ^{1}H NMR **(Table 3, Fig.A1)** the backbone glycerol sn2 proton multiplet was observed at δ5.27. The magnetically nonequivalent sn 1, 3 downfield and upfield protons resonated at δ4.3 (dd, J=4.5) and 4.15 (dd, J=6.12 Hz). The signal for terminal methyl of aliphatic chain was observed as a triplet at δ0.884 (J=6.9Hz). The existence of an aliphatic side chain was evident from the absorption in the region δ1.0-2.4. A broad singlet at δ1.3 (-CH_2 of aliphatic chain), a quintet centered at δ1.62 (4H, β to the ester carbonyl), a multiplet at δ2.1 (4H, α to the olefinic methine), a triplet at δ2.3 (protons adjacent to carbonyl group) and a triplet at δ2.75 is indicative of polyunsaturated fatty chain. The signal at δ2.75 arises from allylic methylene protons within the series of double bonds in the chain

[-CH=CH-CH_2- (CH=CH-CH_2)$_n$] with n≥1. For linoleic acid, n=1, its specific methylene gives a triplet at δ2.75. The signal at δ5.34 was assigned to the olefinic protons.



1, 3-distearoyl-2-palmitoleoyl glycerol

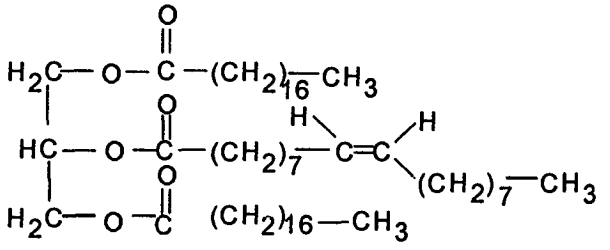
2

The ^{13}C NMR spectrum including DEPT **(Fig.A2 and Fig.A3)** indicated the presence of signals for two carbonyl groups (δ173.29 and 172.84) of α- and β- acyl fatty chain respectively. It also contained signals for terminal methyl at δ 14.01 and methylenes (-CH_2)$_n$ - from δ22.56 to δ34.18 with the most predominant at δ29.69 and 29.65. The most intense signals for double bonds were found at δ130.0 and 129.66 accompanied by less intense signals at δ130.2, 129.69, 128.06 and 127.87. The presence of glycerol moiety was confirmed by the signal at δ62.02 corresponding to the substituents at C_1 and C_3 by o-acyl group and the doublet at δ 68.8 was assigned to C_2 of glycerol substituted by o-acyl chain.

The shift values of the signals at δ129.66 and 130.0, the most intense signals in the region of ethylenic carbon atoms are well in agreement for an oleate (18:1) group in the β-acyl position and with the double bond at Δ^9 (C_9/C_{10}) position having Z configuration. Less intense ethylenic signals at 129.69/130.2 were assigned to palmitoleate group of β-acyl chain with Δ^7 (C_7/C_8) double bond in Z configuration (Marcel *et al.*, 1995a). Signals of moderate intensity at δ129.96, 128.06/127.87 and δ130.2 match more closely with a linoleate (18:2) group in β-position of glycerol with C_9/C_{10} | C_{12}/C_{13} double bonds in Z configuration (Marcel *et al.*, 1995b). From the presence of only two signals (δ173.29 and 172.84) in the carbonyl region and their intensities it is apparent that saturated fatty acids are distributed over the 1, 3 position and unsaturated fatty acids have preference for position 2 of glycerol (Mannina *et al.*, 1999). Interestingly from the intensity of the signals in the olefinic region, among unsaturated acids oleic acid (δ129.6 and 130.0) has even more marked preference for position 2 than palmitoleic or linoleic acids. This means that compound A though TLC pure is a mixture of triglycerides. This is supported by CI-MS (chemical ionization-mass spectra) as well as ESI-MS spectra of the compound.

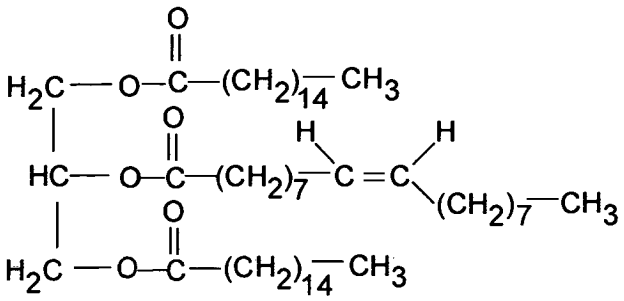
CI-MS spectra of triglycerides when ammonia is used as the reagent gas $[M+NH_4]^+$ ion is recorded as quasimolecular ion QM^+ which is also the base peak, as for the fragment ions only $[M+H-RCO_2H]^+$ ion is

recorded (Murata & Takahashi, 1977). In positive CI-MS of A **(Fig. A4)** QM^+ for the C_{52} group are recorded with a high intensity which means that triglycerides with C_{52} are the main components of A, with the major component having the most intense QM^+ at m/z 878 being the triglyceride where the three fatty acids are $18^0:16^1:18^0$. This is further evidenced by the presence of $[M+H-RCO_2H]^+$ ions at m/z 577 and 607 and of $[M+NH_4]^+$ ions at m/z 272.2 and 302 corresponding to palmitoleic and stearic acids respectively. CI-MS of A is also indicative of the presence of triglycerides with C_{54} and C_{50} evidenced by the presence of QM^+ at m/z 906 and 850 respectively where the main component in C_{54} group has the acyl composition $18^0:18^1:18^0$, the triglycerides being 1,3-distearoyl-2-oleoyl glycerol and in C_{50} group the main constituent is $16^0:18^1:16^0$ (fragmentation at m/z 577 and 551), the corresponding glyceride being 1, 3-dipalmitoyl-2-oleoyl glycerol. Thus compound A is mainly 1, 3-distearoyl-2-palmitoleyl glycerol **(2)** with considerable amounts of 1, 3-distearoyl-2-oleoyl glycerol **(3)** and 1, 3-dipalmitoyl-2-oleoyl glycerol **(4)**.



1, 3-distearoyl-2-oleoyl glycerol

3



1, 3-dipalmitoyl-2-oleolyl Glycerol

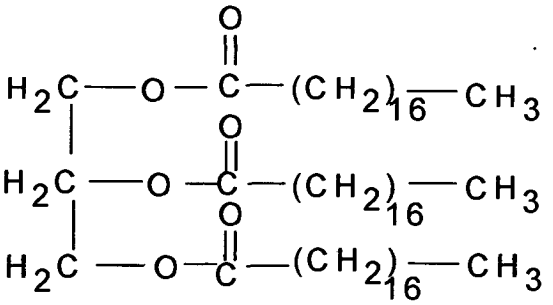
4

Glycerolipids purified from biological sources are often mixtures of several molecular species, which differ in the chain length of fatty acyl group and the double bond position and the degree of unsaturation in the fatty acyl group. As it is difficult and time consuming to separate mixtures into each molecular species, ESI-MS is the latest technique to identify them without purification. Hence, it was further confirmed by ESI-MS.

ESI-MS of the compound A **(Fig.A5)** is in conformity with CI-MS in that it is indicative of A being primarily a mixture of triglycerides with the major component being triglyceride with M^+ 860, evidenced by the most abundant molecular adduct ion at m/z 1743 corresponding to $[2M+Na]^+$ which is also the base peak. Next most abundant molecular adduct ion $[M+Na]^+$ was observed at m/z 914 corresponding to M^+ 890 when all three fatty acids are $C_{18:0}$, well in agreement with CI-MS data where the QM^+ for tristearoyl glycerol **(5)** occurs at m/z 908. Positive ESI spectrum was also indicative of the presence of TAG of C_{56} and

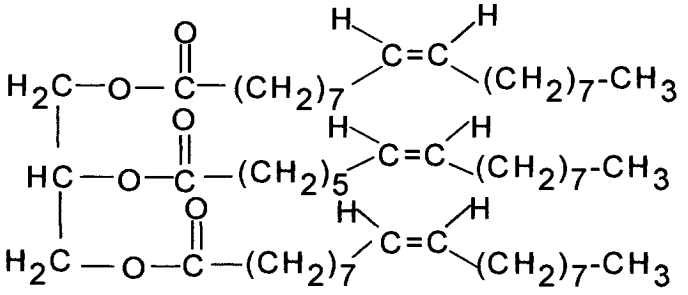
C_{58} group as evidenced by sodiated molecular ion adducts as $[M+Na]^+$ at m/z 942 and 971 respectively. Mass difference of 2 amu is also apparent with the presence of ions at m/z 883, 885, 912, 914, 943, 945, 971 and 973, which indicated that triglycerides in each group differ by only one site of unsaturation (Duffin *et al.*, 1991). This explains the presence of linoleic acid as one of the fatty acyl constituent of triglycerides, which seems to be present in the mixture.

Fig.B1 contains positive ion ESI-MS spectrum of oil B. It is primarily a mixture of TAG belonging to C_{52} and C_{50} groups with the major component being triglycerides with sodiated molecular ion adducts at $[M+ Na]^+$ m/z 879 and 855.7 respectively, the former adduct ion at m/z 879 being the most abundant which is also the base peak. Next most abundant sodiated molecular adduct ion is the odd carbon number molecular adduct ion at m/z 897 corresponding to the C_{53} group. The presence of fragments at m/z 575.4 $[M+H -RCOOH]^+$ i.e. 857-282 and 603.5 $[M+H-RCOOH]^+$ i.e. 857-254, **(Fig.B2)** arising from the loss of oleic and palmitoleic acid moieties, is indicative of the presence in the major glyceride of fatty acids $C_{18:1}$ and $C_{16:1}$ respectively, i.e. the TAG should have the composition $C_{18:1}$, $C_{16:1}$, $C_{18:1}$. It was identified as 1, 3-dioleoyl-2-palmitoleyl glycerol **(6)**.



Tristearoyl glycerol

5



1,3-dioleyl-2-palmitoleyl glycerol

6

Table 3: NMR data of TAGs A & B

Protons	δ_H (multiplicity, coupling constant)	δ_C
Sn2	5.27(m)	68.8 (d)
Sn1, 3	4.3 (dd, J=4.5Hz) 4.15 (dd, J=6,12Hz)	62.02 (t)
Fatty acid residues		
$-CH_3$	0.88 (d, J=6.9Hz)	14.01(q)
$(CH_2)_n$	1.18-1.32 (bs)	22.56-34.18 (t)
$-CH_2-\overset{O}{\overset{\|}{C}}-O$	2.3 (t)	34.18 (t)
$-(CH_2)_2-CH_2-\overset{O}{\overset{\|}{C}}-O$	1.62 (q)	24.8, 29.1(t)
$-CH_2-CH=CH-CH_2-$	2.1 (m)	27.2 (t)
$-CH=CH-CH_2-CH=CH$	2.75 (t)	25.65 (t)
$-CH=CH-CH_2-CH=CH$	-	C_9, C_{10}/C_{12}, C_{13} 129.96 (d), 128.06(d)/ 127.87(d), 130.2 (d)
-CH=CH-	5.34	129.66(d), 130.0(d)

The next glyceride with sodiated molecular adduct ion at m/z 855 corresponding to M^+ 832 and belonging to C_{50} group was identified as having palmitic and oleic acids as component fatty acids; palmitic acid was placed at α - acyl position and $C_{18:1}$ at β–acyl position based on the ^{13}C NMR values for the C-1 of acyl group, the most intense signal being observed at δ173.2 for the α-acyl group and the β-acyl carbonyl was observed at m/z 172.8. The NMR **(Fig.B3 & B4)** data of compound B is almost identical to that of A. It also shows the presence of linoleic acid. Thus compound B, though homogenous on

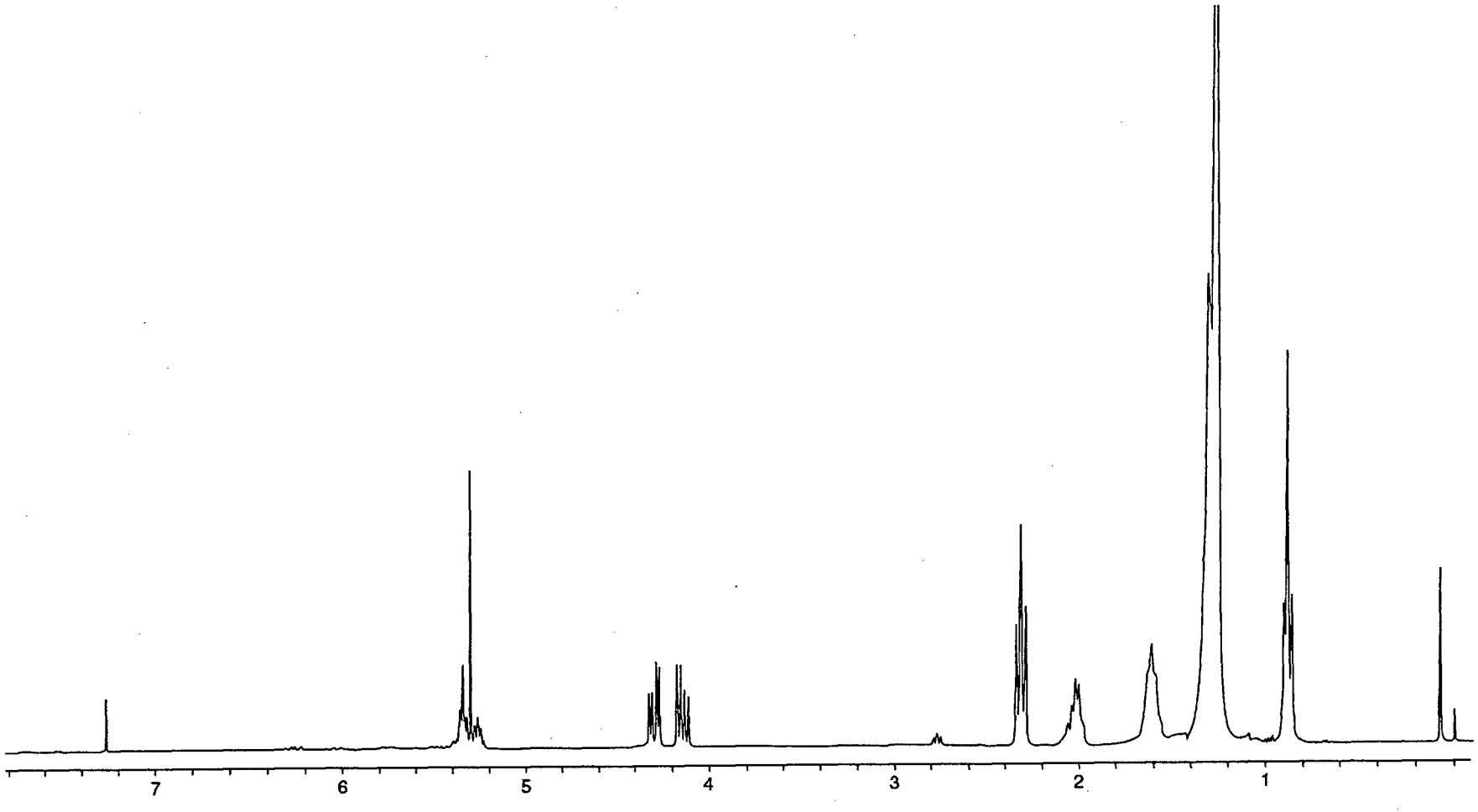


Fig.A1:^{1}H NMR spectrum of compound A

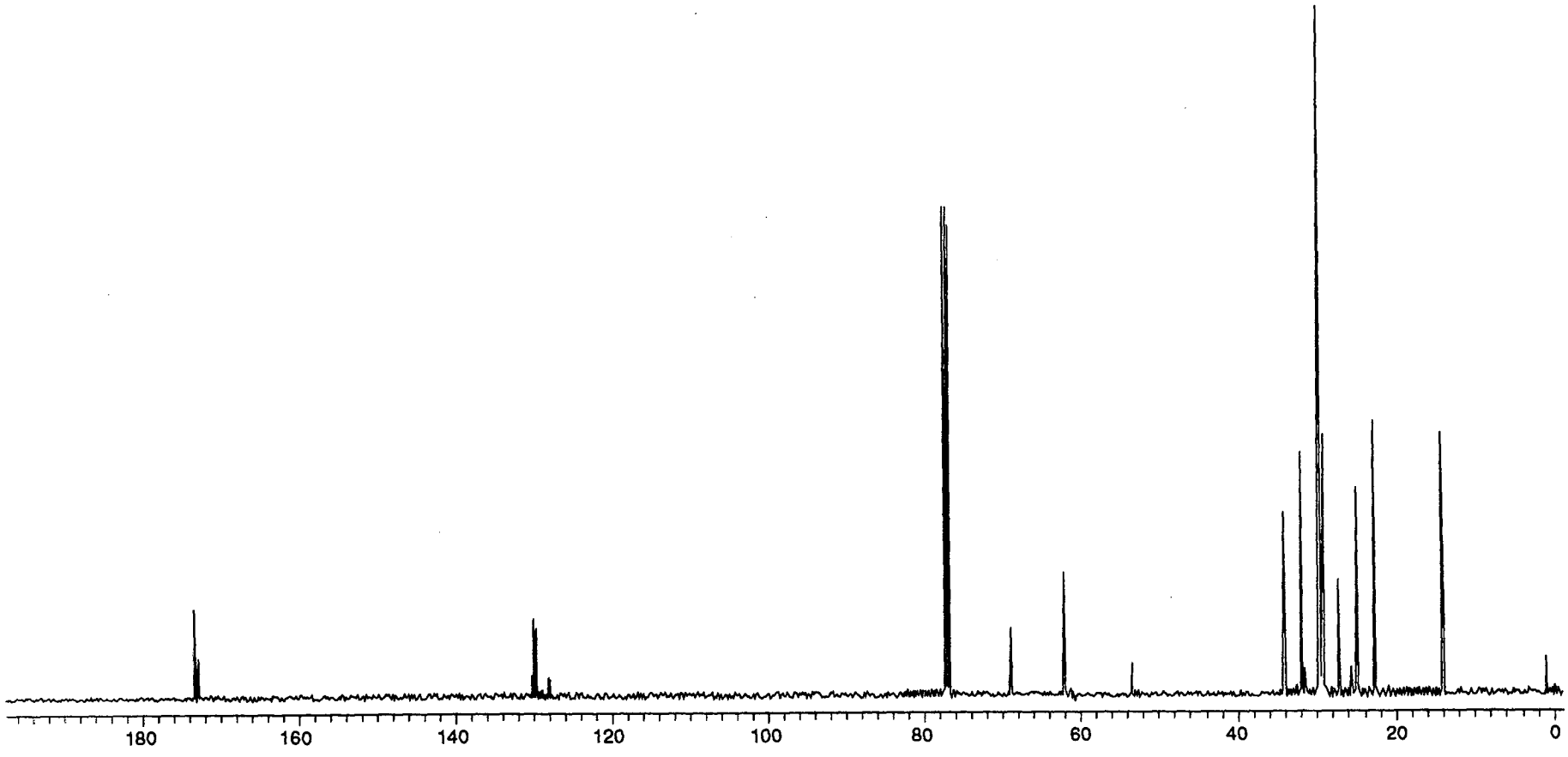


Fig.A2: ^{13} C spectrum of compound A

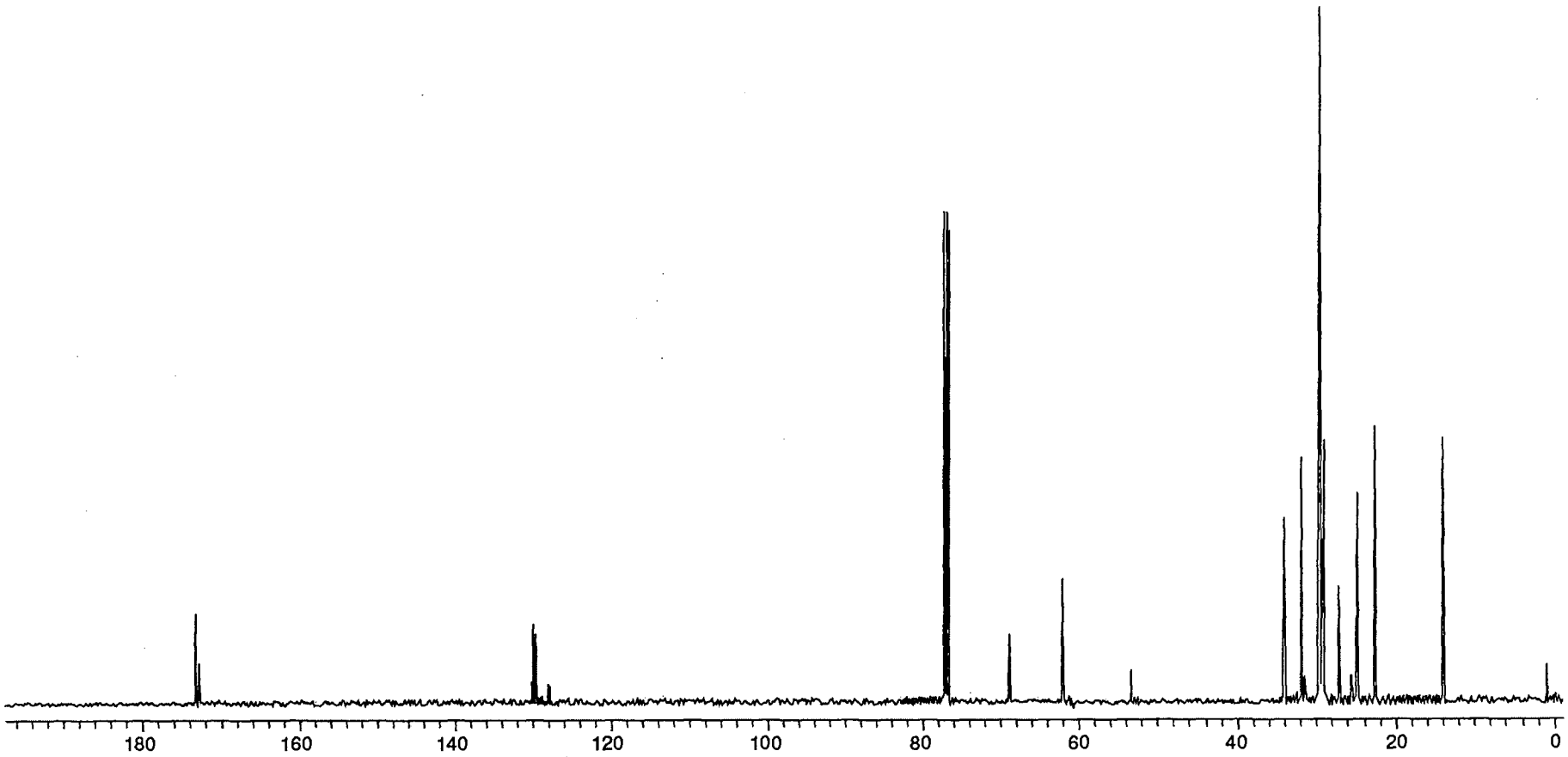


Fig.A2: 13 C spectrum of compound A

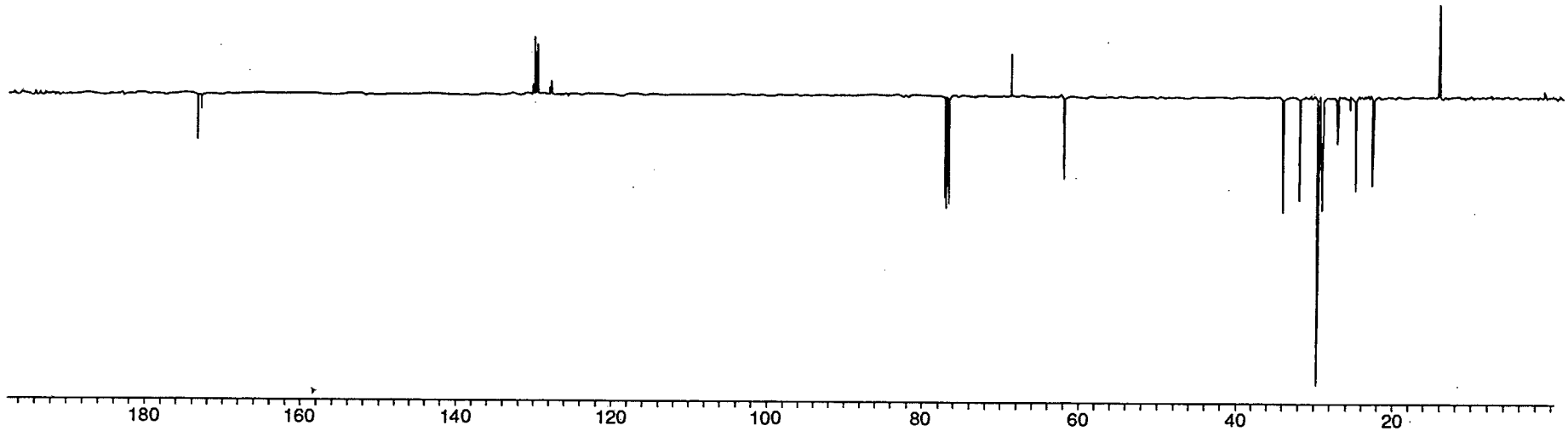


Fig.A3: DEPT spectrum of compound A

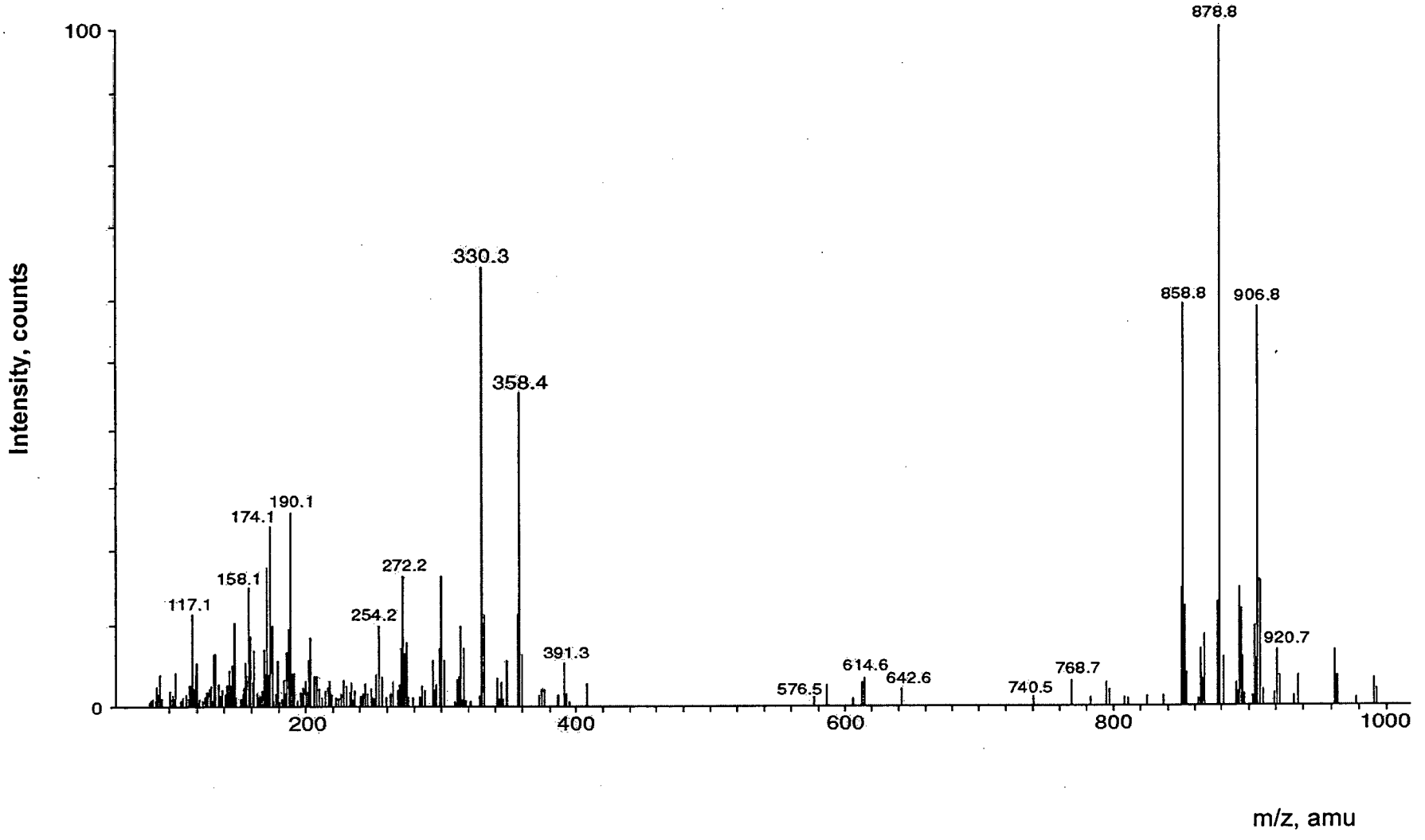


Fig.A4: CI-MS of compound A in positive ion mode

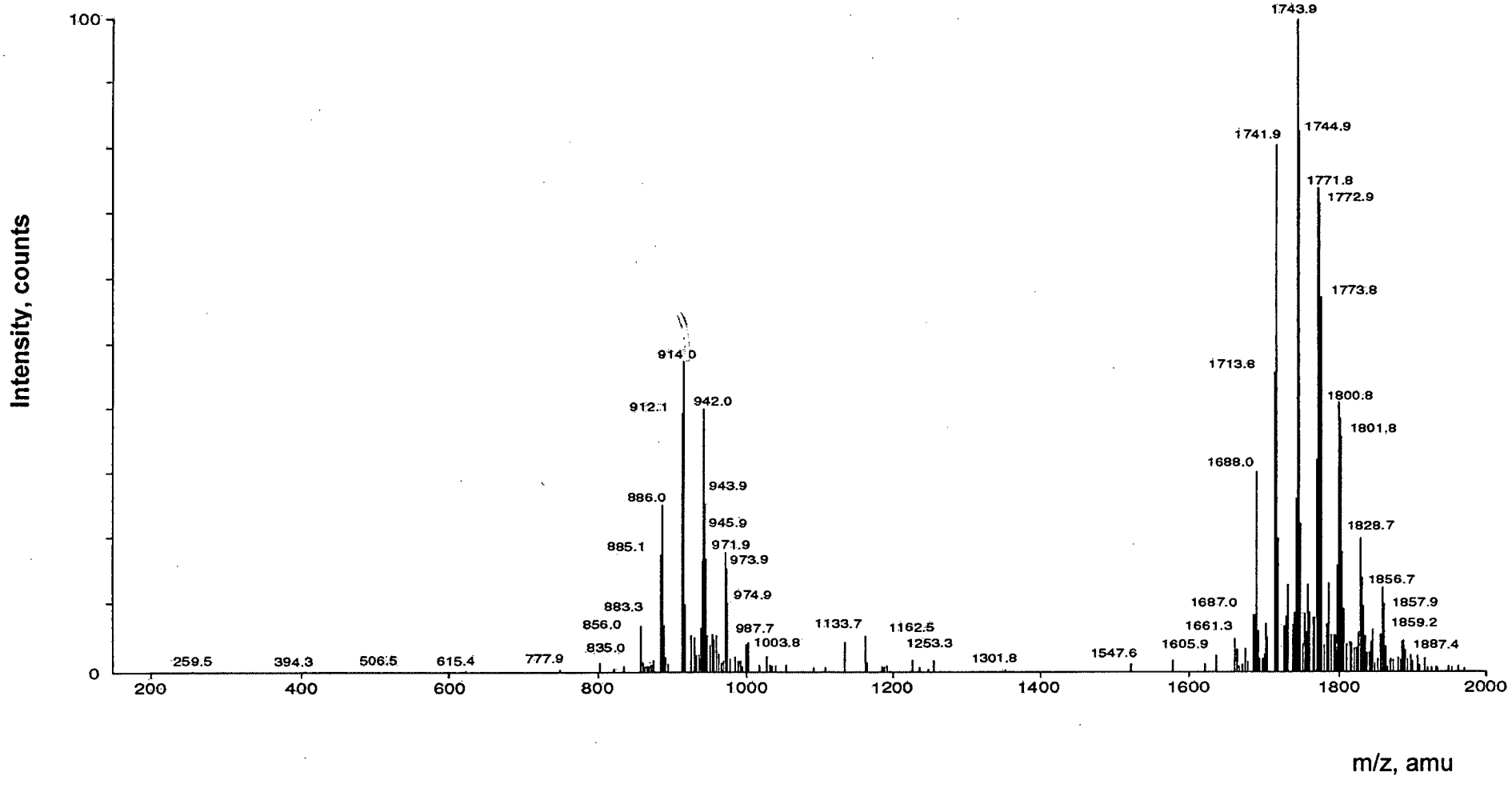


Fig.A5: ESI-MS of compound A in positive ion mode

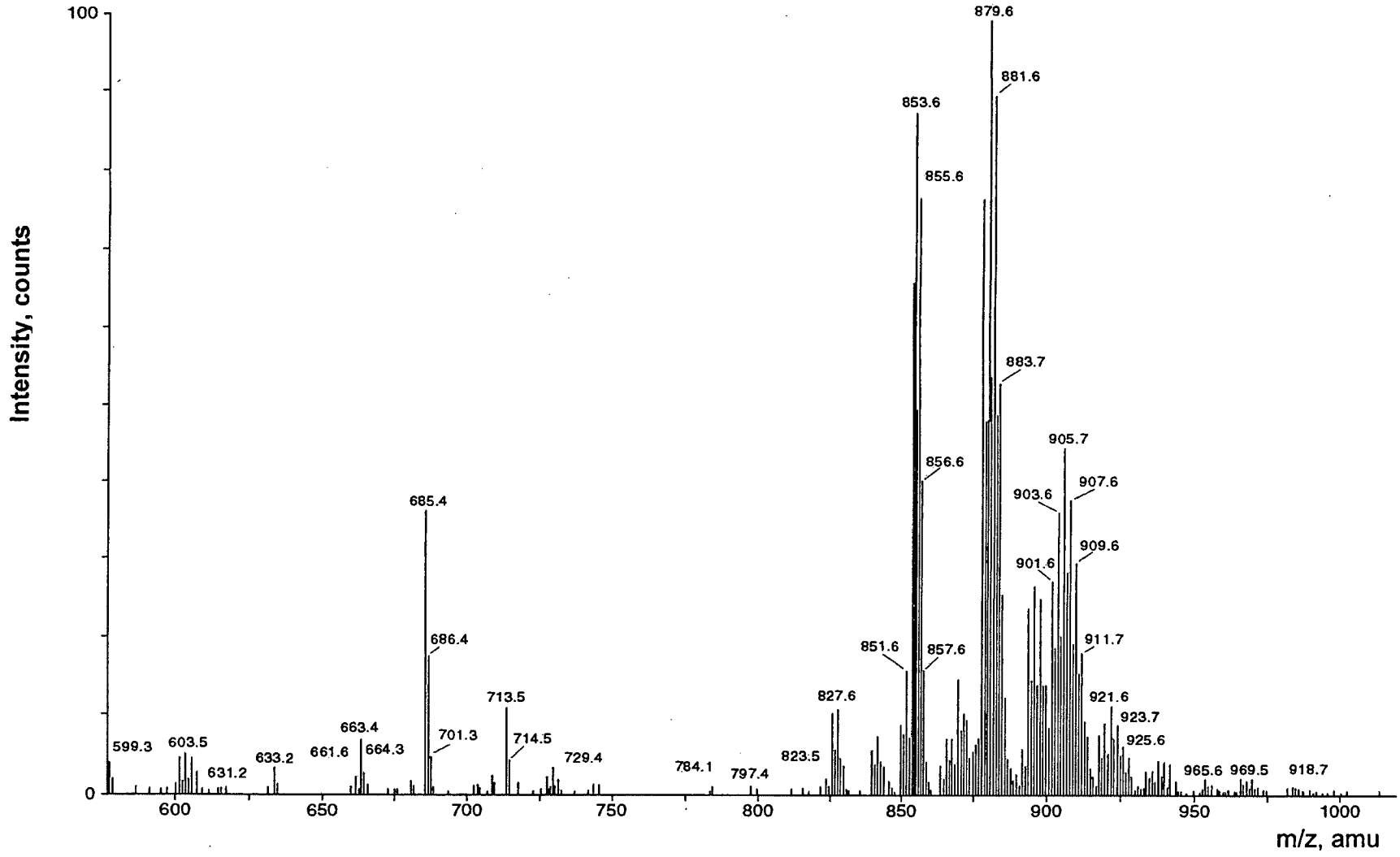


Fig.B1: ESI-MS of compound B in positive ion mode

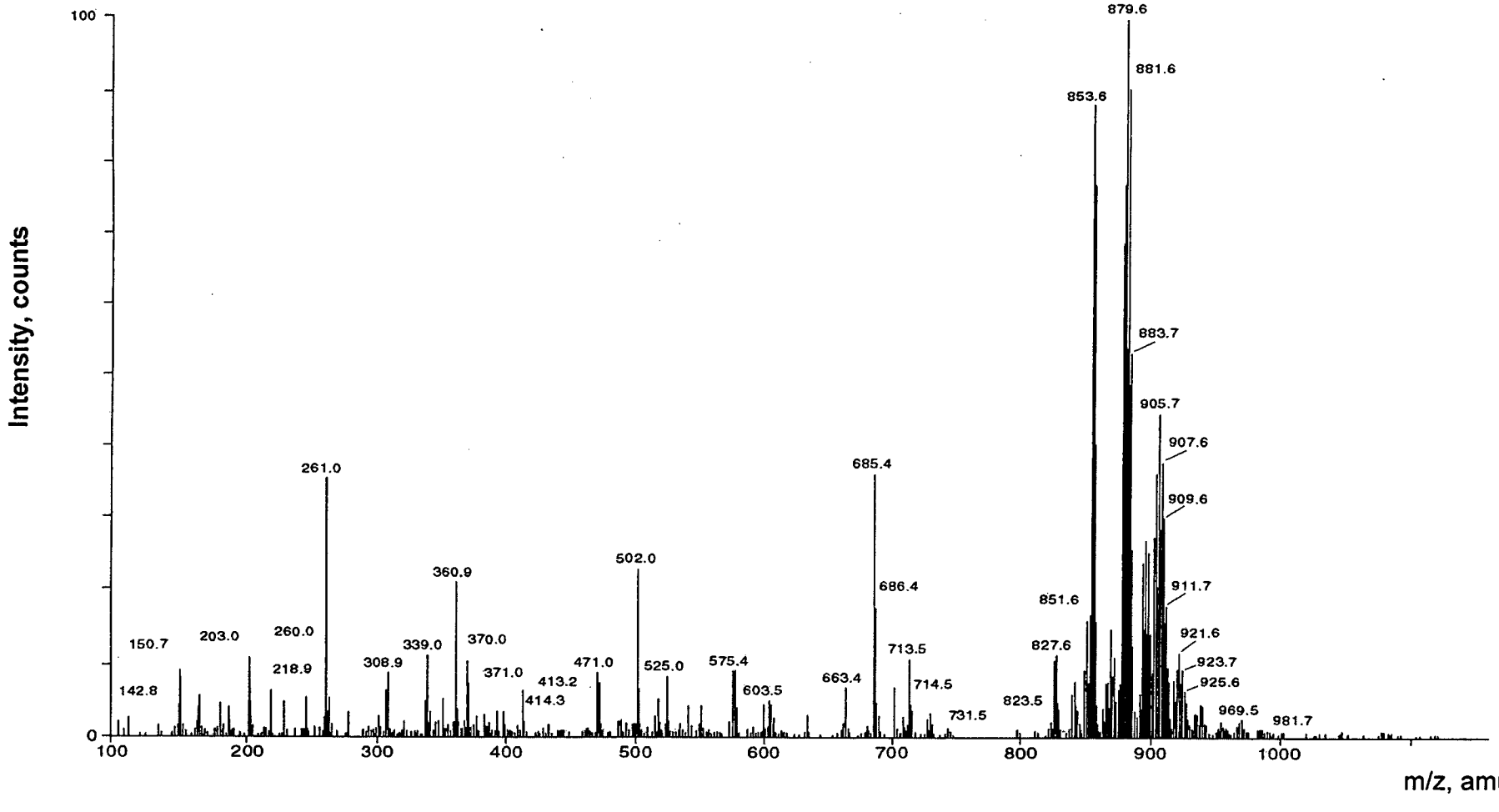


Fig.B2: ESI-MS of compound B in positive ion mode

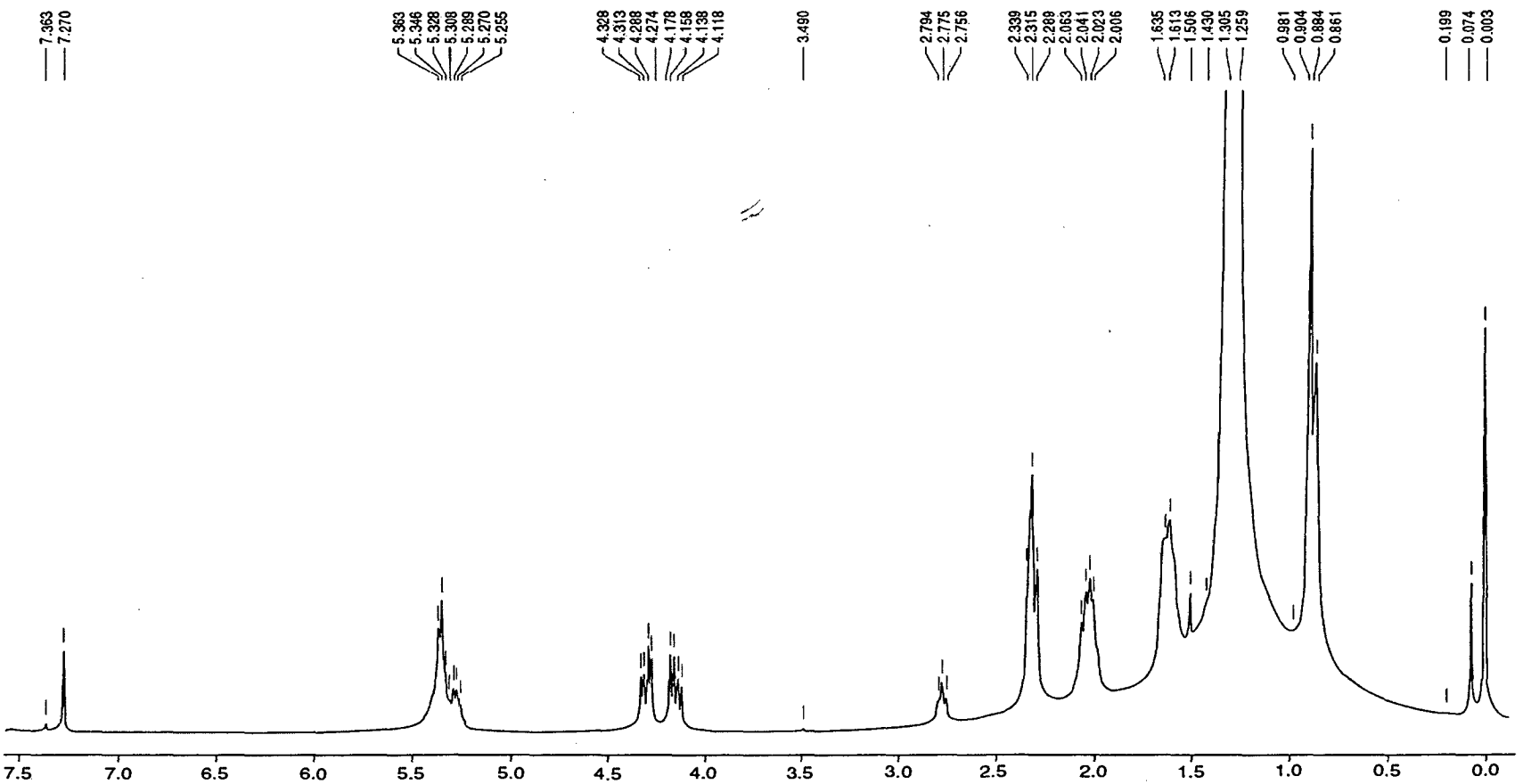


Fig.B3:^{1}H NMR spectrum of compound B

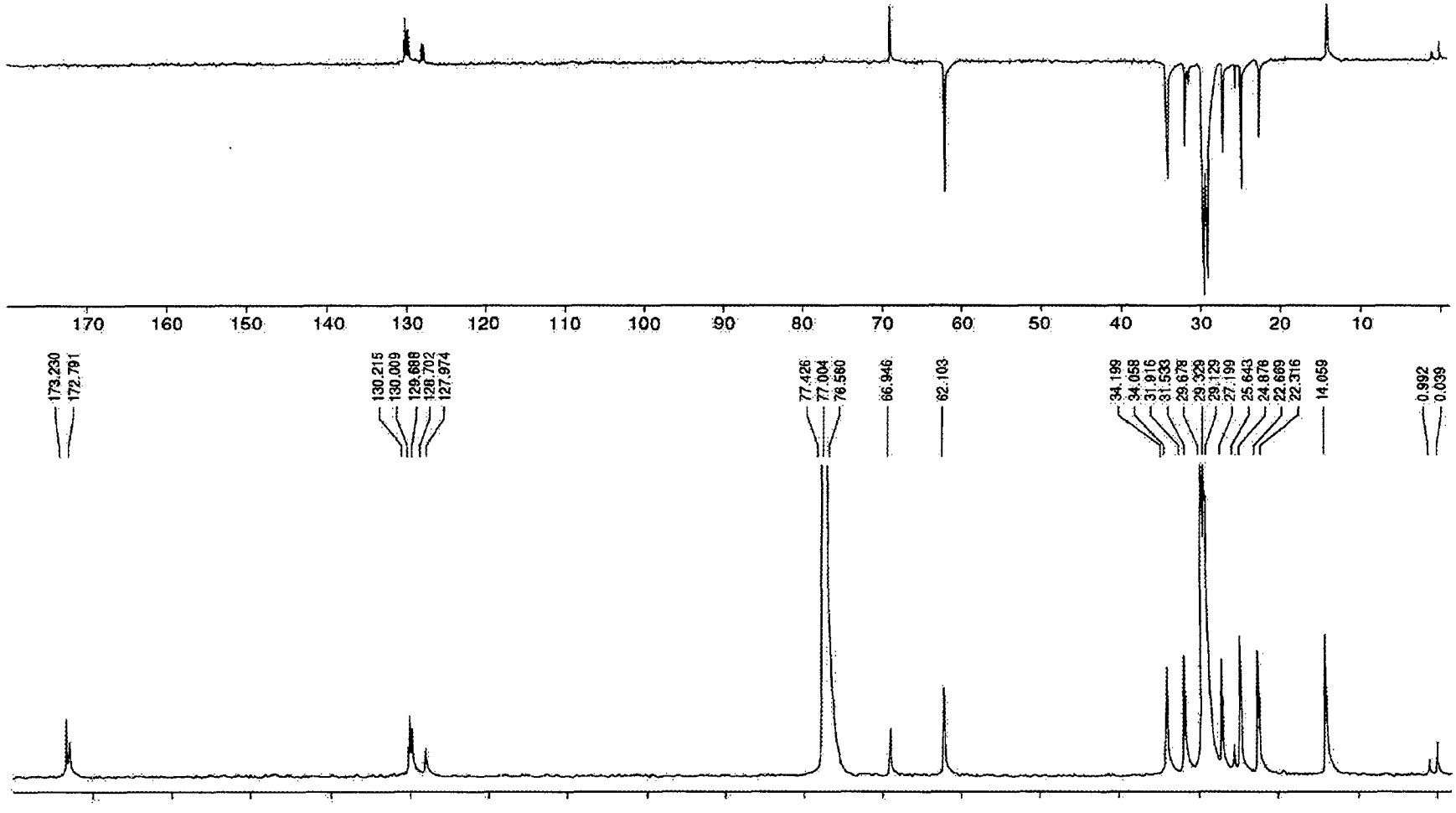


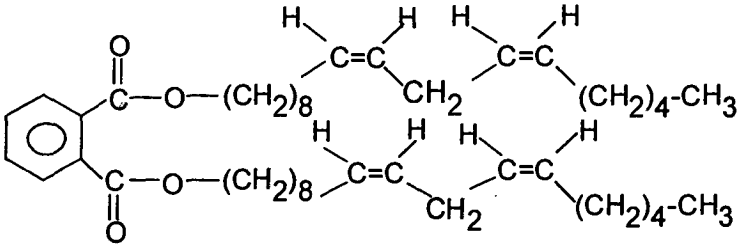
Fig.B4: ^{13} C NMR and DEPT spectra of compound B

TLC, is a mixture of 1, 3-dipalmitoyl-2-oleoyl glycerol **(4)** and 1, 3-dioleyl-2-palmitoleyl glycerol **(6)**.

Compound C, also viscous oil, exhibited ^{1}HNMR **(Table 4, Fig.C1)** typical of a phthalate ester with signals for four aromatic protons at δ7.52 (2H, J= 3.3 Hz) and 7.69 (2H, J= 3.3Hz). The signal for end methyl was evident as a triplet (J=7.2Hz) at δ 0.945. The absorption in the region δ 1.1- 2.75 was attributed to fatty acid chain. The ester methylene protons (4H) appeared as a triplet at δ4.29 and the olefinic protons were seen as multiplet at δ 5.3. Its ^{13}C NMR **(Fig.C2)** was also well in agreement with it being a phthalate ester with less intense peak at δ167.65 assignable to the two equivalent C=O groups, equally intense signal at δ132.2 to the two equivalent substituted aromatic carbons and the large peaks at δ130.83 and 128.75 to the remaining aromatic carbons. The signal at δ 65.5 was assigned to the equivalent ester methylene protons with the olefinic methines of the fatty acid chain being evident at 129.91, 129.63, 127.98 and 127.85, values close to those reported for linoleic group (18:2, 9Z, 12Z).

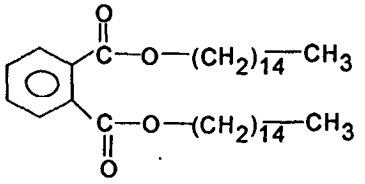
Positive ESI-MS of compound C **(Fig.C3)** exhibited sodiated molecular ion peak at m/z 685 and the $[M+H]^+$ peak at m/z 663. Besides the characteristic peaks at m/z 149 and 167 typical of phthalate ion, fragment ions at m/z 515 and 495 corresponding to M^+-149 and M^+-167 respectively was observed. The fragment at 413.3 $[M-C_{18}H_{33}]^+$ is

indicative of n-octadecyl-9Z, 12Z-diene acyl chain. On the basis of the above spectral data compound C was identified as o-phthalic acid bis (n-octadecyl-9Z, 12Z-diene) ester. The ESI-MS spectra was also indicative of considerable amounts of yet another phthalate ester with sodiated molecular ion peak at m/z 609 corresponding to M^+ 586 in agreement with n-pentadecyl ester. Its ^{1}HNMR **(Fig.C4)** did not exhibit any olefinic signal and the ester methylene protons were evident at δ4.237 and other methylenes were extended from δ1.264-1.694. Thus oil C is a mixture of phthalate esters, o-phthalic acid bis-(n-octadecyl-9, 12-diene) ester **(7)** and o-phthalic acid bis-(n-pentadecyl) ester **(8)**.



o-Phthalic acid bis (n-octadecyl 9z,12z diene) ester

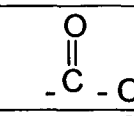
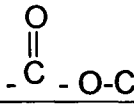
7



o-Phathalic acid (n-pentadecyl) ester

8

Table 4: NMR data for fraction C (Phthalate esters)

Protons position	δ_H (Multiplicity, coupling constant)	Carbon No.	δ_C (Multiplicity)
2	-	2	132.2 (s)
3	7.69 (d,J=3.3Hz)	3	130.83 (d)
4	7.52 (d,J=3.3Hz)	4	128.75 (d)
Fatty acid residues			
		1	167.6 (s)
CH_3-C	0.945 (t, J=7.5)	22	13.59 (q)
-$(CH_2)_n$-	1.28-2.31 (bs)	6-12, 18-21	33.9 -22.56 (t)
	4.29 (t)	5	65.5 (t)
		13-14/ 16-17	129.96,127.98/ 129.63,127.85 (d)

Phthalic acid esters (PAEs) are widely used industrial chemicals, serving as important additives impart flexibility in polyvinyl chloride (PVC) resins. They have been found widespread in environmental samples such as sediments, natural waters, soils, plants and aquatic organisms (Duffin *et al.*, 1991; Sullivan *et al.*, 1982; Giam *et al.*, 1984). In the present study seawater:distilled water (1:1) has been used for the preparation of growth media but it is unlikely that phthalate esters identified in the present investigation, might have their genesis from seawater as fatty chain seems to have been derived from linoleic acid and palmitic acid produced by the fungus *Aspergillus sulphureus*.

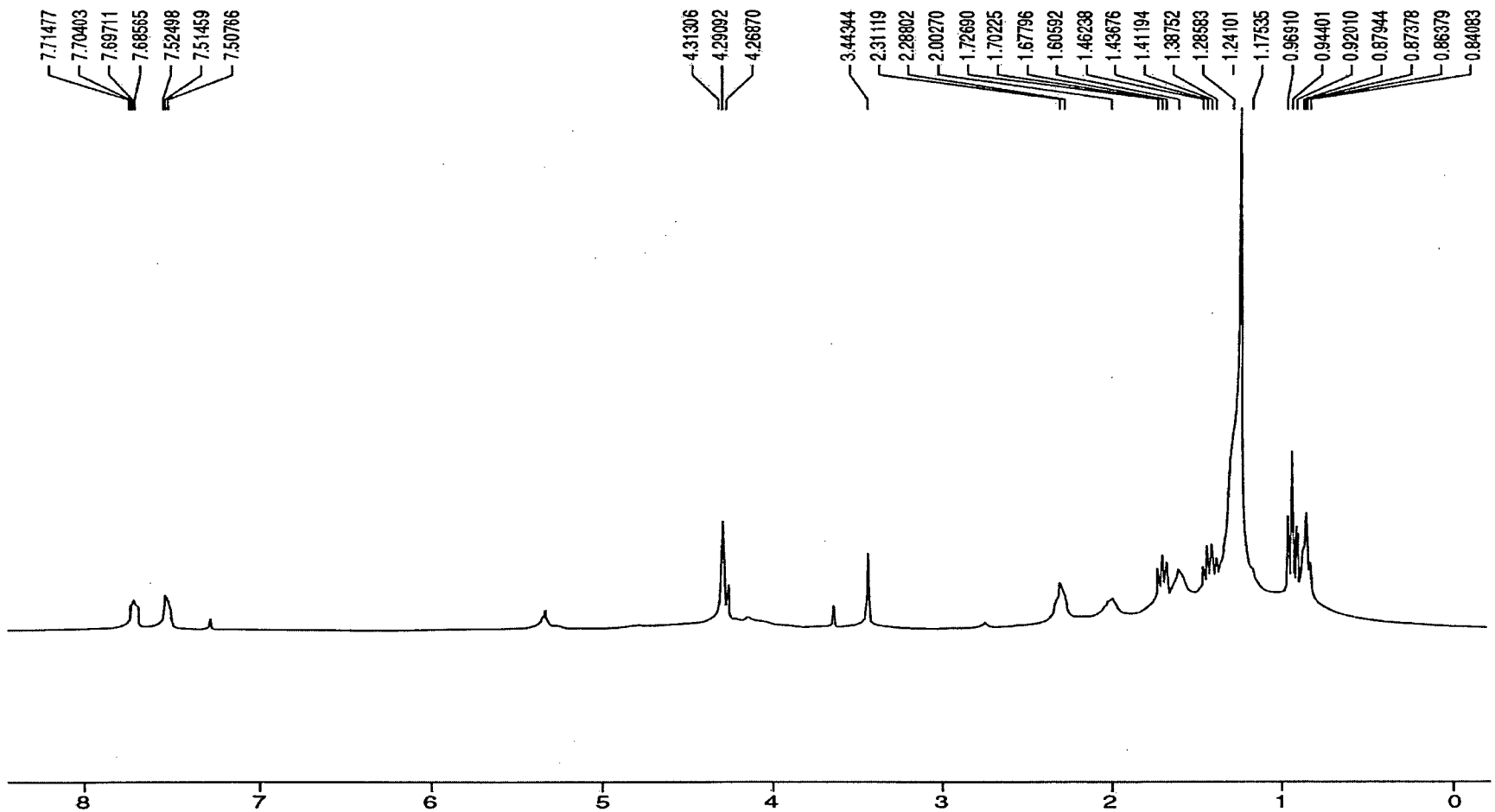


Fig.C1:^{1}H NMR spectrum of compound C

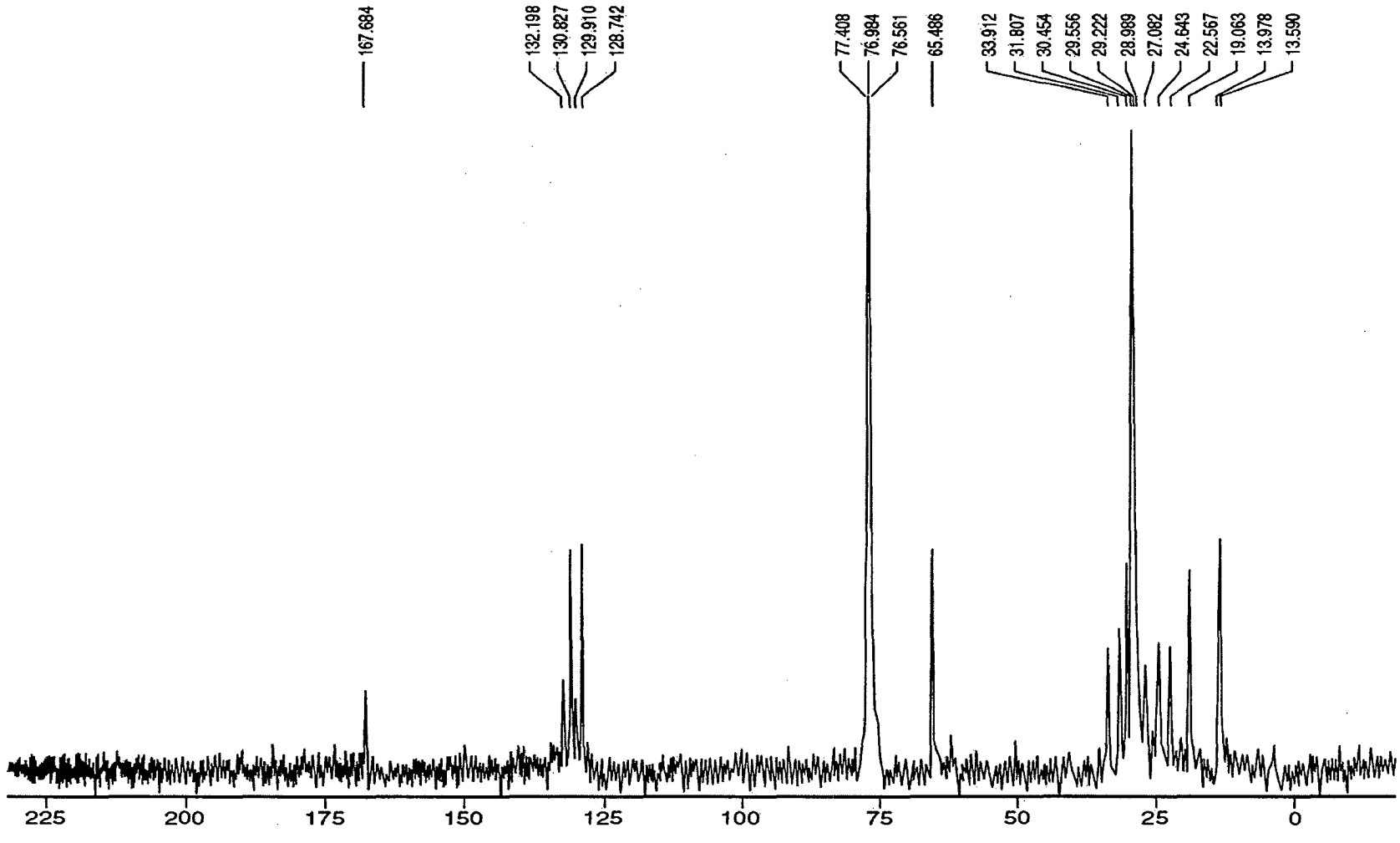


Fig.C2: ^{13} C spectrum of compound c

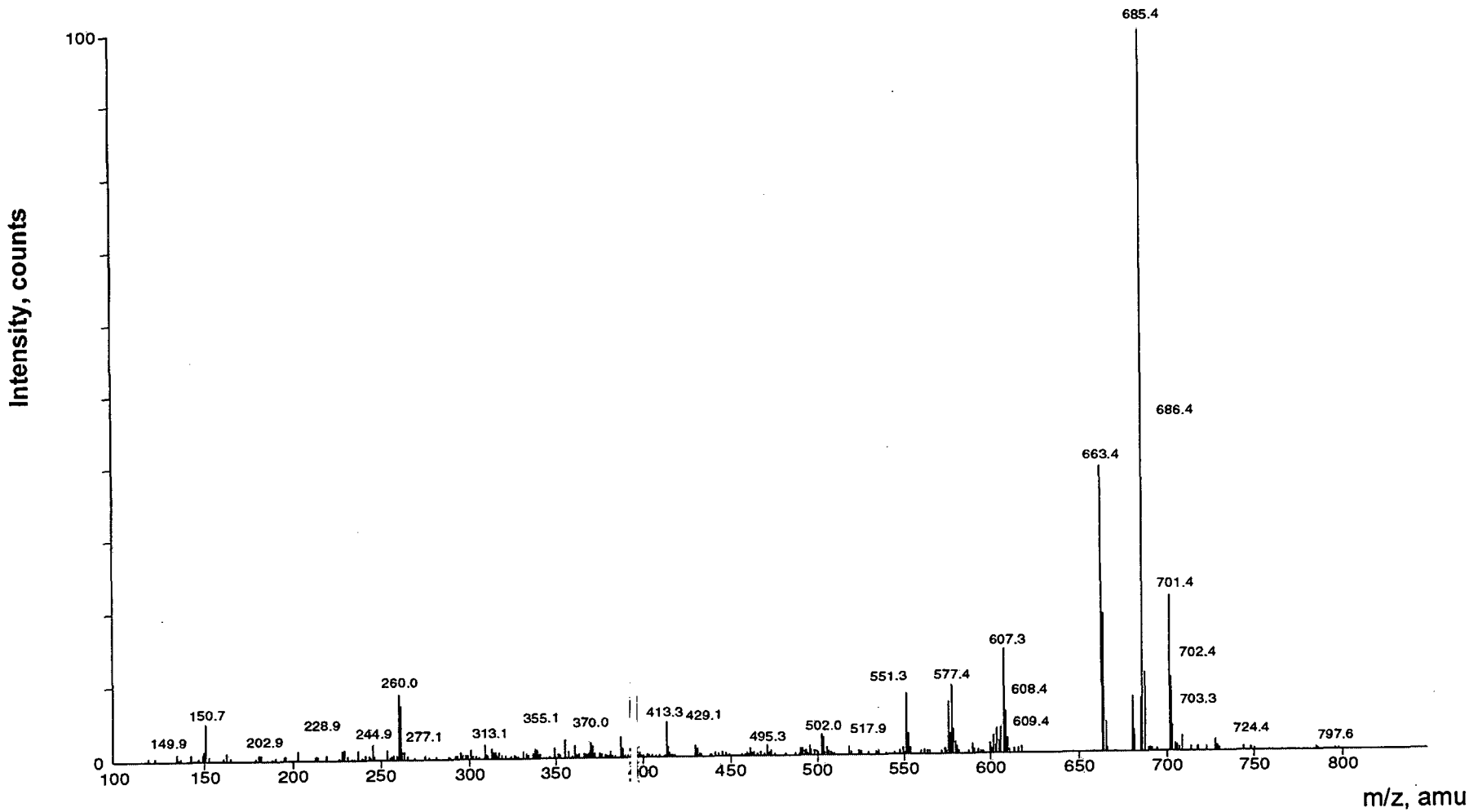


Fig.C3: ESI –MS of compound C in positive ion mode

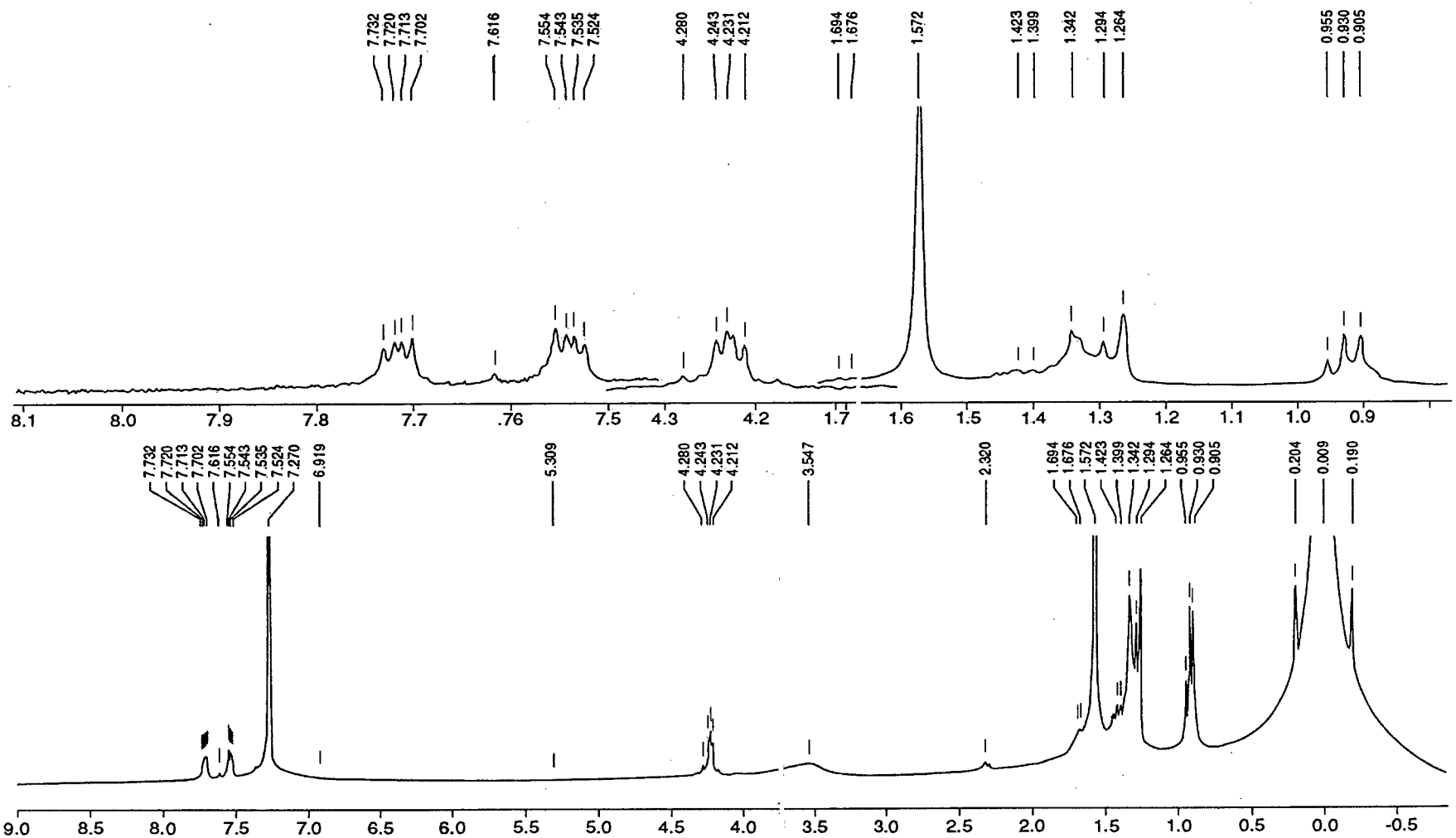
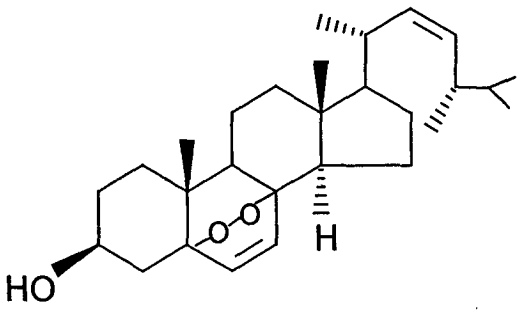


Fig.C4: ^{1}H NMR spectrum of di-n-pentadecyl phthalate

Running a blank experiment, simultaneously, by preparing the media in distilled water alone and checking the contents for the presence of phthalates further confirmed this. Phthalates even if present in the media used (seawater) it would not have yielded the phthalates reported in the present investigation. O-phthalates do not undergo enzymatic hydrolysis at all (Staple *et al.*, 1997; Cipiciani *et al.*, 1996) for further conversion by lipase catalysed transesterification using the fatty acids produced by the fungus, though lipases catalysed synthesis of phthalic acid esters is known (Valiente *et al.*, 1998).

The most polar compound D was identified to be a mixture of E and Z isomers at C-22 of ergosterol peroxide (5α, 8α-epidioxy-ergosta-6, 22-diene-3β-ol) (EP) with the E isomer being the major component. The presence of Z isomer, in minor quantities was evident from the presence in the NMR spectral data **(Table 5, Fig.D1, D2, D3)** of EP with additional doublets, of low intensity, at δ4.9 and δ5.4 due to C-22 olefinic protons.



Ergosterol peroxide

Chemical shift **(Table 5)** for H-6 and H-7 protons in EP were observed at δ6.26 and 6.53 respectively. Low intensity doublets, for H-6 (δ 6.3) and H-7 protons (δ 6.6) were also observed in the ^{1}HNMR of EP. This was attributed to the presence of dehydro-EP, in trace quantities. The double bond is probably present in $\Delta^{9,11}$ positions. It was an inseparable mixture difficult to purify even on acetylation.

Table 5: NMR data for compound D

Carbon No.	δ_H (ppm) (Multiplicity, coupling constant)	δ_C (ppm) (Multiplicity)
1		35.0
2		31.9
3	3.9(1H,m)	66.8
4		37.3
5		82.5
6	6.53(1H,d, J=8.5 Hz)	135.8
7	6.26(1H,d, J=8.5 Hz)	131.1
8		79.8
9		51.4
10		37.1
11		21.0
12		39.7
13		44.9
14		52.0
15		23.0
16		29.0
17		56.6
18	0.84 (3H,s)	13.2
19	0.88 (3H,s)	18.5
20		40.1
21	1.02(3H,d, J=6.5Hz)	21.2
22	5.24(1H,dd, J=15.34,7.5Hz)	135.6
23	5.16(1H,dd, J=15.3Hz, 8.3Hz)	131.2
		43.1
		33.3
26	0.84 (3H,d, J=6.7Hz)	20.3
27	0.85 (3H,d, J=6.6Hz)	20.0
28	0.94 (3H,D, J=6.6 Hz)	17.9

ESI-MS **(Fig.D4)** of the acetylated compound D showed pseudo molecular ions $[M+Na]^+$ at m/z 493 and $[M+K]^+$ at m/z 509 due to EP. Dehydro EP and Dihydro EP pseudo molecular ions were also observed at m/z 491, 507 and m/z 495, 511 respectively.

Ergosterol peroxide can be found in plant (Kim *et al.*, 1997), lichen (Piovano *et al.*, 1999) and fungi such as *Ganoderma lucidum* (Arisawa *et al.*, 1986), *Sporothrix schenckii* (Sgarbi *et al.*, 1997) and *Cordyceps sinensis* (Bok *et al.*, 1999). It has been suggested that it is an artefact produced by photosensitised oxygenation of ergosterol (Gunatilaka *et al.*, 1981) and a precursor of ergosterol biosynthesis. In fungi like *Fusarium monoliforme* (Adams, 1967) and *Cantharellus cibariun* it is reported as true metabolite and could be an intermediate in the biosynthesis of cervisterol (Sbrebryakov *et al.*, 1970).

Nemac *et al.*, 1997 have studied steroid and fatty acids of five industrially important *Aspergillus sp.* They have reported ergosterol peroxide as a product of *Aspergillus sp.* Addition of antioxidant BHT (Butylhydroxytoluene) under dark conditions and working in daylight without the addition of BHT yielded ergosterol peroxide indicating that it is a product of fungal metabolism. This led us to conclude that the sterol isolated in the present investigation is a true metabolite of *Aspergillus sulphureus* and not an artefact.

Various biological activities were known from this compound i.e., ergosterol peroxide has immunosuppressive (Lindequist *et al.*, 1989a,

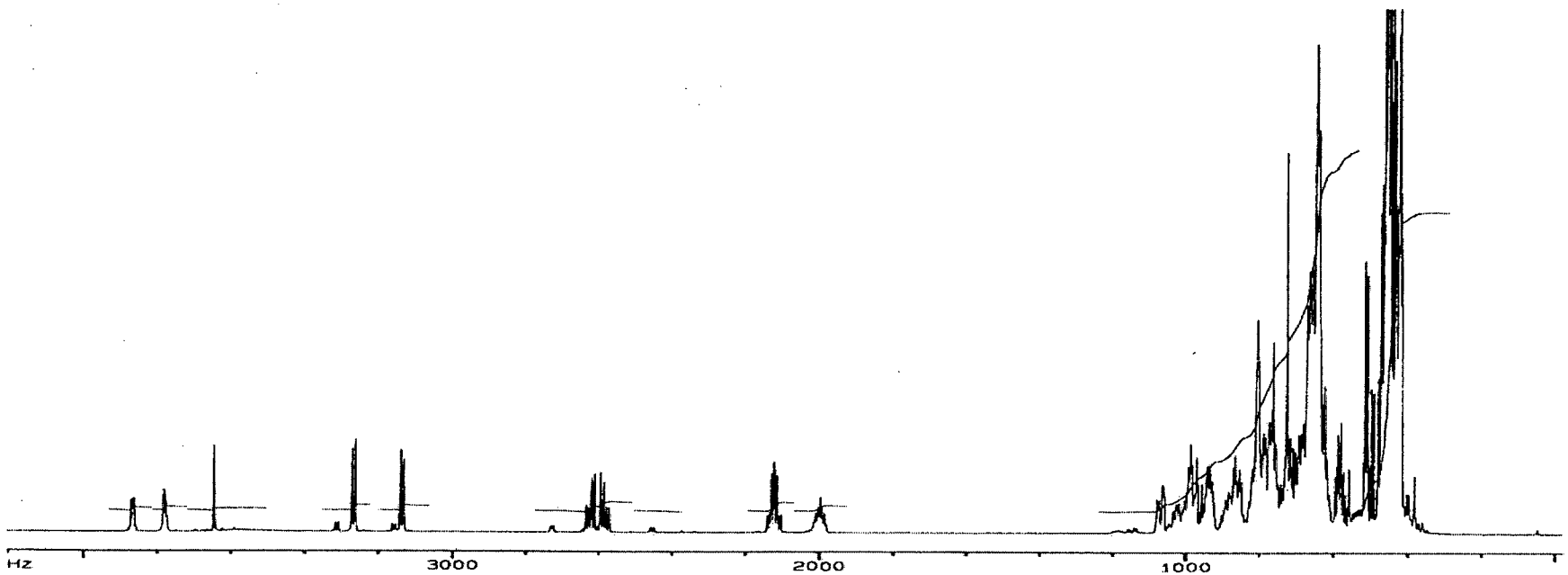


Fig.D1: ^{1}H NMR spectrum of ergosterol peroxide

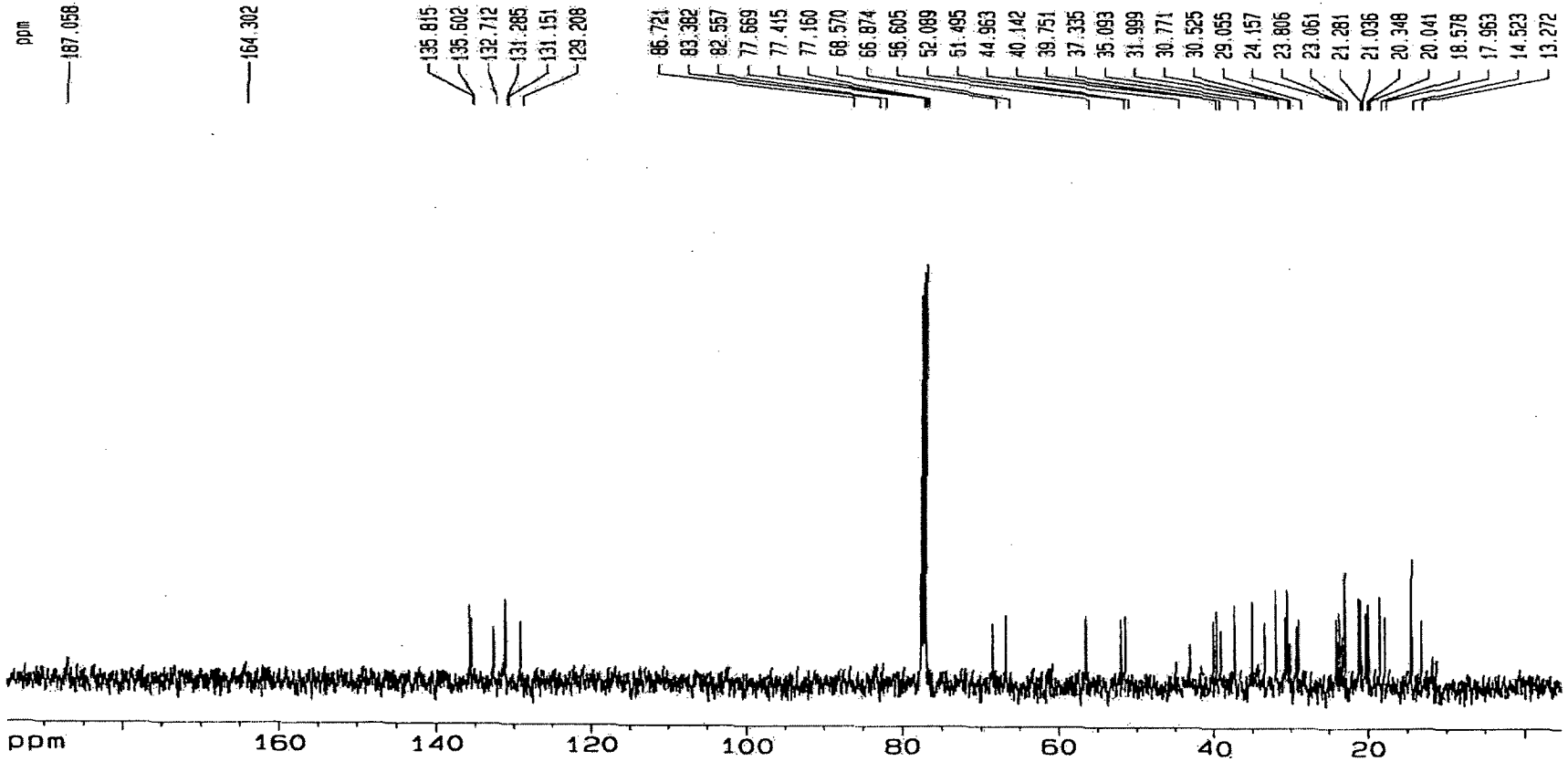


Fig.D2: ^{13}C NMR spectrum of ergosterol peroxide

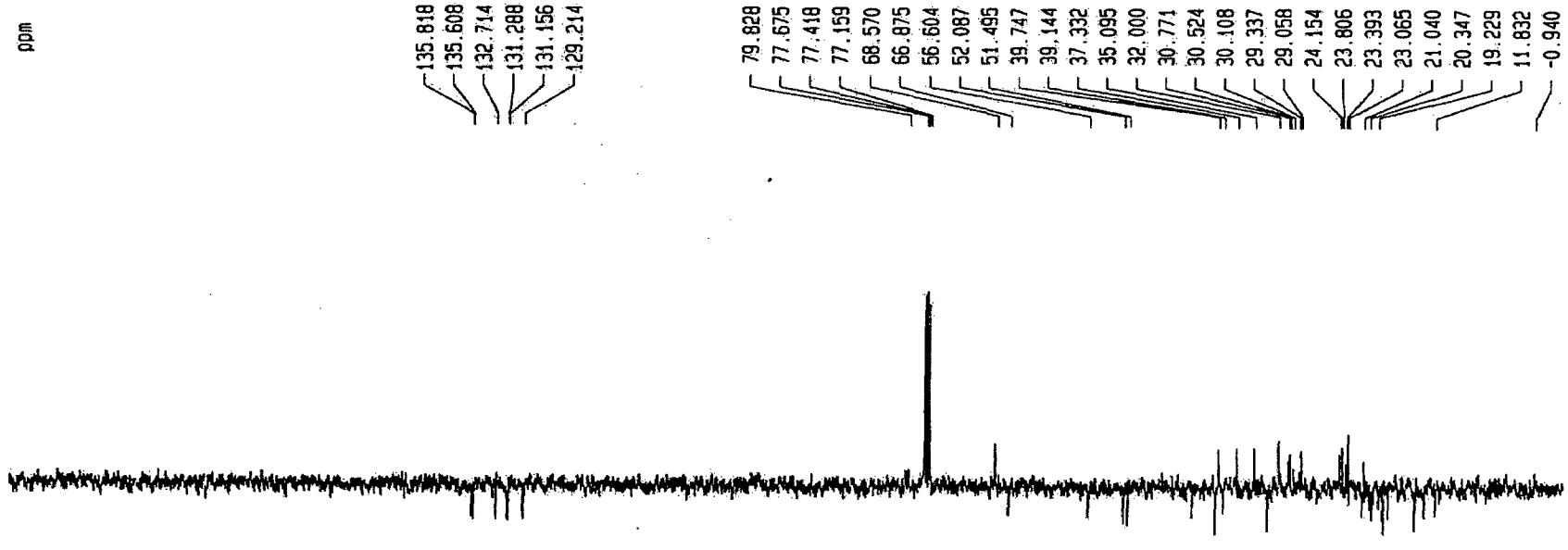


Fig.D3: DEPT spectrum of ergosterol peroxide

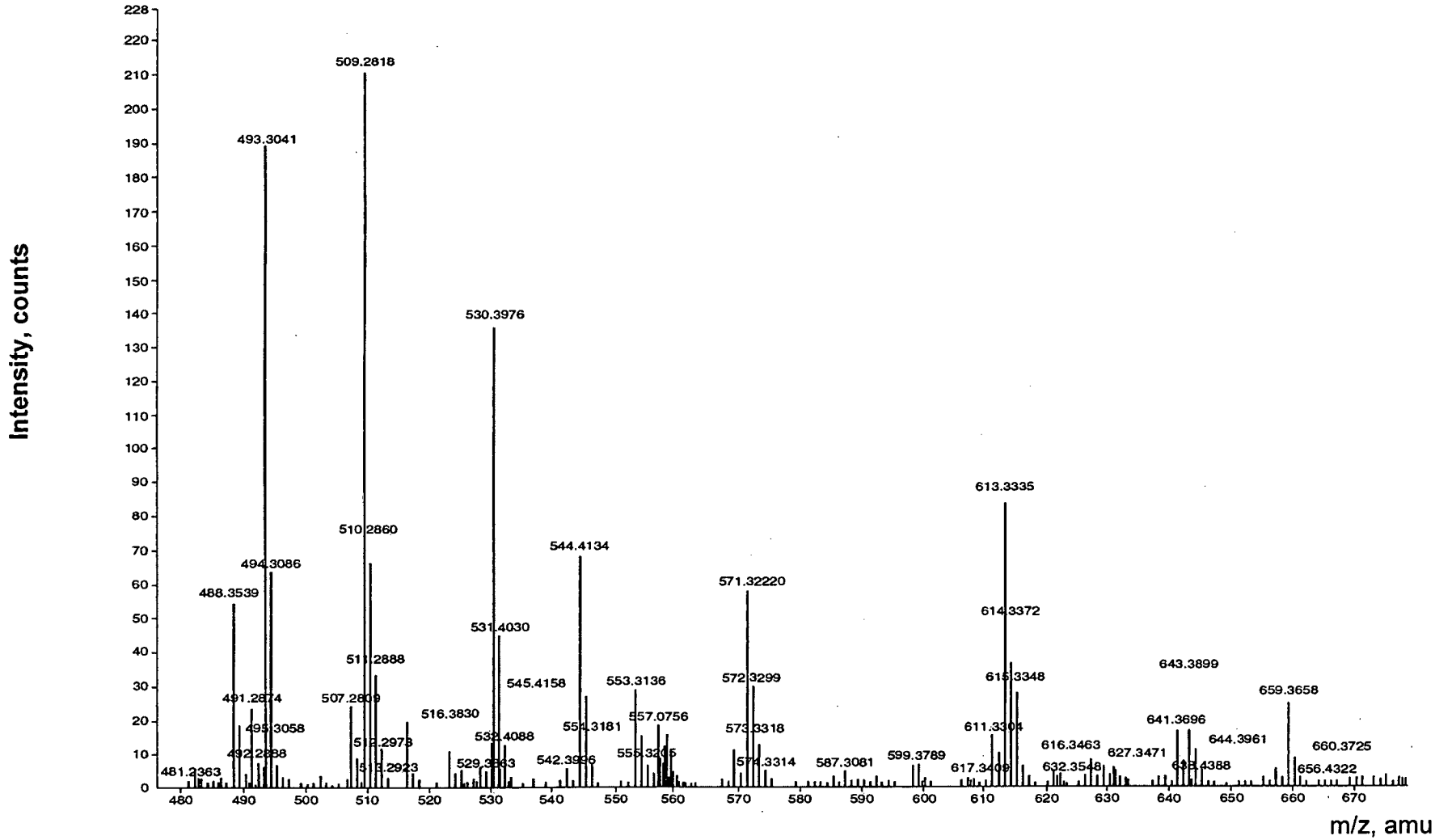


Fig.D4: ESI-MS of compound D

Fujimoto *et al.*, 1994, Kreisel *et al.*, 1990), antiviral (Lindequist *et al.*, 1989b, Nakanishi *et al.*, 1998), anti-inflamatory (Yasukawa *et al.*,1996) and antitumor (Bok *et al.*, 1999) activities. It also shows potent inhibition on lipid peroxidation and exhibits higher antioxidant activity than well known antioxidants α-tocopherol and thiourea (Bok *et al.*, 1999).

The findings described above led to the conclusion that this particular strain of fungus, *A. sulphureus* is a producer of triglycerides with the sn-2 and sn-1, 3 position of glycerol backbone being preferred for substitution with unsaturated and saturated acyl groups respectively. Among the unsaturated acyl groups identified there is preference for oleoyl group over the palmitoleoyl and linoleoyl groups. Saturated fatty chain was attributed to stearic and palmitic acids. Thus, *Aspergillus sulphureus* of the present investigation must be producing non-specific lipase. The production of lipase enzyme by *A. sulphureus* has been confirmed by the hydrolysis of tributyrin (Cruickshank *et al.*, 1975) by the fungus.

Lipase from *A. niger* is known to synthesize glycerides from, not only fatty acids, but dibasic acids and aromatic acids, making ester bonds only at positions 1 and 3 of glycerol (Sang *et al.*, 1999). Phthalates are being reported for the first time as metabolite of fungi.

The production of ergosterol peroxide and the fatty acids oleic, palmitoleic and linoleic acids by the fungi belonging to genus

Aspergillus studied so far points to their use as biochemical marker in taxonomy of *Aspergilli.*

EXPERIMENTAL:

Fungal strain and fermentation conditions:

Aspergillus sulphureus is a species reported by Subramanian from the Indian soil. It was isolated by plate dilution method by taking a known weight of sea grass sediment in sterile seawater followed by a series of dilution with seawater blanks and plating selected dilution in agar. To inhibit the growth of other microorganisms, the antibiotics Streptomycin (0.01 gL^{-1}) and Penicillin G (0.01 gL^{-1}) were used in the medium. The isolated fungus was grown on mycological agar and sub cultured for purification using the method of Collins and Taylor, 1967. It was then inoculated into Hi Media mycological broth in sterilized 1:1 seawater: deionised distilled water. All culture work was conducted under aseptic conditions at room temperature (27°C).

Extraction and fractionation:

A cell mass *(A. sulphureus)* of 19.8 gL^{-1} (dry weight) of broth was filtered, crushed in a mortar and extracted thrice with $CHCl_3$ followed by extraction with MeOH:Water (50:50v/v) at room temperature (27°C) for 12 hours with occasional shaking. Solvent from the combined $CHCl_3$ extracts was removed under reduced pressure on a rotavapour to yield a crude extract of 2.2 g.

The MeOH:Water extracts were combined and concentrated under reduced pressure and then partitioned with butanol. The filtered broth was also concentrated similarly under vacuum at 40°C and then partitioned with butanol. The butanol extract of the cell mass and the broth were combined to yield a crude extract (0.21 g) after removal of the solvent at reduced pressure.

Instrumental analysis:

Infra red spectra was recorded as KBr disc on Fourier Transform Infra Red spectrophotometer (FTIR 8201 PC, Shimadzu).

NMR spectra were taken on a Bruker Avance AMX300 spectrometer, in $CDCl_3$ with TMS as internal standard, spectral width 225 ppm for ^{13}C and δ 8 for ^{1}H NMR. When necessary appropriate resolution enhancement was applied to optimize the separation of signals.

Analysis by ESI-MS (electrospray ionization mass spectrometry) was performed using a Micromass Quatro II triple quadrupole mass analyser. The analyte in MeOH containing 0.01% NaOAc, was introduced by direct infusion. Analysis incorporated single quadrupole scanning over a mass range of m/z 0-2000. The tuning parameters in the +ve ion mode were cone voltage 70V, 40V and ion energy 0.6V which gave rise principally to pseudomolecular $[M+Na]^+$ ions. CI-MS

spectra of triglycerides was recorded with ammonia as the reagent gas yielding QM, $[M+NH_4]^+$ ions; as for the fragments ions only $[M+H - RCO_2H]^+$ ion is recorded (Murata and Takahashi, 1977).

Isolation of 2-hydroxymethyl-5-hydroxy-γ-pyrone:

The crude n-butanol fraction was filtered through Sephadex LH-20 with methanol as the eluent. The fractions were monitored on TLC and were combined according to TLC profile. Thin layer chromatography was carried out on Merck silica gel 60 F_{254} plates and developed using butanol: acetic acid: water (50:40:10, v/v/v) at ambient temperature. A bright yellow spot was visualized with ninhydrin. Further purification was done by column chromatography over silica gel (60-120 mesh), by gradient elution with increasing concentrations of methanol in chloroform. Pure crystals, which were white to yellowish in colour, were obtained which were collected and weighed. The yield was approximately 120 mg.

Isolation of major lipids:

The chloroform crude extract was filtered through Sephadex LH-20 with chloroform: methanol (1:1) as the eluent. The fractions were monitored on TLC and were combined according to TLC profile for further purification on silica gel by gradient elution with ethyl acetate in petroleum ether. It yielded four TLC homogeneous compounds A, B, C, and D in order of their increasing polarity. Thin layer

chromatography was performed on Merck silica gel 60 F_{254} plates in hexane: diethyl ether: formic acid (80:20:2, v/v/v) and visualized with iodine vapours and methanolic sulphuric acid.

Acetylation of compound D:

Acetic anhydride (1 ml) in dry pyridine (2ml) was added to compound D (20 mg) in dry pyridine (2ml). The solution was left overnight, taken in chloroform, washed first with dilute HCl to remove pyridine and then with water. On removal of the solvent it gave a residue (18 mg), which was purified on silica column with gradient elution of ethyl acetate: petroleum ether. The acetylated compound (yield 12 mg) was used for ESI-MS.

Lipase test - Hydrolysis of Tributyrin:

The test for the production of lipase by the fungus was done by the method described by Cruickshank *et al.*, 1975. The fungus is spot inoculated on a solid medium made up of peptone 5 gL^{-1}, yeast extract 3 gL^{-1}, tributyrin (glyceryl tributyrate) 10 gL^{-1} and agar 20 gL^{-1}. An emulsion of micro droplets of the fat, tributyrin in the solid medium makes it opaque. Lipolytic organisms remove the opacity by converting the fat to water-soluble butyric acid. After incubating the plates for 72 hours under room temperature, it was examined by transmitted light. Colonies of lipolytic organisms were surrounded by wide zones of clearing.

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Section III

Elicitor Enhanced Production of Kojic Acid in cell suspension culture of Aspergillus sulphureus

INTRODUCTION

Kojic acid, 2-hydroxymethyl-5-hydroxy-γ- pyrone, is an organic acid produced by various fungal or bacterial strains, such as *Aspergillus oryzae, Penicillium* or *Acetobacter* species (Gerhard *et al.*, 2004) during fermentation of various carbon containing substrates (Chen *et al.*, 1991a). Kojic acid continues to draw attention due to its varied application in the field of medicine as anti-inflammatory and pain killer (Chen *et al.*, 1991a), food science as precursor for flavor enhancers (Le Blanc & Akers, 1989) and anti-browning agent (Chen *et al.*, 1991b) and in cosmetic industry as whitening agent (Ohyama & Mishima, 1990). Kojic acid has many biological functions such as insecticide and antibiotic. In addition it may have other uses in the future for the production of novel biodegradable plastics from kojic acid, if it can be produced industrially at reasonable cost (Futamura *et al.*, 2001).

Kojic acid may be produced by aerobic fermentation of *Aspergillus sp.* using various sources of carbon, such as glucose, sucrose, acetate, ethanol, arabinose and xylose (Beelik, 1956, Presscot & Dunn, 1959). However, glucose is the best carbon source and acts as a precursor for Kojic acid synthesis (Arnstein & Bentley, 1953). During the fermentation, kojic acid is formed directly from glucose through a multistep reaction without cleavage of carbon chain (Kitada & Fukimbara, 1971; Bajpai *et al.*, 1981).

For the industrial production of fermentation products, it is necessary to satisfy the following conditions: breeding of the strain producing bio-products with high yield from a raw material, using a raw material, which can be obtained easily and cheaply, inexpensive and simple process (Futamura *et al.*, 2001). Several methods are reported for optimizing the production yield and rates of microbial processes (Kazuhiro *et al.*, 1996) so as to reduce the product cost but none has tried elicitor for enhancement of kojic acid yields.

In recent years, the function of oligosaccharides as elicitors has been well studied in production of secondary metabolites by plant cell cultures (Darvill *et al.*, 1992; Fry *et al.*, 1993). These studies were focused firstly on the biosynthetic processes related to plant defense mechanisms and gradually extended into the production of bioactive substances for pharmaceuticals, agricultural and industrial chemicals.

Alginate oligosaccharides have been implicated in the elicitation of secondary metabolites in cultures of *Lithospermum erythrorhizon* (Kim & Chang ,1990). Other secondary metabolites produced in increased yields through the influence of carbohydrate fragments in plant cell cultures include indole alkaloids such as acridone (Smith *et al.*, 1987; Eilert *et al.*, 1984), the phytoalexin pisatin (Walker-Simmons *et al.*, 1983) and steroids such as solasodine and diosgenin (Holden *et al.*, 1988; Rokem *et al.*, 1984). Chitinase activity has been reported to be

stimulated in plants contacted with chitosan and chitosan oligosaccharides (Inui *et al.*, 1991). Messiaen *et al* (1993) have achieved prolonged increase in cytosolic calcium in carrot protoplasts by use of oligogalacturonides from fungal cell walls. In the studies carried out on fungal cell cultures, chitosan was found to induce profound ultrastructural and morphological changes in the filamentous fungus *Fusarium oxysporium* (Benhamou, 1995). Yonemoto *et al* (1993) and Tomoda *et al* (1994) have also reported growth promoting effects of alginate oligosaccharides on plants. In animals, chitin oligosaccharides have been shown to have anti- tumor activity (Suzuki *et al.*, 1986) and chitosan oligosaccharides are reported to enhance serum lysozyme activity (Hirano *et al.*, 1991). In cultures of *Bifidobacteria*, alginate oligosaccharides amongst other oligosaccharides tested, accelerated growth when added as a supplement to the culture medium (Akiyama *et al.*, 1992)

Many commercially important secondary metabolites have been produced by microbial cultures. Given the notable role of fungal cultures in the production of secondary metabolites such as citric acid, itaconic acid, antibiotics such as penicillin it is clearly of practical as well as theoretical interest to seek the evidence of enhancement of secondary metabolites yields in fungal cultures. One of the most extensively studied microbial fermentation processes has been the production of kojic acid by the filamentous fungi *Aspergillus oryzae*

and *A. flavus*. The production of kojic acid is well characterized and therefore forms a suitable model system for studying the effects of oligosaccharides on the physiology of secondary metabolites in fungi.

In the present study, commercially available different culture media were evaluated for kojic acid production. The effect of different nitrogen source and elicitation by the addition of commercial alginic acid (BDH, Bombay) as sodium alginate in culture media on the yields was also investigated. Based on the results to make the process economical, aqueous 0.03% Na_2CO_3 extracts of brown algae have been studied as carbon source and for elicitation of kojic acid production, in stationary flask cultures of *Aspergillus sulphureus* isolated from sea grass environment. This is a first report on the strain of *Aspergillus sulphureus* as producer of kojic acid.

Fermentation profile **(Table 1)** of *Aspergillus sulphureus* shows that fermentation medium is the best medium for kojic acid production. The addition of alginate oligosaccharides inhibited the production of the metabolite but pronounced effect on kojic acid yields were observed when fermentation media is supplemented with a combination of elicitor and peptone. The yields are 3 times higher than control when fermentation medium alone was used without peptone and oligosaccharide.

RESULTS AND DISCUSSION:

Kojic acid is produced from carbohydrate sources in an aerobic process by a variety of organisms mainly belonging to the genus *Aspergillus*, the most widely studied strains being *A. flavus* and *A. oryzae* as they are known to produce a substantially high level of kojic acid. In the present study *Aspergillus sulphureus* isolated from sea grass environment was found to produce kojic acid as one of the metabolite, when grown in mycological broth. So far there are no reports of this species yielding the organic acid.

General strategies for optimization of secondary metabolites production in plant cell culture, such as elicitation, immobilization, cell wall permeabilization and feeding of precursors, have been successful for improving product yields in a number of cases.

Since we had a source of pharmaceutically useful molecule in hand we attempted to increase its productivity using different commercially available media and different elicitors. The results obtained are shown in **Table 1 & 2.**

In the first set of experiments fermentation media and commercially available culture media (Hi Media) Potato Dextrose broth (PDB) and Nutrient broth were tested. As evident from **Table 1**, fermentation

medium optimized for kojic acid production described by Ariff *et al* (1997) was found to give highest yield (15.42 gL^{-1}). In PDB, kojic acid production was decreased to 1.02 gL^{-1} and was nil in nutrient broth. This is expected because glucose is the best source of carbon and acts as a precursor for kojic acid synthesis (Arnstein & Bentley, 1953). PDB does contain some dextrose so one can still expect some kojic acid, but potato starch is a poor source of carbon for kojic acid production unless the starch is hydrolysed to glucose. No kojic acid was produced when starch was used as a carbon source by *Aspergillus oryzae* (Kitada *et al.*, 1967). Nutrient broth as expected did not yield any kojic acid, because it did not contain glucose as carbon source, though production of kojic acid also depends on the concentration of nitrogen source. In nutrient broth besides peptic digest of animal tissue, yeast extract is also present as a source of nitrogen. Moulds require a nitrogen source to maintain enzymatic activity (Ogawa *et al.*, 1995). It is also evident that in fermentation medium, the carbon source has been consumed mainly for the production of the acid (15.40 gL^{-1}) as compared to its utilization for the cell growth (5.8 gL^{-1}). On the other hand with PDB, the carbon source has been utilized mainly for the growth of mycelia (11.80 gL^{-1}); the kojic acid formed being only 1.00 gL^{-1}. Nutrient broth was found to be neither suitable for the growth of the strain (1.22 gL^{-1} dry wt) nor did it produce any kojic acid.

It is always desirable to harvest secondary metabolites without destroying the cells. Therefore the results of **Table 1** show that kojic acid is an extracellular product and as such its concentration in the broth is several fold higher compared to its concentration in the cell mass irrespective of the medium used.

Table 1: Amount of Kojic acid produced per litre of media

Media	Cell mass dry weight (g/l)	Amount of Kojic acid in cell mass (g/l)	Amount of Kojic acid in the broth (g/l)	Total Amount of Kojic acid produced per litre of media (g)
Potato Dextrose broth	11.80	0.07	0.95	1.03
Potato Dextrose broth + Peptone	27.46	0.09	0.57	0.66
Potato Dextrose broth + Alginic acid	16.00	0.65	5.06	5.71
Potato Dextrose broth + Peptone + Alginic acid	25.32	0.21	13.50	13.72
Fermentation medium	5.82	1.86	13.56	15.42
Fermentation medium+ Peptone	15.99	3.79	25.09	**28.88**
Fermentation medium+ Alginic acid	8.58	0.09	3.533	3.62
Fermentation medium+ Peptone + Alginic acid	28.25	0.24	48.71	**48.95**
Nutrient broth	1.22	-	-	-
Nutrient broth + Alginic acid	11.05	0.04	0.10	0.14

Effect of peptone as nitrogen source on kojic acid production:

Since nutrient medium did not yield any kojic acid it was not used for further studies. As evident from **Table 1**, the addition of 0.6% peptone as a nitrogen source with a reduction in the amount of glucose to 50 % to fermentation media resulted in 87 % increase in the production of kojic acid (28.80 gL^{-1}) with a corresponding increase in dry cell weight to 15.99 gL^{-1} which is 2.7 times higher than that with fermentation medium alone. On the other hand addition of peptone to PDB reduced the kojic acid yield to 0.66 gL^{-1} from 1.00 gL^{-1} and the dry cell weight increased to 27.46 gL^{-1}. This means that carbon source tends not to be utilized for kojic acid production but predominantly for mycelial growth. Fermentation medium already contains yeast extract (5.00 gL^{-1}) as the nitrogen source and when supplemented with additional nitrogen in the form of peptone further enhancement in the yields of kojic acid was observed.

Effect of alginate elicitor on the production of Kojic acid:

Elicitors that can induce the accumulation of secondary metabolites have received wide acceptance because of their ability to significantly improve the productivity of plant cell system. In order to evaluate further enhancement of kojic acid production alginate elicitor (alginic acid in the form of sodium alginate) was investigated using fermentation medium and PDB. The results are as shown in **Table 1**.

In order to study the influence of alginate elicitor, fermentations were carried out in stationary Erlenmeyer flasks using sodium alginate. The growth profile of *Aspergillus sulphureus* and production of kojic acid was investigated using 1% alginic acid converted into sodium salt by the addition of Na_2CO_3. When PDB was supplemented with the elicitor a marked increase from 1.00 gL^{-1} to 5.70 gL^{-1} yield of kojic acid was observed when compared to control cultures without the oligosaccharide. This was accompanied by 33% increase in the biomass. This could be explained by the elicitor activating the secretion of cell bond amylolytic enzymes for the hydrolysis of potato starch into glucose. Fungal strains of genus *Aspergillus* are known to secrete amylolytic as well as glucoamylase enzymes during its growth (Ellaiah *et al.*, 2002; Rosfarizan *et al.*, 1998).

Fermentation medium with glucose as carbon source supplemented with sodium alginate resulted in a dramatic decrease from 15.4 gL^{-1} to 3.64 gL^{-1} in kojic acid production as compared to the control. There was comparatively better fungal growth with the dry cell weight showing an increase from 5.84 gL^{-1} to 8.54 gL^{-1}. An important aspect of kojic acid fermentation process is its sensitivity to changes in pH of the fermentation medium. Addition of Na_2CO_3 for conversion of alginic

acid to sodium alginate affected the pH and hence the observed decrease in the yields of kojic acid.

Fermentation medium contains yeast extract that is known to stimulate production of certain secondary metabolites like prenylated flavanone (Yamamota et al., 1995). Harn & Albersheim (1978) were the first to report a glucan elicitor in yeast extract. Song *et al* (1995) isolated a mannan as the elicitor that induces the accumulation of *p*-coumaroylamino acids in *Ephedra distachya* cell cultures. The same authors (Song et al., 1994) claim that commercial yeast mannan (Sigma chemicals, USA) has no elicitation activity.

Combined effect of addition of elicitor and peptone as nitrogen source:

The above experiment was repeated to study the effect of addition of peptone along with elicitor alginate. As evident from the **Table 1**, it greatly enhanced the growth of the mycelia and in turn increased kojic acid production. The dry cell mass with fermentation medium and PDB increased to more than three fold (28.25 $gL^{-1}$from 8.54 gL^{-1}) and 1 ½ times respectively corresponding an increase of 70% (48.95 gL^{-1} from 28.87 gL^{-1}) and 140% (13.74 gL^{-1} from 5.74 gL^{-1}) respectively.

Maximum production and cell growth observed on the combined addition of peptone and elicitor might be due to pH and nitrogen supply being favorable for the production of enzymes needed for the biosynthesis of kojic acid from glucose in the case of PDB.

Dissolved oxygen also greatly influences the secretion of amylolytic enzymes, especially α-amylase which would further enhance the availability of carbon source in the culture and hence production of kojic acid. It has been reported that α-amylase and glucoamylase production by *Aspergillus sp.* was greatly influenced by the aeration condition (Rousset & Schlich, 1989).

In conclusion, fermentation medium is the best media yielding maximum kojic acid. Addition of alginate elicitor (1%) along with peptone further enhanced the yield more than three fold. Aeration is expected to further enhance the yield by increasing the secretion of enzymes.

Effect of 0.03% aqueous Na_2CO_3 extracts of some selected seaweeds on kojic acid production.

As evident from **Table 1** maximum kojic acid production, from the strain of *Aspergillus sulphureus* was obtained when fermentation

medium was supplied with 1% alginate elicitor and 0.6 % peptone as nitrogen supplement. To make process economical the effect of brown seaweeds which are known as source of polysaccharides (alginates) on the production of kojic acid was evaluated. Aqueous 0.03 % Na_2CO_3 extracts of three brown seaweeds *Sargassum tenerrimum*, *Dictyota dichotoma* and *Padina tetrastromatica* were investigated using the maximum yielding fermentation medium with peptone as the nitrogen source. The results obtained are shown in **Table 2**.

Table 2: Amount of Kojic acid produced in the media containing seaweed extract

Media	Cell mass dry weight (g/l)	Amount of Kojic acid in cell mass (g/l)	Amount of Kojic acid in the broth (g/l)	Total Amount of Kojic acid produced per litre of media (g)
Sargassum extract + Fermentation medium	13.96	1.48	10.13	**11.62**
Sargassum extract + Fermentation medium+ peptone	26.02	0.52	0.70	1.22
Dictyota extract + Fermentation medium	13.27	1.52	15.01	**16.53**
Dictyota extract + Fermentation medium+ peptone	17.96	0.33	0.87	1.20
Padina Extract + Fermentation medium	11.36	1.08	4.39	5.47
Padina Extract + Fermentation medium+ peptone	15.77	0.94	3.86	4.80

As mentioned above, brown seaweeds are known to be a source of alginates. Hence, it was presumed that aqueous 0.03 % Na_2CO_3 extract of the algae would contain the elicitor. Extract of 300 g of seaweed was used. The results show that, in the absence of peptone, maximum yield of kojic acid was obtained from *Dictyota dichotoma* (16.54 gL^{-1}) followed by *Sargassum tenerrimum* (11.64 gL^{-1}) *Padina tetrastromatica* extract yield being the lowest. This is well in agreement with the alginic acid content of the seaweeds reported by Solimabi & Naqvi (1975). Further, the alga contains polysaccharides, other than alginates, which might get hydrolysed under the conditions used helping in the production of kojic acid.

EXPERIMENTAL:

Microorganism and media:

The strain of *A. sulphureus* used in this study for Kojic acid production was isolated from sea grass environment. The strain was maintained on potato dextrose agar (Hi Media) at (-20°C) and was sub- cultured every two months.

Kojic acid fermentation:

Experiments with different media were conducted under the same laboratory conditions and by using methodology given under **chapter 1, section II**. Two parts of experiments were conducted:

Estimation of mould growth:

The amount of mould growth was established by determining the dry weight of mycelium formed. The contents of each flask were transferred to a weighed Whatman paper 1; washed three times with water and the mycelium dried at 60° C for 24 hours.

Experiment 1:

In experiment 1 different culture media were used. Potato dextrose broth (PDB) (Hi Media)[Potatoes infusion (200g/l) from dextrose (20g/l)], fermentation medium [Glucose 100 gL^{-1}, yeast extract 5 gL^{-1}, KH_2PO_4 1gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}] and nutrient broth (Hi Media) (peptic digest of animal tissue 5 gL^{-1}, NaCl 5 gL^{-1}, beef extract 1.5 gL^{-1}, yeast extract 1.5 gL^{-1}) were used in the first set. In the second set 1% of alginic acid was added to each of the culture medium, i.e., PDB, fermentation medium and nutrient broth. Alginic acid is insoluble in aqueous media. As the pH is raised above 3, the alginic acid is partly converted into a soluble alginate salt. This is done by slow additions of minute quantities of sodium carbonate. Addition of Sodium carbonate to the medium is done with vigorous shaking till all the alginic acid goes into solution. Gelation or precipitation of the alginate can occur at pH values below 4, as the salt is converted to the insoluble alginic acid. Alginate solutions are stable in the pH range of around 4-10. In the third set peptone 0.6% was added to PDB and fermentation medium [Glucose 50 gL^{-1}, Peptone 6 gL^{-1}, KH_2PO_4 1gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}]. Glucose

concentration was reduced by 50% in order to monitor the effect of peptone, a nitrogen source under lesser concentrations of carbon. Nutrient broth was not used here, as it did not yield Kojic acid in the first set due to the absence of glucose and its yield with alginic acid was not significant. In the fourth set, Peptone (0.6 %) and Alginic acid (1%) were added to PDB and fermentation medium.

Experiment 2:

In experiment 2 different seaweed extracts were used. Brown algae *Sargassum tenerrimum*, *Dictyota sp.* and *Padina tetrastromatica* were collected from Anjuna, Goa. Approx 300 g of seaweed was washed with tap water and then with distilled water. It was ground in a grinder and extracted thrice by ultra sonication (Transsonic 460/H) with 300 ml distilled water (approximately 100 ml each time) containing 0.03% of anhydrous sodium carbonate to attain a pH of around 8.0-8.5. The extract was filtered and diluted to 1000 ml with distilled water. The seaweed extract was used in the following combinations to prepare the media for culturing the fungus.

Set 1: 100% Seaweed (*Sargassum tenerrimum*, *Dictyota sp.* and *Padina tetrastromatica*) extracts.

Set 2: 50% Seaweed extract + 50% Fermentation medium (Glucose 100 gL^{-1}, yeast extract 5 gL^{-1}, KH_2PO_4 1gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1})

Set 3: 50% Seaweed extract + 50% Fermentation medium (Glucose 100 gL^{-1}, yeast extract 5 gL^{-1}, KH_2PO_4 1gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}) + 0.6% peptone.

All the media were sterilized in an autoclave (121°C) for 15 minutes. One litre of each of the above media was inoculated with *Aspergillus sulphureus*. The culture was incubated at 30°C for 20 days. After 20 days the cell mass was harvested. The cell mass was separated from the broth by filtration and extracted. The cell mass was ground and extracted with distilled water. The broth and the cell mass extract were used for the estimation of Kojic acid. Plain seaweed extract in set 1 did not support the growth of fungus.

Analytical techniques

Estimation of Kojic acid:

Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) was determined colorimetrically according to Bentley (1957) with slight modification. In this case UV-VIS recording spectrophotometer (UV-2401 PC) was used in place of a colorimeter. Bentley method is based on the intense red colour produced by kojic acid with dilute solutions of ferric chloride.

Kojic acid standard solutions

0.5 g of recrystallized kojic acid dissolved in 500 ml of water. Dilute 1:10 for calibration.

Ferric Chloride solution

1.0 g of $FeCl_3.6H_2O$ is dissolved in 100 ml of 0.1 N HCl.

Procedure:

A suitable aliquot (1ml) of culture medium is treated with 4 ml of 1% ferric chloride and diluted to 10 ml. The color develops immediately and is stable for some hours. The absorbance is read at 505 nm on UV-VIS spectrophotometer (UV-2401 PC UV-Vis spectrophotometer SHIMADZU). A blank is prepared from the same volume of uninoculated culture medium and 4 ml of ferric chloride. Calibration is carried out in the same way with aliquots of stock kojic acid solution.

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Section IV

A Novel Antifungal Metabolite Acetyl Cyclopiazonic Acid

INTRODUCTION

Although the distributions and ecological roles of marine fungi are not well understood, there are enough reports that suggest that fungi from diverse marine substrates are good source of new secondary metabolites. During the course of investigations on the enhancement of kojic acid production by *Aspergillus sulphureus, which* was isolated from sea grass environment, we have tried peptone bile sorbitol broth as one of the culture medium for fermentation. Though there was cell growth it did not yield any kojic acid. Hence, the broth was investigated for the presence of other secondary metabolites.

In this section we describe the isolation and structural elucidation of a novel molecule, acetyl cyclopiazonic acid **(1)** an antifungal metabolite from the culture filtrate of *Aspergillus sulphureus* grown on peptone bile sorbitol broth.

RESULTS AND DISCUSSION:

The fungus *A. sulphureus* was grown on peptone bile sorbitol broth for 20 days at 28°C. The broth (1 L) was extracted with ethyl acetate and the ethyl acetate soluble fraction was subjected to gel filtration through Sephadex LH-20 followed by silica gel column chromatography to afford compound **1**. This new indole derivative designated as acetyl cyclopiazonic acid was identified on the basis of spectral data.

Compound **1** has the molecular formula of $C_{22}H_{22}N_2O_4$ as revealed by ESI-MS m/z 379.09 $[M+H]^+$. IR absorption bands **(Fig.1)** at 3310 and 1703 cm^{-1} were attributed to –NH and carbonyl group(s) respectively. The amide carbonyl was evident at 1682 cm^{-1}. Absorption observed at 1770 and 1175 cm^{-1} are typical of enol acetate. Its UV spectrum **(Fig.2)** in MeOH showed absorption at 263 nm (small hump), 293 nm (sharp) and 379 nm (small hump) indicative of aromatic chromophore or extended conjugation. Its ^{1}HNMR **(Table 1, Fig.3)** with signals very similar to those of cyclopiazonic acid except for the signal at δ 12.4 due to hydrogen bonded proton reported for cyclopiazonic acid (Holzapfel, 1968;Yokota *et al.*, 1981) was absent and an additional singlet at δ 1.25 was observed.

Cyclopiazonic acid is considered to be an equilibrium mixture of the four possible enolic tautomers with the Z-exo-enol form predominating (Steyn & Wessels, 1978; Nolte *et al.*, 1980), which is also evident from IR absorption at 3310 cm^{-1}. The presence of indole nucleus unsubstituted at either the α- or β- position was suggested by purple–blue Ehrlich colour reaction.

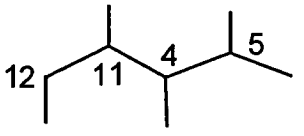
Table 1: Spectral data for acetyl cyclopiazonic acid

CARBON NUMBER	^{13}C δ (ppm)	DEPT	^{1}H δ (ppm)	COSY	HMBC
NH-1			8.3, s	7.16	127
2	124.3	CH	7.16, s		111,127,134.7, 117.8,129
3	111.3	C			7.13,7.14,124.3
4	37.4	CH	3.62-3.68, dd (J=5.7Hz)	2.64, 4.04	54.4
5	73.1	CH	4.04-4.07, d (J=10.8Hz)		37.4,110, 196
6	196.5	C			
7	106.9	C			
8	176.6	C			
10	64.7	C			
11	54.4	CH	2.64, m		
12	27.8	CH_2	3.0, d (J=10.8Hz)	2.64, 6.8	129,54.4,37.4, 117,127
13	129.9	C			
14	117.8	CH	6.8-6.9, d (J=6.9Hz)	7.16, 7.22	110,122,127
15	122.2	CH	7.16, s		111,127,134.7, 117.8,129
16	110.0	CH	7.22, s		6.9,117.8
17	134.7	C			
18	127.2	C			
19,23	186.2	C			
20	21.0	CH_3	2.44, s		106.9,186.2
21	25.7	CH_3	1.63, s		25.7,27.7/27.8, 54.4,64.7
22	27.7	CH_3	1.67, s		54.4,25.7,64.7
24	31.0	CH_3	1.25, s		31.0,64.7

In the high field region the presence of three tertiary methyl groups as singlets at δ 1.63, 1.63 and 2.44 is evident. Methine protons attached to nitrogen/carbon bearing oxygen function appeared as a multiplet at δ 2.64, a quartet at δ 3.62 (J=5.7, 5.7Hz) and as a doublet at 4.04

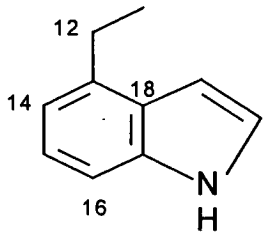
(J=10.8Hz). In the low field region the four aromatic protons between δ 6.89 -7.22 indicated the presence of trisubstituted benzene ring. The ^{13}C NMR data **(Table 1, Fig.4)** including DEPT (135°) experiments disclosed the presence of 3 methyls, one methylene, seven methine and nine quarternary carbons of which those observed at δ 196.5, 186.2 and 176.6 were attributed to ketonic carbonyl. All the protons were connected to carbons by HMQC experiments **(Fig. 5)**.

^{1}H-^{1}H COSY experiments **(Fig.6)** revealed the proton connectivities for the partial structure **A**. For the partial structure **A** the nitrogen bearing methine (δ 4.04, C-5) exhibited clear correlation with the adjacent methine (δ 3.62, C-4) which in turn was further correlated with the methine at δ 2.64 (H-11) which was also coupled to methylene doublet at δ 3.0 (H-12).



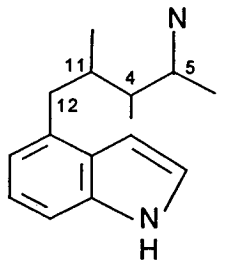
A

Further ^{1}H-^{1}H COSY correlation of H-12 was observed with the aromatic methine doublet at δ 6.9 (H-14), which was in turn coupled to aromatic methines at δ 7.22 (H-16) and δ 7.16 (H-15). Diagnostic long range ^{1}H -^{13}C coupling in HMBC **(Fig.7)** correlation of H-12 with C-11, C-13, C-14 and C-18 established part structure **B**.



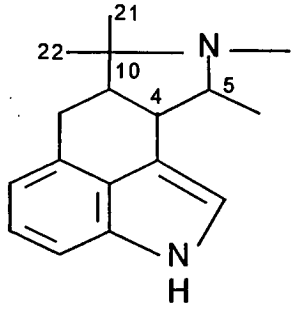
B

HMBC experiment allowed to connect the part structure **A** and **B** leading to part structure **C**. HMBC correlation of proton at δ 7.16 (H-2) with the quarternary carbon at δ 111.3 (C-3) led us to the connectivity of C-3 with C-4 and hence the formation of ring **C**.



C

HMBC correlation of H-21 and H-22 to C-10 and C-11 led to the five member ring part structure **D**.



D

Further long range coupling of H-5 proton at δ 4.04 with the C-6 carbonyl at δ 196 and the (C-7) coupled with the HMBC **(Fig.9)** of H-

20 methyl protons with C-7 carbons at 106.9 and the carbonyl at δ 186.2 led to the structure **1** for the compound.

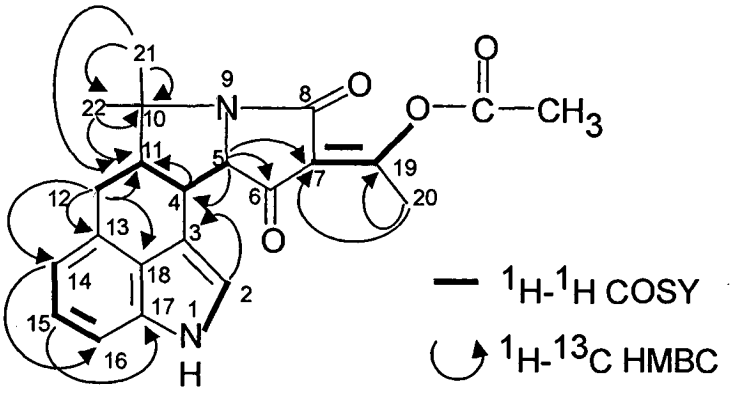
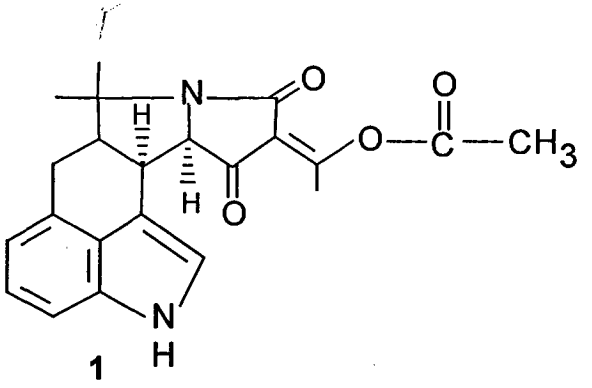


Fig.9: Selected 2D NMR correlations for compound 1



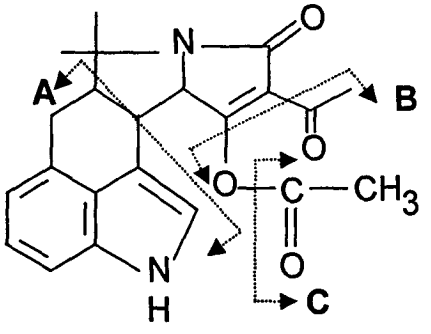
1

Since the carbon chemical shifts of C-6, C-7, C-8, C-19 and C-20 were close to those of cyclopiazonic acid, the geometry of C-7- C-19 double bond in structure **1** was assigned as the same as that of cyclopiazonic acid, which is considered to be an equilibrium mixture of the four possible tautomers with the Z-exo-enol form predominating.

derivative of cyclopiazonic acid it was concluded the compound **1** is a new molecule, identified as acetyl cyclopiazonic acid.

This molecule is not reported as a fungal metabolite but has been prepared by Holzapfel (1968) while elucidating the structure of cyclopiazonic acid and only few IR absorption bands are reported. The anti-relation for H-4/H-5 was deduced from the coupling constant J=10.8 Hz.

The mass spectral fragmentation pattern **(Fig.10)** observed is easily rationalized by the following cleavage patterns:



$[M+H]^+$ m/z 379 ⟶ m/z 379

Cleavage A: $[C_{11}H_9N+2H]^+$= m/z 157

Cleavage B: $[M-(Ac+OAc)]^+ = [M-102]^+$= m/z 277

Cleavage C: $[M-(2Ac-2H)]^+$= M-84= m/z 295

Fig.10: Fragmentation of Compound 1

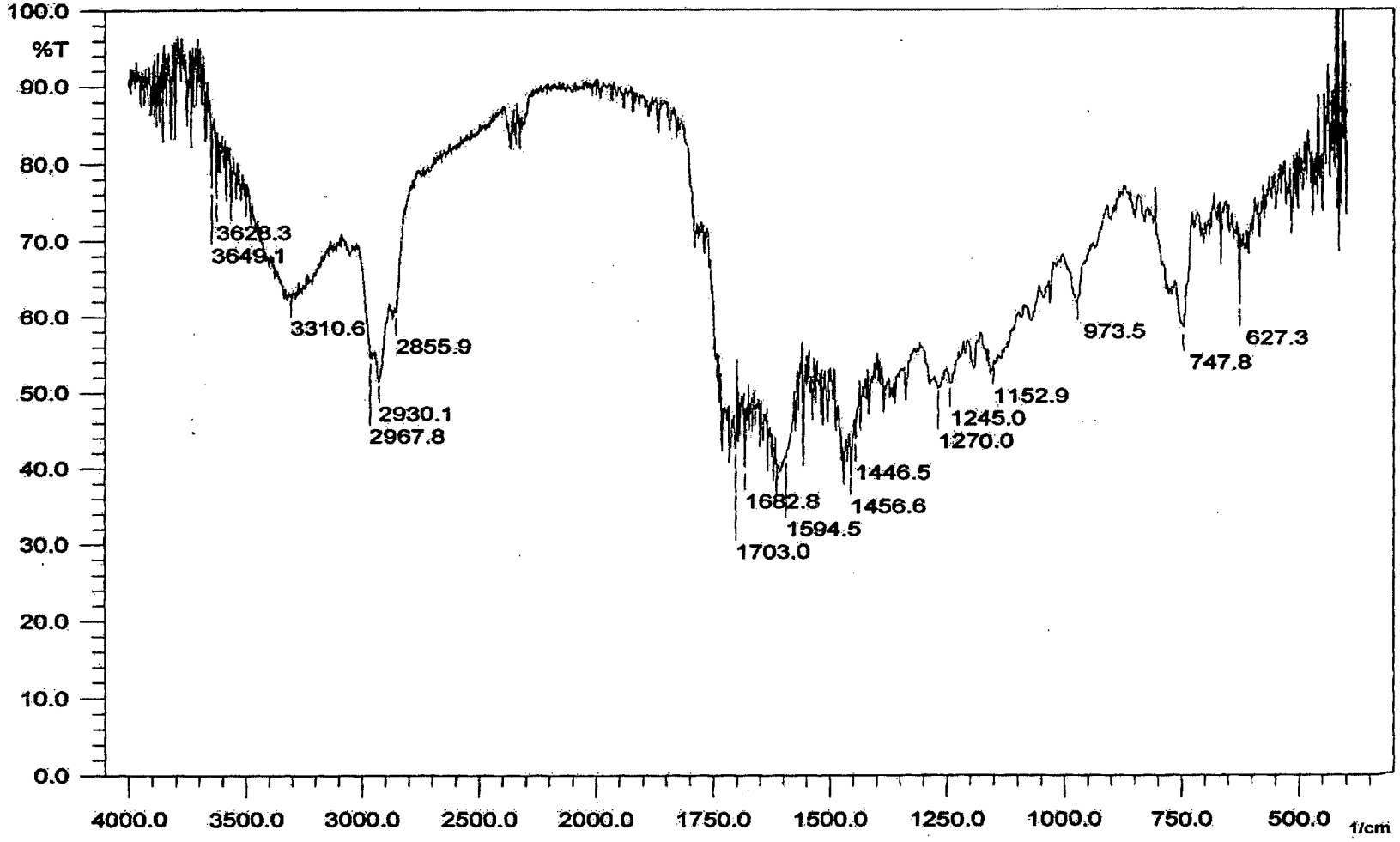


Fig.1: IR spectrum of acetyl cyclopiazonic acid

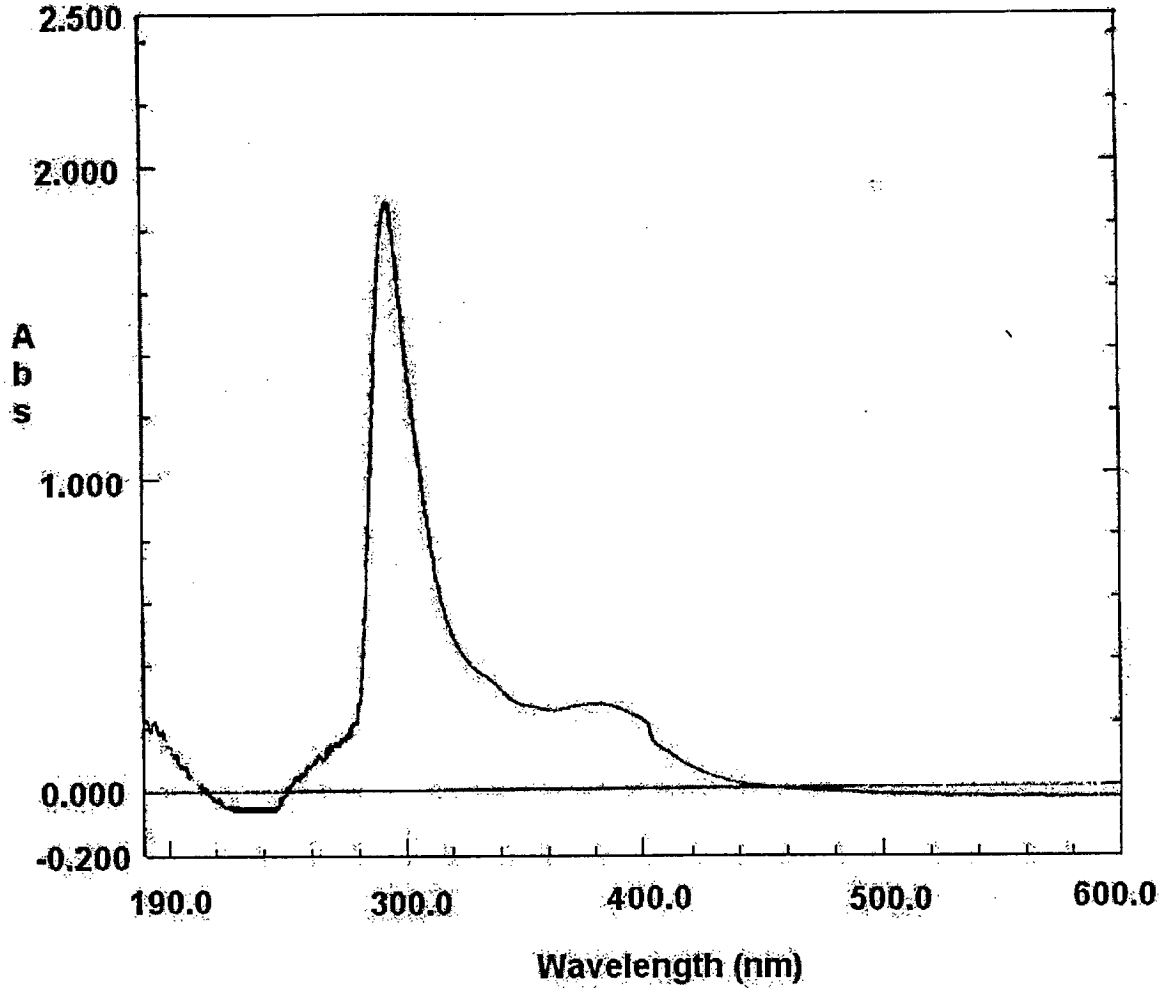


Fig.2: UV spectrum of acetyl cyclopiazonic acid

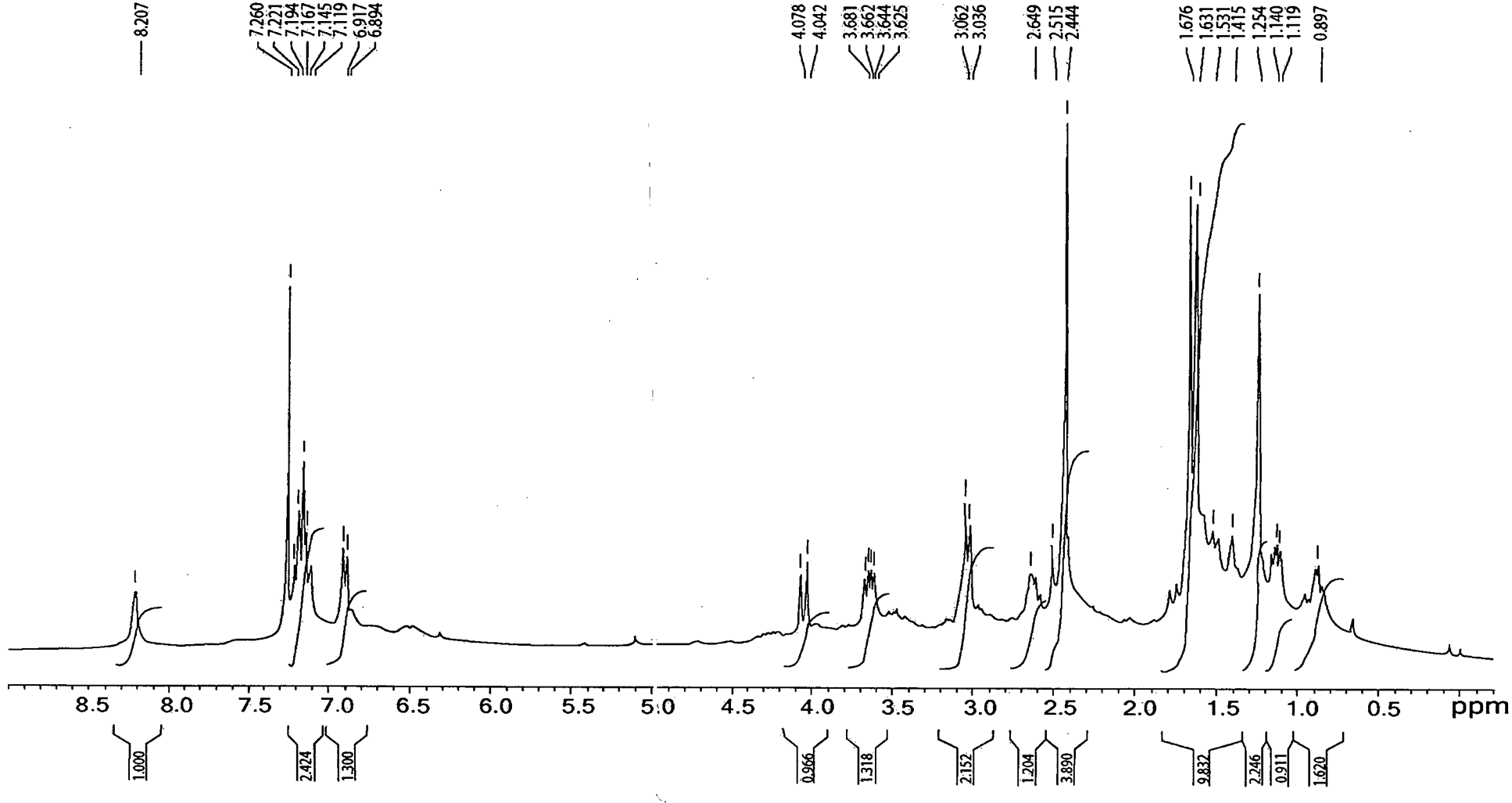


Fig.3: ^{1}H NMR spectrum of acetyl cyclopiazonic acid

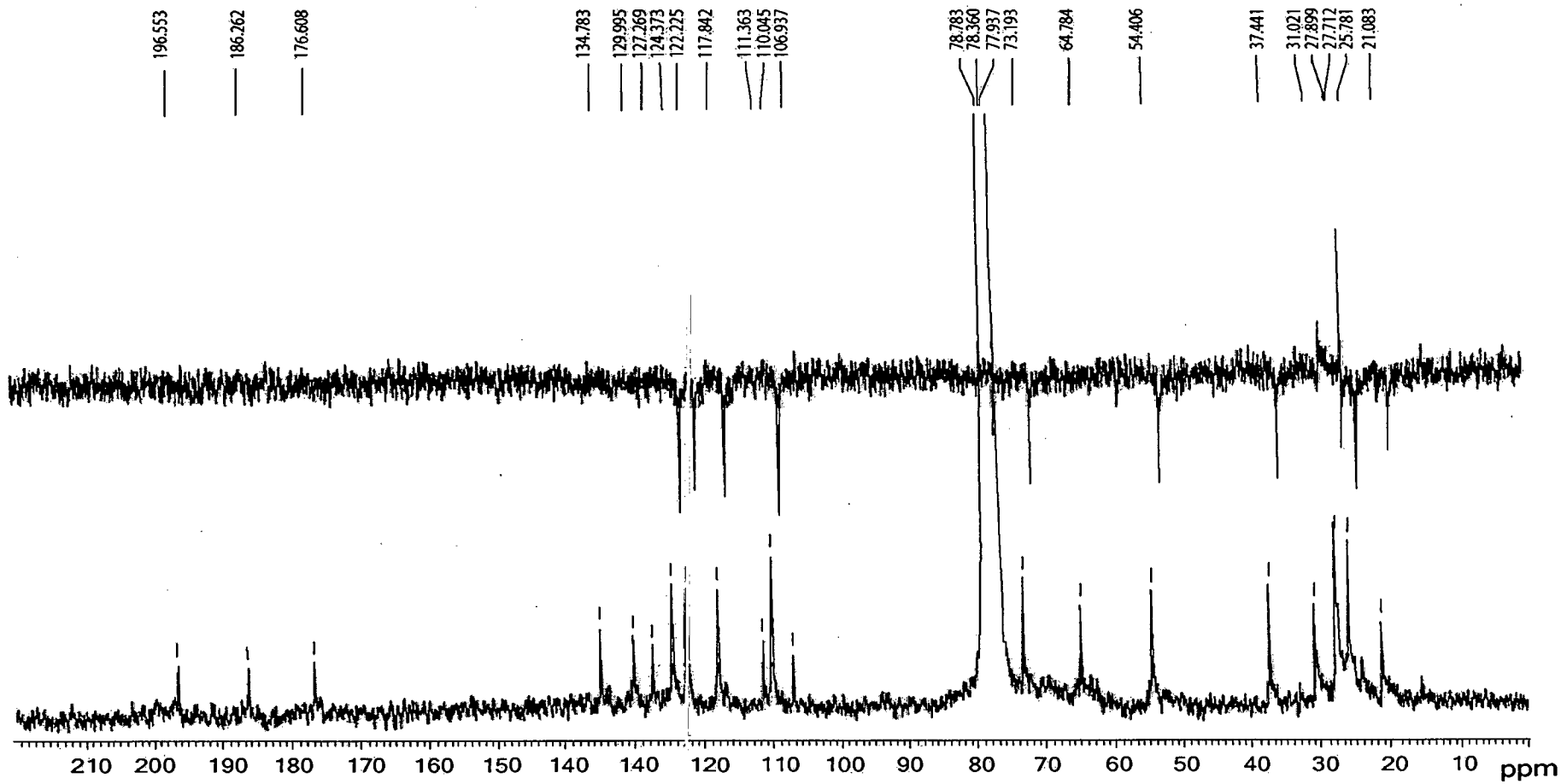


Fig.4: ^{13}C NMR & DEPT spectra of acetyl cyclopiazonic acid

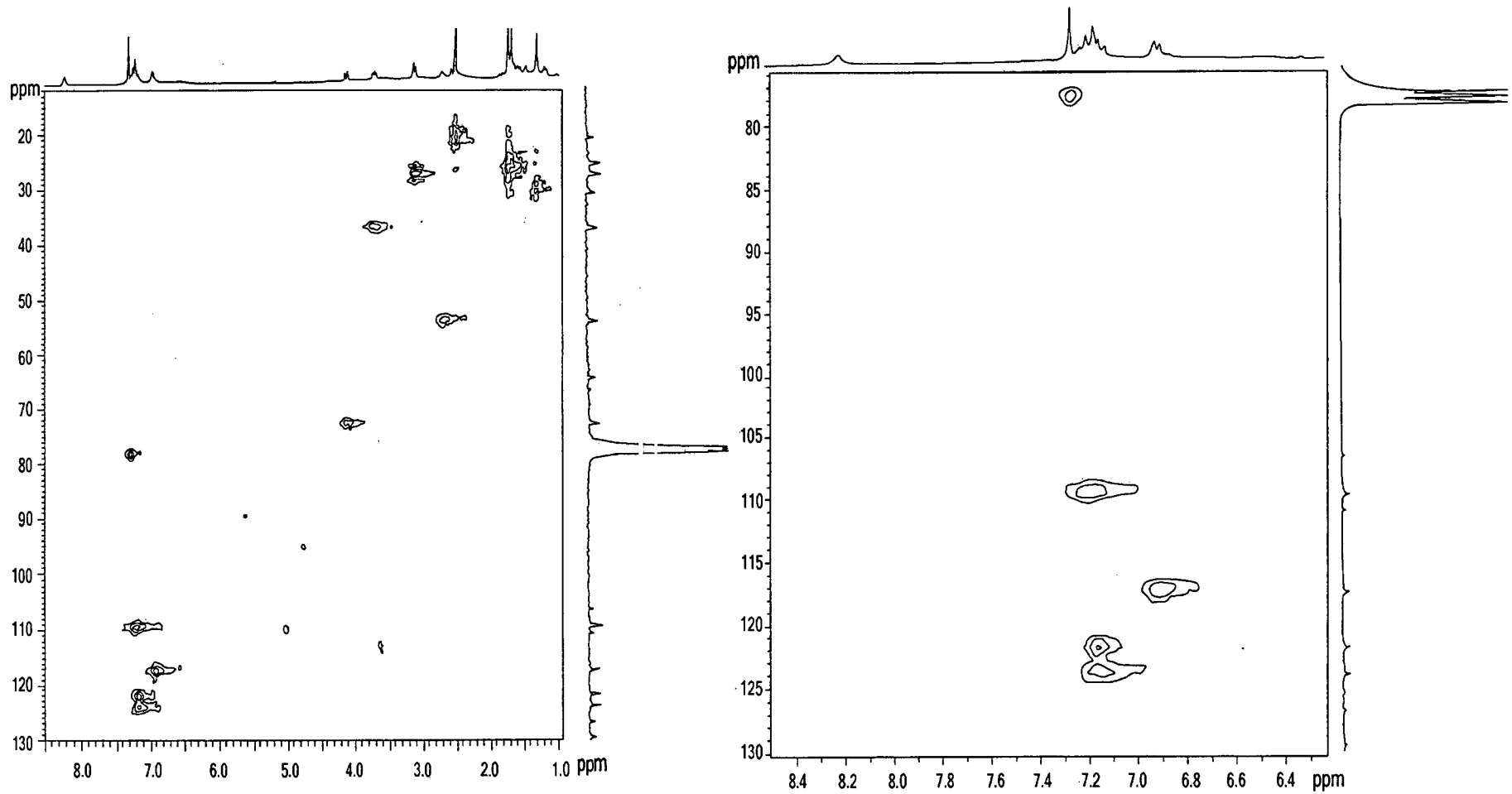


Fig.5: HMQC spectra of acetyl cyclopiazonic acid

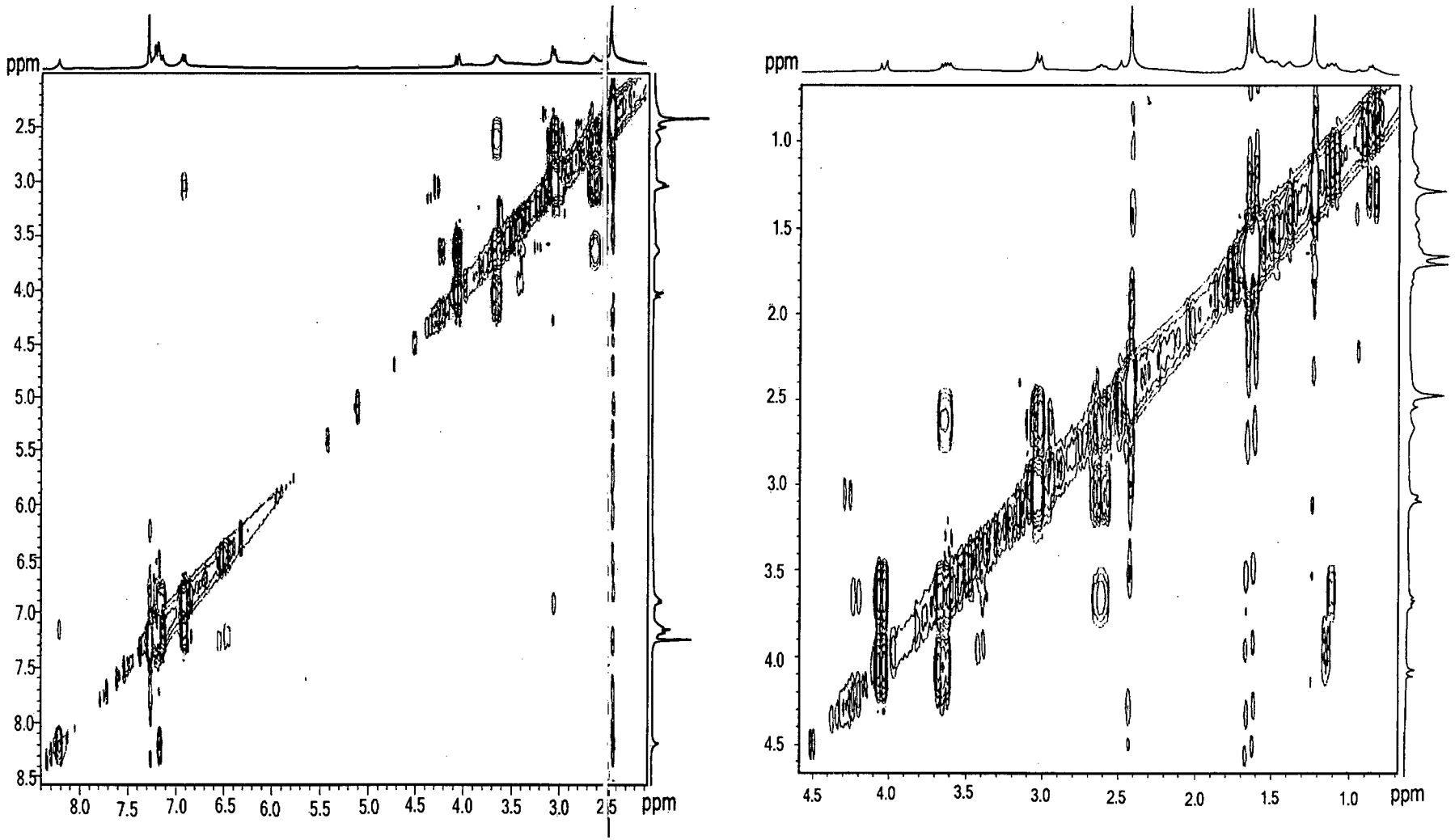


Fig.6: COSY spectra of acetyl cyclopiazonic acid

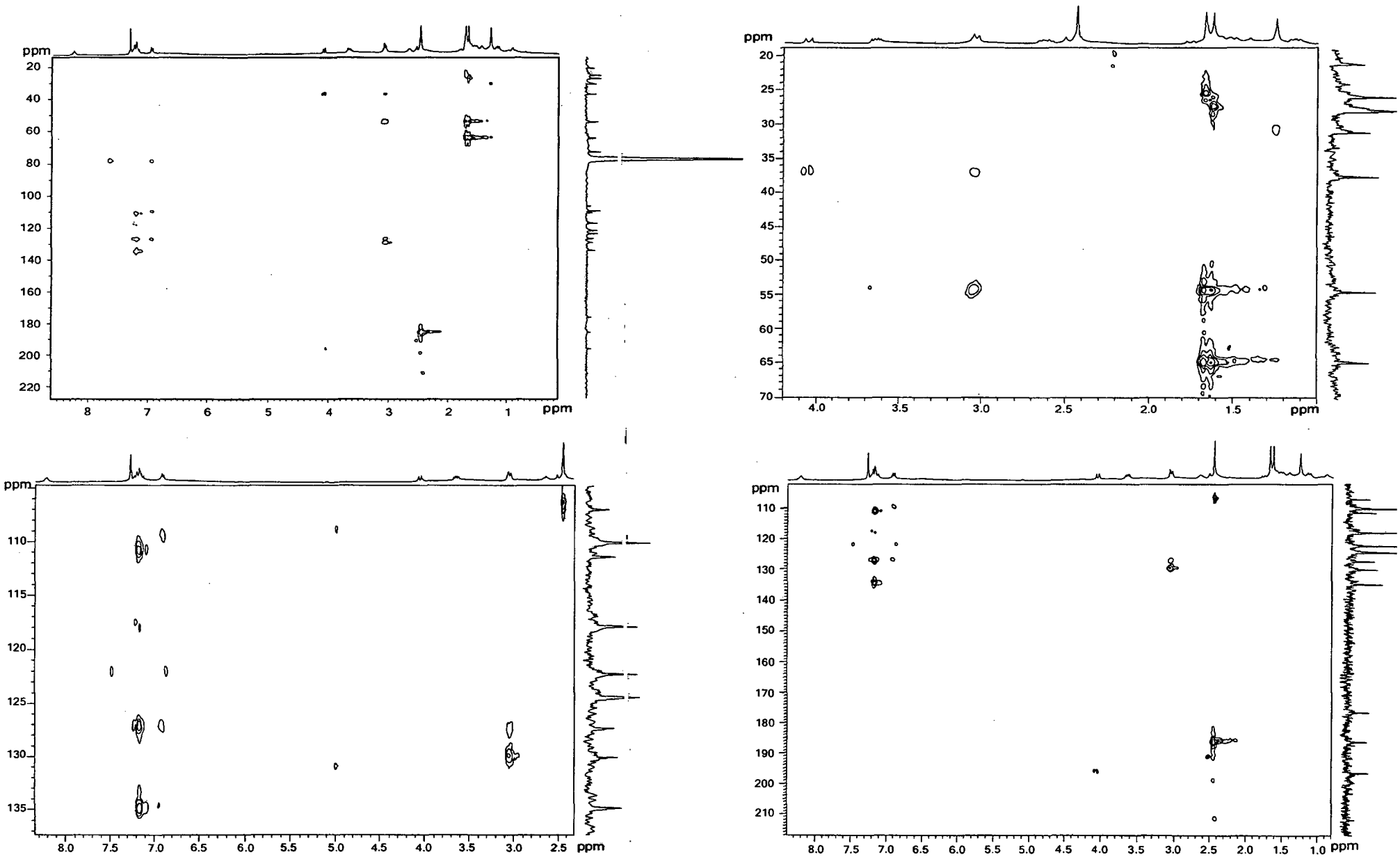


Fig.7: HMBC spectra of acetyl cyclopiazonic acid

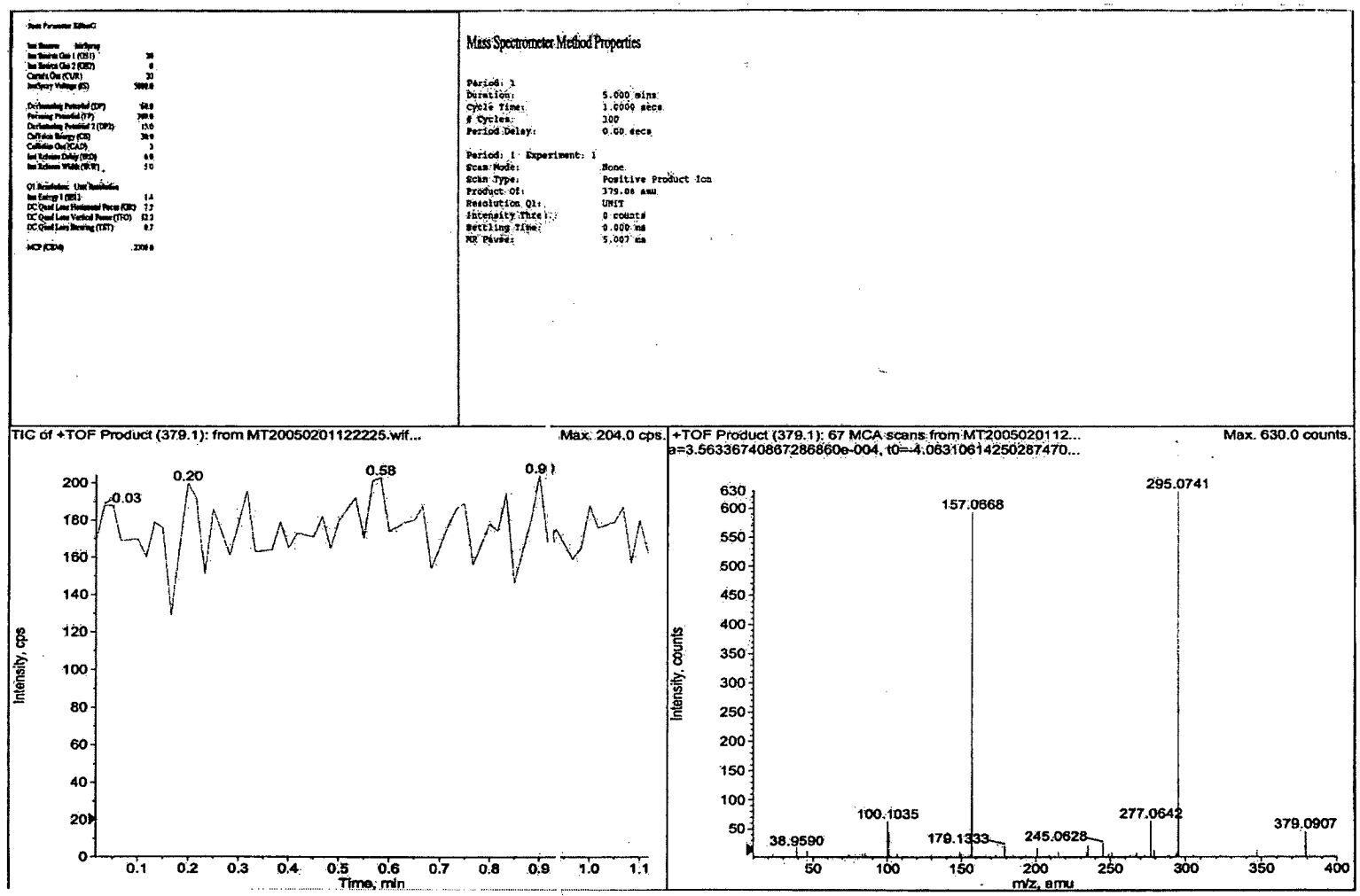
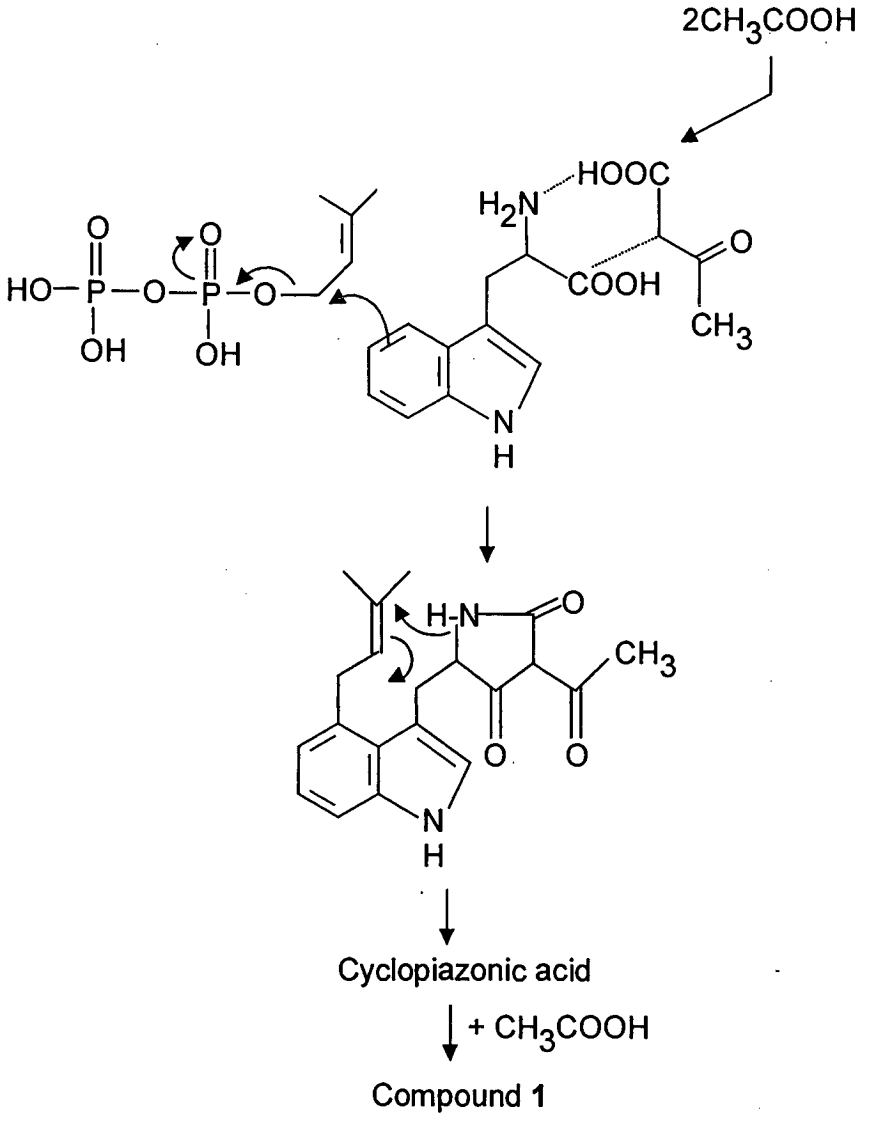


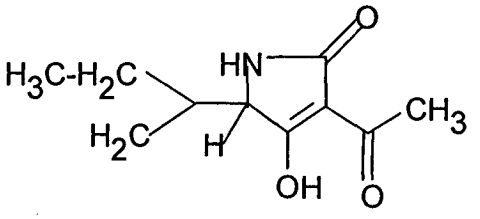
Fig.8: Mass spectrum of acetyl cyclopiazonic acid

Structure analysis of its skeleton shows that biogenetically the compound is probably derived from tryptophan a C-5 unit derived from mevalonic acid (the precursor of active isoprene, e.g. γ,γ-dimethyl allyl pyrophosphate) and three molecules of acetic acid as outlined in **scheme 1.**



Scheme 1: Biogenesis of compound 1

The biological introduction of mevalonate derived C5 units in 4–position of an indole system is known to occur, e.g. in the biosynthesis of lysergic acid portion of ergot alkaloids in *Claviceps* species (Bhattacharji *et al.*, 1962). The condensation of tryptophan and two molecules of acetic acid (or acetoacetate) to form a substituted tetramic acid would be analogous to the formation of tenuazonic acid from L-isoleucine and two molecules of acetic acid in *Alternaria tenuis* Auct. (Stickings & Townsend, 1961).



Tenuazonic acid

Speradine A with a 1-N-methy-2-oxindole ring a congener of cyclopiazonic acid has been recently reported from a marine derived fungus *Aspergillus tamarii* (Masahi Tsuda *et al.*, 2003)

This is the first report of natural occurrence of acetyl cyclopiazonic acid. It was screened for anti microbial activity and it was found to show strong anti fungal activity (6-7mm) against *Candida albicans* **(Table 3)**. It also showed weak antibacterial activity against *Shigella*

flexineri and *Klebsiella sp.* **(Table 2)** and a multi drug resistant bacterial strain *Acinetobacter* **(Table 4)**. Compounds exhibiting strong antifungal activity against *Candida albicans* are invariably reported to be anti- tumor. This is being investigated.

Table 2: *In vitro* antibacterial activity of acetyl cyclopiazonic acid

Bacterial Culture	Compound (acetyl cyclopiazonic acid)	Standard (Streptomycin)
E. coli	-	+ (7mm)
Ps. aeringinosa	-	+ (3mm)
S. aureus	-	+ (2mm)
S. typhi	-	+ (1mm)
Sh. flexineri	+ (3mm)	+ (7mm)
Klebsiella sp.	+ (2mm)	+ (6mm)
V. cholerae	-	+ (1mm)

Table 3: *In vitro* antifungal activity of acetyl cyclopiazonic acid

Fungal Culture	Compound (acetyl cyclopiazonic acid)	Standard (Nystatin)
A. fumigatus	-	-
Fusarium	-	-
Cry. neoformans	-	-
A. niger	-	-
Rhodotorula	-	-
Nocardia	-	-
C. albicans	+ (6-7mm)	-

Table 4: *In vitro* antibacterial activity of acetyl cyclopiazonic acid against multi drug resistant strains.

Bacterial Culture	Compound (acetyl cyclopiazonic acid)	Standard (Streptomycin)
S. pyogenes	-	+ (8mm)
Acinetobacter sp.	+ (1-2mm)	+ (4mm)
S. typhi	-	+ (4mm)

- Inactive
+ Active

EXPERIMENTAL

Fungal strain and fermentation conditions:

Methods of isolation, culturing and growth of *Aspergillus sulphureus* is explained in **Chapter 1, section** II. The isolated *fungus* was inoculated into Peptone bile sorbitol broth (Hi Media) in sterilized distilled water. All culture work was conducted under aseptic conditions at room temperature (27°C).

Extraction and fractionation

A cell mass *(A. sulphureus)* of 9 gL^{-1} of broth was filtered and the filtrate was extracted thrice with ethyl acetate in a separating funnel at room temperature (27°C). Solvent from the combined Ethyl acetate extracts was removed under reduced pressure on a rotavapour to yield a crude extract of approximately 350 mg of crude extract.

Isolation of acetyl cyclopiazonic acid

The crude ethyl acetate extract was filtered through Sephadex LH_{20} with methanol as the eluent. The fractions were monitored on TLC and were combined according to TLC profile. Thin layer chromatography was carried out on Merck silica gel 60 F_{254} plates and developed using ethyl acetate: petroleum ether (10:90 v/v) at ambient temperature. A bluish purple spot was visualized with 5% methanolic sulphuric acid. Further purification was done by column chromatography over silica gel (60-120 mesh), by gradient elution with increasing concentrations of ethyl actetate in petroleum ether. Pure orange solid were obtained which were collected and weighed. The yield was approximately 15 mg.

Instrumental analysis

Analysis by IR, NMR and ESI-MS was performed as explained in **Chapter 1, section II**.

Bioassay:

Bacterial and fungal strains:

Bioassays were done using seven test bacteria, seven test fungi and three multi drug resistant strains. These are clinical isolates obtained from Goa Medical College, Goa. The test bacteria include *Escherichia*

coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi, Shigella flexineri, Klebsiella sp. and *Vibrio cholerae.* Three multiple drug resistant strains include *Staphylococcus pyogenes, Acinetobacter* and *Salmonella typhi* while the seven test fungi include *Aspergillus fumigatus, Fusarium sp., Cryptococcus neoformans, Aspergillus niger, Rhodotorula, Nocardia and Candida albicans.*

Antibacterial Assay:

Antibacterial assays were carried out using disc diffusion method (NCCL, 2003). The compound was dissolved in ethyl acetate and sterile filter paper discs of Whatman filter paper No. 1 (6 mm diameter) were impregnated with the sample (0.5 mg/disc). Agar plates were spread plated (100 µl) with the test organism to allow an established lawn. On solvent evaporation, the discs were placed on agar plates. Negative control included the disc containing solvent on evaporation while the positive control included streptomycin discs (10 µg/disc, Hi Media, Mumbai). The plates were incubated at 37°C, 24 hours and zones of inhibition halos were observed and measured in mm. The assay was done in triplicates and average values are presented.

Antifungal assay:

Antifungal assays were carried out using disc diffusion method. Sterile filter paper discs impregnated with the sample (0.5 mg/disc) was

placed on agar plates containing the test fungus and were incubated at room temperature for 24-48 hours. Nystatin (100 units/disc, Hi Media, Mumbai) was used as a positive control while the solvent was used as a negative control.

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Chapter 2

Study of biliary PAH metabolites from Oreochromis mossambicus

Section I

Introduction to Polycyclic Aromatic Hydrocarbons (PAHs) in the Marine Environment

INTRODUCTION:

Industrialization of our civilization has resulted in an increased contamination of our environment with a number of cancer producing chemicals. Polynuclear aromatic hydrocarbons are of particular concern because of their demonstrated carcinogenic activity (IARC, 1973; Suess, 1970), wide distribution and persistence in the environment (Radding *et al.*, 1976).

It has become increasingly evident in recent years that extremely complex mixtures of hydrocarbons are ubiquitous trace components of the marine environment. In most cases these hydrocarbon mixtures contain complex homologous and isomeric series of alkanes, olefins and aromatic hydrocarbons (Anderson *et al.*, 1974a, b; Moore and Dwyer, 1974). The greatest concern has been expressed in marine ecological studies about the possible impact of pollutants on coastal ecosystems. Aromatic hydrocarbons particularly polynuclear aromatic hydrocarbons (PAHs), are of priority interest here because of their persistence in the environment, high bioaccumulation and high toxic potential, including their mutagenic and carcinogenic properties (GESAMP, 1993) and because of the potential health hazard they may pose to consumers of marine foods. Every year 230000 tonnes of PAHs reach the marine environment and are ubiquitously distributed

worldwide (Law, 1986). Therefore investigation of these compounds is of great scientific interest (Lipiatou & Saliot, 1991).

The concept of "polynuclear aromatic hydrocarbons" (PAHs) covers a wide range of compounds characterized by the presence of two or more condensed aromatic rings in their structure, and may be the parent unsubstituted compounds or their alkyl derivatives. PAHs are ubiquitous constituents of the natural environment, and thus also of the sea. PAHs are exceedingly toxic to aquatic organisms at concentrations of about 0.2-10 ppm. Deleterious sublethal responses are sometimes observed in aquatic organisms at concentrations in the range of 5-100 ppb (Neff, 1985). As several PAHs are known to have toxic and carcinogenic properties, they are classified as priority environmental pollutants (Schuster, 1994; Fitzmaurice, 1992; Andrulewicz & Rhode, 1987). These compounds have therefore been subjected to intensive study, primarily as markers of anthropogenic input into the sea.

The four-, five-, and six ring PAHs appear to be more carcinogenic than PAHs with smaller or larger ring systems (Neff, 1985). PAHs with small molecular weight have been shown to exhibit acute toxicity to aquatic organisms. Higher molecular weight PAHs are not highly toxic to aquatic organisms; however, in the presence of

solar ultraviolet radiation many of them may become acutely toxic. The acute toxicity of PAHs with solar ultraviolet radiation has been demonstrated in juvenile fish (Oris & Giesy, 1985, 1986; Tilghman & Oris, 1991). Parent compounds of PAHs themselves are not carcinogenic; they require metabolic activation to become reactive electrophilic metabolites (Neff, 1985).

The major environmental sources of PAHs are technological processes involving combustion or pyrolysis of material, such as heat and power generation, refuse burning, miscellaneous industrial processes and emissions from vehicular transportation media. PAHs in small quantities also appear in the environment from natural sources such as forest fires, volcanic activities and endogenous synthesis by some living organisms (Suess, 1976). These carcinogenic substances may enter natural waters, and thereby public water supplies in a variety of ways including the release of industrial effluents, direct fallout from atmospheric particulate matter, road run-offs, discharge from urban and domestic sewage and runoff or leaching from soils (Suess, 1976).

Since the occurrence of PAHs in the environment is due mainly to the combustion and pyrolysis of fossil fuels or wood, and to the release into the environment of petroleum products (Law & Nicholson, 1995) PAHs originating from combustion are dominated

by non–alkylated compounds and those from petroleum–by the alkyl derivatives. Unsubstituted PAH compounds mainly of pyrogenic origin are transported to the sea principally by rivers or the atmosphere (Lipiatou & Albaiges, 1994). They are composed of two or more fused aromatic rings. On the basis of their properties and their molecular weight, two classes of PAHs can be distinguished, i.e., the two and three ring aromatics from naphthalene to anthracene and the four to six ring aromatics from fluoranthene to indeno (1,2,3-c, d) pyrene.

PAHs occurring in the marine environment can also be formed in dia-genetic processes in coastal sediments or on land (Bouloubassi & Saliot, 1993). Many PAHs arise naturally from such sources as volcanoes, oil seeps and forest fires (NRCC, 1983). Numerous authors have suggested, moreover, that at least some PAHs can be synthesized by unicellular algae, higher plants or bacteria, but at the same time others have concluded that organisms accumulate PAHs rather than synthesise them (Cripps & Priddle, 1991; Cripps, 1992). Despite these controversies, one thing is certain: PAHs production resulting from natural processes is generally very low compared to PAHs originating from anthropogenic sources (Lipiatou & Albaiges, 1994). Anthropogenic sources of PAH are the major current input to the environment, and it has been estimated that total PAH inputs to water from all

sources (natural and anthropogenic) now amount to more than 80000 tonnes yr^{-1} (NRCC, 1983).

The solubility of PAHs in water is low and decreases with increasing molecular weight. Due to their hydrophobic nature the concentrations of dissolved PAHs in seawater are very low. Otherwise, the PAHs are easily associated with particulate matter and finally deposited in the sediment. Microbial degradation, photo-oxidation and chemical oxidation are thought to be the major processes contributing to the decomposition of PAHs (Lee & Ryan, 1983; Readman *et al.*, 1984). Fish living in PAH-contaminated environments absorb these compounds through the body surface and gills or by ingestion of contaminated sediments or food (Varanasi *et al.*, 1989).

Parent PAHs often predominate in sediments, deriving both from combustion sources and from oil. They are quite persistent, particularly in aerobic sediments and can accumulate to high concentrations (Witt, 1995). Although, the lower molecular weight PAHs can be acutely toxic, however, the major concern is for some of the higher molecular weight compounds that when ingested by marine animals can form metabolites that are active carcinogens. These compounds are absorbed across the body surface (especially the gill epithelium), or are taken up from the alimentary

canal following the ingestion of contaminated sediment or food (Varanasi *et al.*, 1989). The incidence of liver neoplasms and other abnormalities in bottom dwelling fish has been linked with PAH concentrations in the sediments over which they feed (Malins *et al.*, 1988; Vethaak & Rheinallt, 1992).

In common with all vertebrates, fish have a well developed mixed function oxygenase (MFO) system that can rapidly metabolise PAHs into hydrophilic products that are more easily excreted. Consequently only minor concentrations of parent compounds are usually detectable in fish tissues (Ariese *et al.*, 1993b, Di Giulio *et al.*, 1995; Araujo *et al.*, 2000). Nevertheless depending on the chemical structure and level of exposure, PAHs and their metabolites have the potential to produce toxic mutagenic and/or carcinogenic effects in fish and other vertebrates including humans (Mac Rae & Hall, 1998; Monteiro *et al.*, 2000) and for this reason, the increase in levels of PAH contamination in aquatic systems that has occurred in recent decades is a cause for concern (Weber & Lanno, 2001).

Aromatic hydrocarbons exposure in marine organisms is often assessed by measuring the concentration of PAHs in their tissues however detection of PAHs accumulation levels in fish tissue is usually not feasible due to rapid transformation (metabolism) of

PAH into more polar and more easily excretable forms (Varanasi *et al.*, 1989). Fish caught at highly polluted sites often showed only trace levels of PAHs in the tissue, due to the ability to metabolise the compounds (Varanasi *et al.*, 1989). PAHs within the organism are subjected to oxidation and conjugation reactions that will facilitate their excretion. Exposure to PAH induces the production of detoxifying enzymes that are present in many tissues and that results in the formation of hydrophilic metabolites (Di Giulio *et al.*, 1995). These biotransformations can be separated into two main phases. In phase I reactions, the catalytic activity of mixed-function oxidases are especially important and often involve the addition of one or more hydroxyl groups to the parent molecule. In phase II reactions, various polar moieties are conjugated to the phase I product (Di Giulio *et al.*, 1995). The liver is an important site of PAH metabolism and metabolites produced here are secreted into the bile and strored in the gall bladder before being excreted in the faeces (Varanasi & Stein, 1991; Britvic *et al.*, 1993). The gall bladder bile is a major excretion route for PAHs in fish. After biotransformation, PAH metabolites are excreted into the bile and concentrated (Aas *et al.*, 1998).

The occurrence of biotransformation does not however imply that PAHs are relatively harmless to metabolizing species. On the contrary, during metabolism some reactive intermediates can be

formed that can bind to proteins and DNA (Conney, 1982; Cavalieri & Rogan, 1992; De Maagd & Vethaak, 1998). An alternative method to assess the uptake of PAHs by metabolizing species is the analysis of biotransformation products (metabolites). In fish this is possible by analyzing the bile fluid (Krahn *et al.*, 1987; Johnston & Bauman, 1987; Ariese *et al*; 1993a,b; Van der Oost *et al.*, 1994, Vethaak *et al.*, 1996).

The use of fish bile metabolites to monitor contamination in aquatic systems is now well established (Stegeman & Lech, 1991; Aas *et al.*, 2000). They are easily detected by sensitive fluorometric techniques either directly (Cormier *et al.*, 2000) or after deconjugation to regenerate phase I metabolites (Ariese *et al.*, 1993b, Stroomberg *et al.*, 1999).

Laboratory studies have demonstrated that the presence of PAH metabolites in bile is well correlated with levels of exposure (Collier & Varanasi, 1991; Britvic *et al.*, 1993; Upshall *et al.*, 1993; Yu *et al.*, 1995). PAH metabolites detected in fish bile have been applied as a biomarker of exposure to both pyrogenic and petrogenic PAHs (Hellou & Payne, 1987; Krahn *et al.*, 1987; Ariese *et al.*, 1993b; Lin *et al.*, 1996). It has been proven to be a simple and sensitive method for screening PAH contamination in fish (Goksoyr & Forlin, 1992; Bucheli & Fent, 1995). By determining PAH metabolites it is

possible to investigate links between exposure levels and effects such as DNA damage and tumour formation (Ariese *et al.*, 1997). Thus, the bile can be used as an indicator of PAH exposure. Many PAHs and their metabolites display strong and characteristic fluorescent properties (Aas *et al.*, 1998).

Since bile metabolites can easily be detected fluorometrically, either directly (Cormier *et al.*, 2000) or after deconjugation (Ariese *et al.*, 1993b), they provide a sensitive technique for detecting and monitoring PAH contamination. The assessment of total bile metabolites by high-performance liquid chromatography (HPLC) has proved useful in biomonitoring programs; although in most cases individual metabolites have not been identified (Krahn *et al.*, 1984; Deshpande, 1989; Upshall *et al.*, 1993). The HPLC-F method for estimating bile metabolites has been successfully used to monitor field exposure to PAHs both in marine (Krahn *et al.*, 1986) and in fresh water fish (Johnston & Baumann, 1989; Maccubbin *et al.*, 1988).

By and large, pollution research is centred on the effects of discrete contaminants on organisms. A major issue, however, is that organisms do not respond to a single factor at a time but to a suite of stresses, including multiple environmental factors, acting together. Under field conditions the metabolism and toxicity of

contaminants are liable to be altered by the presence of multiple contaminants / stresses (Addison, 1988). Reports indicate that PAHs could have more than additive (synergistic) effects with other PAH mixtures and with other pollutants (Chaloupka *et al.*, 1993).

The concentration of NO_2^- in aquatic ecosystems can be elevated much above the normal as a result of pollution with nitrogenous waste and/or an imbalance in the bacterial nitrification and denitrification processes. Several estuarine and coastal areas worldwide are increasingly experiencing eutrophication and subsequent oxygen depletion from an over-supply of nutrients (Nixon, 1990).

Besides, in some upwelling areas, waters periodically have naturally high nutrient content and low dissolved oxygen. Hypoxia alters the biogeochemical cycling of nutrients in the affected waters through denitrification, leading to short-term accumulation of nitrite (as well as formation of nitrous oxide, a green house gas) (Shailaja *et al.*, unpublished)

Naqvi *et al* (2000, 2002) have reported severe oxygen depletion in the Arabian Sea bordering the west coast of India during the SW monsoon on account of high organic production fuelled by nutrients supplied through upwelling and increased fertilizer runoff from land. The development of hypoxia leads to intense water column

denitrification and accumulation of nitrite (upto 16 μM) at shallow depths (Naqvi *et al.*, 2002). Nitrite is a highly reactive chemical, which is more toxic to freshwater organisms than to sea water organisms (Eddy *et al.*, 1983). Brackish and marine fish are not particularly susceptible to nitrite toxicity since chloride salts compete with nitrite for uptake at the gill lamellar surface. Nevertheless, nitrite has been found to significantly increase the toxicity of PAHs such as phenanthrene leading to severe hepatic damage in estuarine fish (Shailaja & Rodrigues, 2003).

The objective of this study was to investigate the fate and biological impact of PAHs in fish in the presence of nitrite. The effect of nitrite on the metabolism and impact of Phenanthrene was investigated in the estuarine fish, *Oreochromis mossambicus*. Biotransformation of PAH was followed by high-performance liquid chromatography of the total PAH metabolites in the bile (Deshpande, 1989) and by analysis of the bile by Electrospray ionization mass spectrometry (ESI-MS). This technique had proved to be a suitable analytical technique for identifying DNA adducts in biological systems. The identification of molecular species is further supported by the literature reports. The formation of toxic metabolites and DNA adducts is also supported by the physiological status and DNA damage which was assessed by single cell gel electrophoresis (Comet assay).

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Section II

Preliminary Analysis of Biliary PAH Metabolites of Estuarine Fish Oreochromis mossambicus

INTRODUCTION

The biotransformation of a hydrophobic xenobiotic in fish is a major determinant of its toxicity, distribution and ability to be excreted. The biological half-lives of lipophilic xenobiotics would be markedly prolonged without biochemical processes that convert lipophilic compounds to more readily water-soluble and excretable products. The major PAHs metabolization pathways involve cytochrome P450 monooxygenase, epoxide hydrolase and several conjugating enzymes (Jimenez & Stegeman, 1990; Pritchard, 1993). The oxidative metabolism of PAHs proceeds through highly electrophilic intermediate arene oxides, some of which are covalently bound to cellular macromolecules such as DNA, RNA and protein (Miller & Miller, 1981).

Eutrophication-related hypoxia is one of the major stresses experienced in the marine environment (Nixon, 1990). Hypoxia alters the biogeochemical cycling of nutrients in the affected waters through denitrification, leading to short-term accumulation of nitrite (NO_2^-). NO_2^- is a highly reactive chemical, which is more toxic to freshwater organisms than to sea water organisms (Eddy *et al.*, 1983).

The mechanism by which nitrite plays a role in the biotransformation of PAH is not completely clear. The HPLC analysis of the biliary PAH

metabolites was carried out to study the synergistic effect of PAH and nitrites on the metabolism in fish. The liver somatic index (LSI) was used to determine the physiological status of the exposed fish. Genotoxicity of the metabolites of PAH formed by the administration of a combination of PAH and nitrite was assessed by modified single cell gel electrophoresis (Singh *et al.*, 1988).

RESULTS AND DISCUSSION:

Reverse-phase HPLC analysis with fluorescence detection of diluted 1:100 bile for conjugated PAH metabolites formed in the presence of nitrite showed distinct peaks which were not present in the bile exposed to nitrite alone (control). In the bile sample of *O. mossambicus* exposed to phenanthrene and nitrite, the presence of new metabolites was observed (**Fig.1c**). This occurred simultaneously with the decrease of one of the major metabolite formed with phenanthrene alone and eluting around Rt=37 minutes.

Similarly, the bile of *O. mossambicus* exposed to chrysene showed only one major peak (Rt=24.37 minutes) that is indicative of chrysene metabolite (**Fig.2b**) whereas there was a marked absence of this peak in the HPLC profile of bile of fish exposed to chrysene plus nitrite (**Fig.2c**). The latter showed three new metabolites (Rt=28.5058, 29.235 and 31.500).

Since identification of the above metabolites was not possible on the basis of HPLC due to non availability of standards, analysis of the bile was further conducted by electrospray ionization mass spectrometry (ESI-MS) **(Chapter 2, Section III)**.

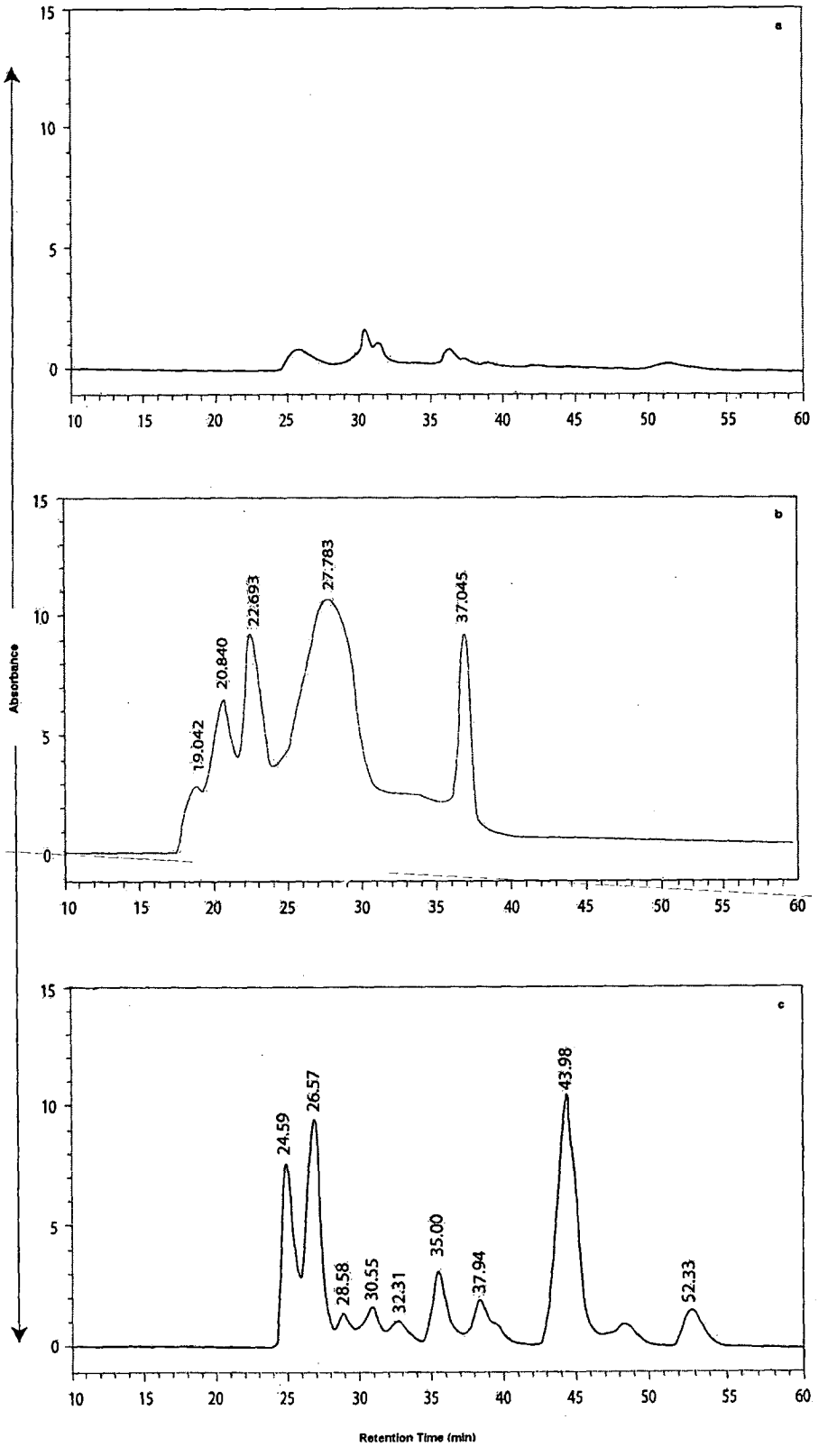

Fig.1: HPLC chromatograms of biliary PAH metabolites of fish exposed to phenanthrene and nitrite

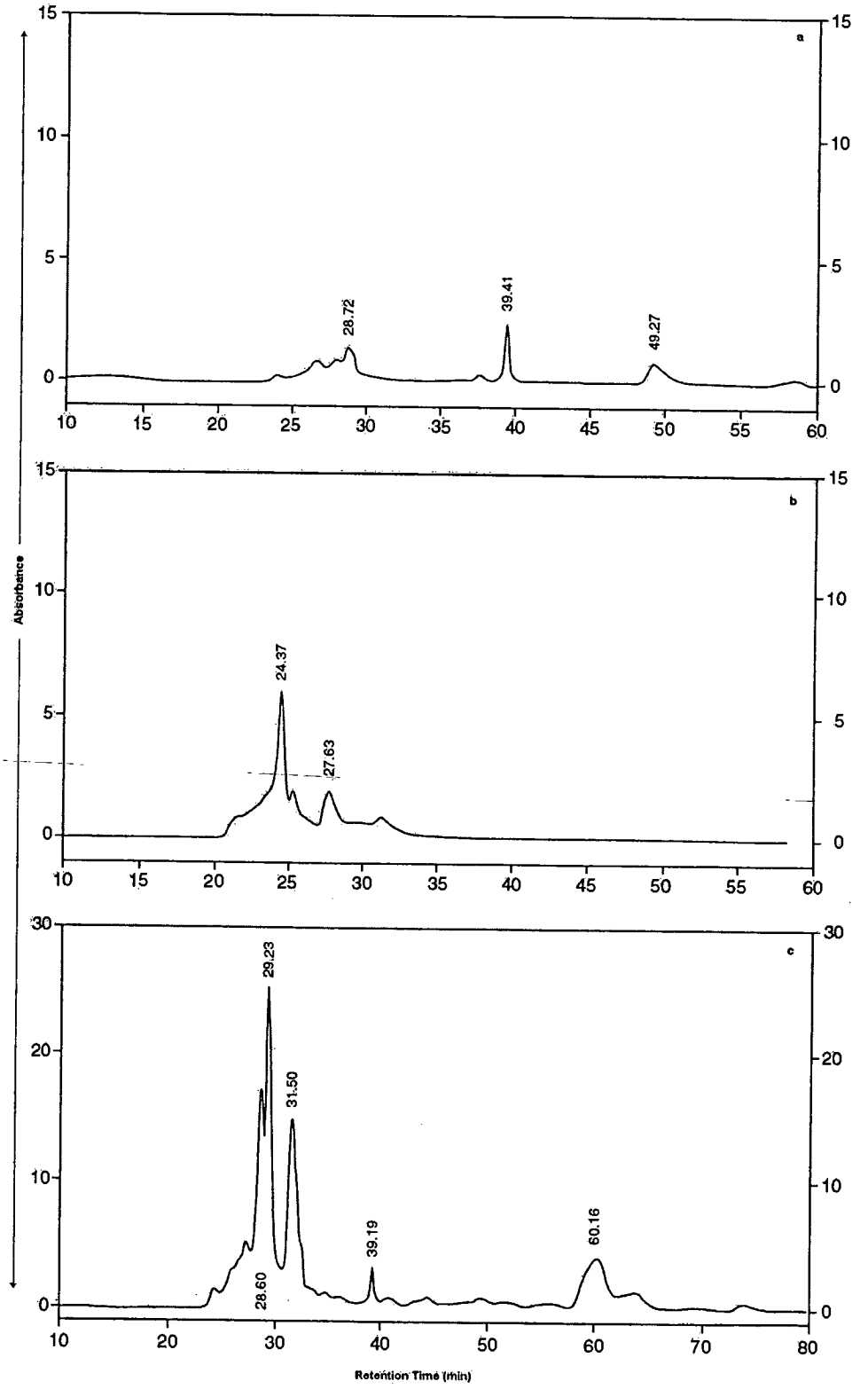

Fig.2: HPLC chromatograms of biliary PAH metabolites of fish exposed to chrysene and nitrite

Liver somatic index (LSI) has frequently been used as a biomarker for examining fish exposed to environmental contaminants. LSI values are generally elevated in vertebrates experiencing induction of hepatic microsomal P-450 for the detoxification of organic compounds (Huuskonen and Lindström-Seppa, 1995). Exposure to pollutants (PCBs, PAHs etc) causes the LSI, to increase as the liver increases in size to allow greater detoxification of pollutants. This is in contrast to the effect of other stresses, such as acidification, petroleum hydrocarbons and other chemical inputs that result in a decrease in LSI exposure. This decrease is presumably caused by a reduction in liver glycogen due to increased metabolic cost, reducing the size of the liver. The LSI values for the present study are given in **Table 1**. The LSI for phenanthrene-exposed fish was decreased to 8.67 ± 0.25 against a control of 14.8 ± 0.23, whereas in chrysene it increased to 24.3 (± 0.8). In case of fish exposed to phenanthrene and chrysene in presence of nitrite LSI values were enhanced to 21.46 (± 0.49) and 42.94 (± 1.12) respectively. From the present investigation it is also apparent that exposure to PAH in the presence of NO_2^- could cause lasting, sublethal physiological deterioration of the fish.

Table 1: Liver somatic index

Experiment	LSI %
Control	14.80(± 0.23)
Phenanthrene	8.67(± 0.25)
Phenanthrene + Nitrite	21.46(± 0.49)
Chrysene	24.30(± 0.8)
Chrysene + Nitrite	42.94(± 1.12)

One of the sensitive approaches for detecting genotoxic damage is the use of a DNA strand breakage marker (Mitchelmore & Chipman, 1998). DNA strand breaks are potentially pre-mutagenic lesions (Kammann *et al.*, 2001). Many genotoxic agents can produce strand breaks even if they do not induce metabolic enzymes (such as Cytochrome P450) and do not form DNA adducts (Kammann *et al.*, 2001). The most commonly used techniques for DNA strand break detection is the single cell gel electrophoresis or the comet assay, developed by (Singh *et al* 1988). In this assay, cells are suspended in agarose gel, lysed and nuclear DNA is subjected to high pH (>13) to allow unwinding and then exposed to alkaline electrophoresis. Broken DNA fragments migrate from the nucleus towards the anode. The resulting images, which are named for their appearance as 'comets' are then measured to determine the extent of DNA damage (Fairbairn *et al.*, 1995; Boeck *et al.*, 2000).

DNA adducts and DNA strand breaks are potentially mutagenic lesions that have been proposed as genotoxicity biomarkers for the biomonitoring of the marine environment (Mitchelmore & Chipman, 1998, Lyons *et al.*, 1999). It has also been hypothesized the DNA damage can produce a suite of health effects referred to as genotoxic disease syndrome, which includes reduced productive and reproductive capacity (Kurelec, 1993). The potential ecological impact of such health effects to individuals includes disturbances in population and community level dynamics. Because of the broad ecological implications associated with genotoxicity, the detection and quantification of genetic damage is of interest in environmental studies. Genotoxicants produce chemical or physical modifications to DNA, commonly measured as DNA adducts or DNA strand breaks respectively (Nacci *et al.*, 1996).

The results of comet assay are represented in **Fig. A (control) and B (comets).** In the present study, liver cells of *O. mossambicus*, which had been exposed simultaneously to non-carcinogenic phenanthrene and nitrite showed quite severe DNA damage. DNA strand break was observed in nearly 84% of the cells of the exposed fish. Similarly, liver cells of *O. mossambicus* exposed to chrysene in the presence of nitrite showed extensive type 4 DNA damage (Kobayashi *et al.*, 1995). More than 70% DNA strand breaks was observed. Chrysene is a weakly

Fig. A: Undamaged DNA (Control)

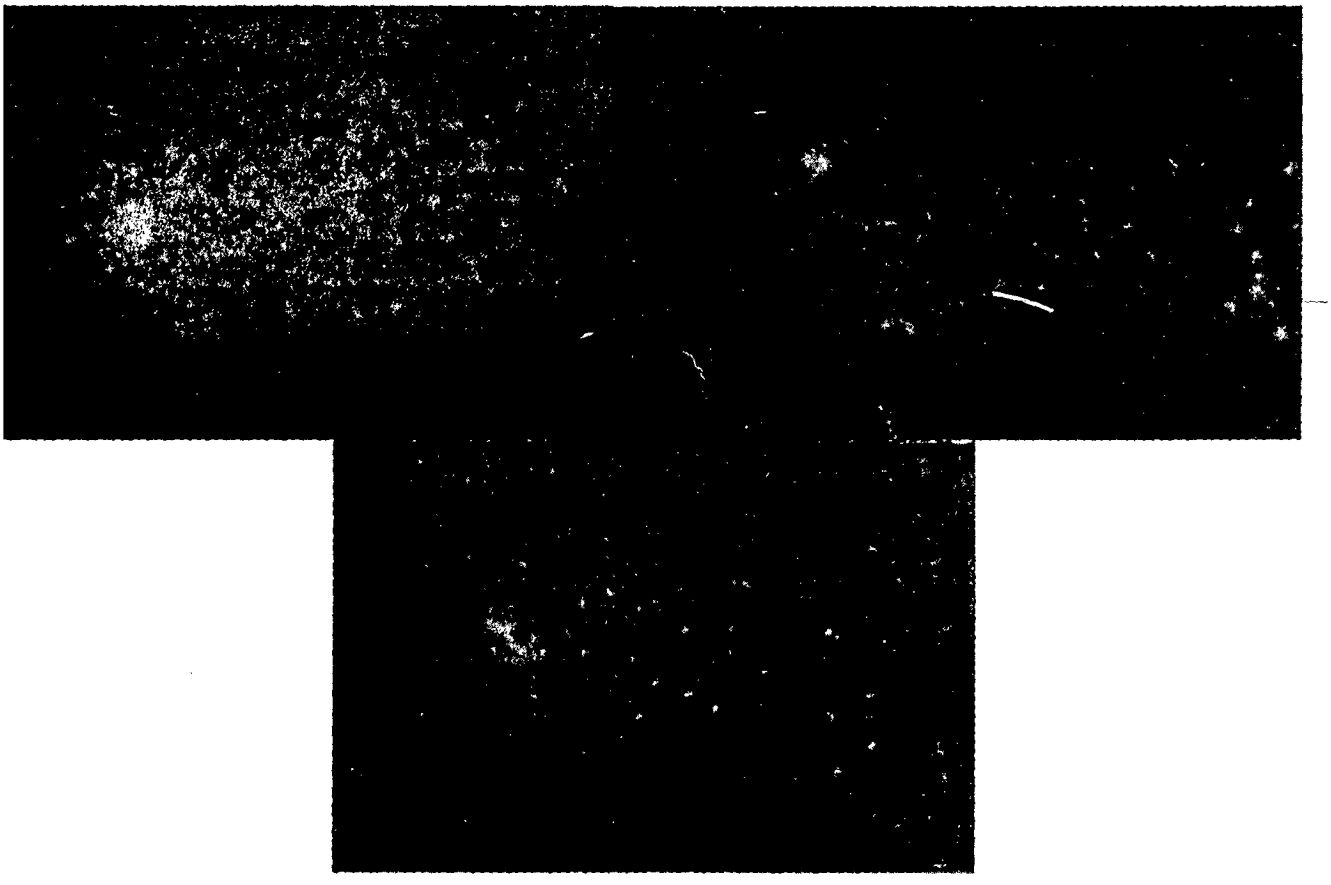

Fig. B: Comets (damaged DNA)

carcinogenic PAH. In association with nitrite its genotoxic effect increases 5-fold.

Nitrite is a highly reactive chemical species but little is known about how it affects the response of biota exposed to abundant xenobiotics in the environment such as PAHs. In both the above studies, i.e. phenanthrene and chrysene, nitrite was found to significantly enhance PAH metabolism.

In coastal waters experiencing reducing conditions, non-lethal concentrations of nitrite showed significant impact on the metabolism of phenanthrene in the euryhaline fish *O.mossambicus* leading to deleterious physiological & biochemical effects (Shailaja & Rodrigues, 2003). Benzo-ring diols (1, 2-diol and 3, 4-diol) plus phenols (1-and 3-hydroxychrysene) have been reported to be the major chrysene metabolites formed by brown bullhead liver microsomes (Pangrekar *et al.*, 2003). Based on the polarity, elution times, genotoxicity and the physiological status of the fish, the metabolites in the present study, we tentatively identified the new metabolites as the nitro derivatives of the oxygenated PAH metabolites, considering the reported possibility of enzymatic conversion of phenols to nitrated compounds by the action of several peroxidases (Budde *et al.*, 2001).

Interest in nitrated polycyclic aromatic hydrocarbons (nitro-PAHS) has grown ever since it was shown that PAHs react with oxides of nitrogen to form nitro –PAHs (Pitts *et al.*, 1978); although the role of nitrite in PAH metabolism and biotransformation in fish is not clearly understood.

Many nitro-PAHs, which are environmental contaminants, are highly mutagenic and some of these have shown to be tumurogenic (IARC, 1989; Howard, 1990; *Fu et al.*, 1988). They originate primarily as direct or indirect products of incomplete combustion. Nitro-PAHs have been detected in diesel exhaust notably 1-nitropyrene, 2-nitrofluorene and 3-nitrofluoranthene. The highest levels of nitro-PAHs in the environment have been found in urban air level. But it has been shown that some nitro-PAHs, notably 2-nitrofluoranthene and 2-nitropyrene, which are not found in diesel exhaust, have been detected in urban, sub urban, forest as well as remote areas. The ubiquitous occurrence of these nitro-PAHs is probably due to their photochemical origin from parent PAHs, which attached to carbon particles, have been widely dispersed in the troposphere (Kielhorn, 2003).

In the atmosphere, gaseous PAHs react with hydroxyl and nitrate radicals, in the presence of NO_x to form a range of nitro PAHs, transformation products. The reactions of PAHs with the hydroxyl radical occurs during day light, while the reaction with the nitrate radical occurs during night (Atkinson *et al.*, 1988, Arey *et al.*,

1989a,Biermann *et al.*, 1985, Atkinson *et al*, 1990,Atkinson *et al.*, 1994, Arey *et al.*, 1989b).

Many nitro-PAHs are mutagenic in bacteria and mammalian systems and also carcinogenic in rodents (IARC, 1989, Tokiwa & Ohnishi, 1985); they are implicated to play a role in the etiology of some human cancers (El-Bayoumy, 1992, Hecht & El-Bayoumy, 1990). Though concentrations of nitro-PAHs are typically 1-2 orders of magnitude lower than analogous unsubstituted PAHs (Atkinson, 1988), their mutagenic potency can be several orders of magnitude greater (Nishioka *et al.*, 1988; Helmig *et al.*, 1992).

Comparatively little is known about most of these nitro-PAHs and how they metabolize and form premutagenic lesions. In particular, the role of oxidation in *in vivo* metabolism and identification of DNA adducts after administration of nitro PAHs need to be further investigated. To our knowledge there are no reports on the studies made to characterize *in vivo* formed PAH metabolites in a medium containing NO_2^- ions. PAHs in fish can be metabolized rapidly to intermediates that either bind to liver DNA or form conjugates for ultimate transfer to bile (Varanasi, 1989; Collier and Varanasi, 1991; D'Adamo *et al.*, 1997; Livingstone, 1998; Broman *et al.*, 1990).

The above results led us to further investigate the biliary metabolites for the formation of DNA adducts.

EXPERIMENTAL

Exposure Protocol:

Oreochromis mossambicus (6.8-18.6g) was obtained from a local estuarine fish farm. The fish were acclimatized in the laboratory for 7 days in stored tap water amended with reagent grade NaCl (final concentration 15 gL^{-1}) to nullify the effect of nitrite in the experimental tanks. The exposure experiments (9-12 fish per tank) were conducted for 15 days in 30 L glass aquaria with continuous aeration. The control experiments with 6 fish each were conducted in clean water as described above and with equivalent amounts of added NO_2^- and dimethylformamide (DMF).

In another set of experiments *O. mossambicus* (n=9) were treated with pure phenanthrene (Sigma, USA) in DMF with and without NO_2^- for 15 days. The test medium was replaced every 24 hours. The final concentration phenanthrene and nitrite was 1mg l^{-1} and 1 μM l^{-1} respectively. The above experiment was repeated by treating *O. mossambicus* (n=12) with pure chrysene (Sigma, USA) in DMF with and without NO_2^- for 15 days.

Hepatic Biomarkers:

At the conclusion of each experiment, livers were excised, weighed and the liver somatic index (LSI) determined according to Sloof *et al.* (1983) as

$$LSI = [\text{liver weight (g)} / \text{whole body weight (g)}] \times 100$$

HPLC analysis of biliary PAH metabolites:

Reagents:

Methanol (HPLC grade), Water (HPLC grade)

Procedure:

After 15 days the fish were harvested and dissected. The gall bladder was detached from the liver and the bile was collected with a syringe. The bile samples were stored at –20°C until analysis. Before analysis the bile samples were diluted 100 times (10μl to 1000μl) with distilled water/methanol 50:50. High-performance liquid chromatography (HPLC) (Thermo Quest Spectra system) separation was used to analyse PAH metabolites in the bile samples without preliminary deconjugation. The metabolites were separated on a reverse-phase C18 column (Chromspher 5) using methanol / water (50:50 v/v) as the solvent. The standard wavelength pairs used to measure phenanthrene metabolites were 306/377 and to measure chrysene metabolites were 308/368 nm (Ruddock *et al.*, 2003).

Comet Assay for DNA strand-break determination:

Isolated hepatic cells of *Oreochromis mossambicus* (control and exposed) were subjected to single cell gel electrophoresis or comet assay (Singh *et al.*, 1988;Fairbairn *et al.*, 1995).

Reagents:

Dimethylsulphoxide (DMSO)- Sigma

Disodium (EDTA)- Sigma

Disodium hydrogen phosphate (Na_2HPO_4)

Ethidium Bromide (Merck)

Potassium Chloride (KCl),

Potassium dihydrogen phosphate (KH_2PO_4)

Sodium Chloride (NaCl)

Sodium lauryl sarcosinate (Sigma)

Sodium hydroxide (NaOH),

Triton X-100 (Sigma)

Tris (Sigma)

I. Preparation of reagents:

A. PBS (Phosphate Buffered Saline) Ca^{++} & Mg^{++} free

137mM NaCl (8g), 2.7mM KCl (200mg), 65mM Na_2HPO_4 (11.5 g), 1.5 mM KH_2PO_4 (200mg) dissolved in 1000 ml of distilled water and pH to 7.4.

B. Lysing solution

2.5 M NaCl (146.1g), 100mM EDTA (37.2 g), 10 mM Tris (1.2 g) is dissolved in 700ml of distilled water and stirred for 20 minutes. About 12 g of NaOH is then added and the mixture is allowed to dissolve. Once dissolved in the solution, 10 g Sodium lauryl sarcosinate is added and the mixture is stirred again to dissolve the ingredients. The pH is then adjusted to 10 using concentrated HCl or NaOH and the volume to 890 ml with distilled water. The lysing stock solution is filtered and stored at room temperature. To the above lysing solution, 1% Triton X-100 and 10 % DMSO are added fresh and the solution is refrigerated for 30- 60 minutes prior to slide addition.

C. Electrophoresis Buffer:

Preparation of stock solution:

(a) 10N NaOH (200 g/500ml distilled water)

(b) 200mM EDTA (14.89 g/200ml distilled water, pH 10)

The working buffer solution is made fresh before each run by mixing 30 ml NaOH solution with 5ml EDTA solution and the volume is adjusted to 1000ml with distilled water.

D. Neutralisation buffer:

0.4 M Tris (48.5 g) is mixed with 1000 ml distilled water and the pH is set to 7.5 with concentrated HCl.

E. Ethidium Bromide stock-20μg/ml:

To 10 mg Ethidium Bromide 50 ml distilled water is added. For the working solution, 1ml stain is mixed with 9 ml distilled water.

II. Preparation of slides:

Materials:

Microscope slides with tray

Cover slips (24 x50mm),

Coplin jars (opaque)

Low melting point agarose (LMPA)-Sigma

(Melting temperature: 65°C, geling temperature: 30°C)

Normal melting point agarose (NMPA)-Sigma

(Melting temperature: 88°C, geling temperature: 36°C)

Microtip pipettes with tips

Procedure:

Cell Isolation:

The fish was dissected and the liver was removed and placed in 1 ml of cold mincing solution i.e., HBSS (Hank's balance salt solution) containing 20mM EDTA/ 10% DMSO. The liver was minced into large pieces and allowed to settle. The mincing solution is aspirated several times to get a fine cell suspension. For the preparation of the slide 5 μl of the cell suspension was mixed with 75 μl LMPA. The slides were prepared as follows.

Preparation of gel slides:

0.5% LMPA and 0.75% NMPA were prepared in PBS (125mg LMPA or 187 mg NMPA in 25 ml PBS). The gels were heated in a microwave or by another heating source until near boiling, to dissolve the agarose. Melted agarose is stored in 5 ml aliquots at 4°C. When needed, heating briefly melted the gels. For layering the gels, about 110-150 μl of NMPA was added onto fully frosted microscope slide and a cover slip was placed over it immediately. The agarose was allowed to set at 4°C for 5-10 minutes. Once NMPA was set, the cover slip was gently removed by sliding, and 75μl LMPA (37°C) mixed with 20μl of cell suspension (fish liver), was added onto the NMPA layer. The cover slip was replaced and the gel was again allowed to set at 4°C for 5-10 minutes. For the third agarose layer, the cover slip was gently removed and 75 μl of LMPA is added onto the second agarose layer. The cover slip was replaced and agarose is allowed to set again at 4° C for 5-10 minutes. The coverslip was removed again and the slides were gently lowered into Coplin jars (opaque) containing cold lysing solution and refrigerated overnight till electrophoresis.

Electrophoresis:

Following lysis on the next day, the slides were gently removed from the lysing solution and placed side by side in the horizontal electrophoretic box nearer the anode (+). The slides were placed as close together as possible, with their gels touching each other. The

electrophoretic reservoir was filled with fresh electrophoretic buffer until the buffer level completely covers the slides. Care was taken to avoid air bubbles over the agarose gel. The slides were allowed to sit in the alkaline buffer for 20 minutes to allow unwinding of DNA the expression of alkali labile damage. Power supply was turned on to 25 volts and the current is adjusted to 300 milli amperes by raising or lowering the buffer level. Electrophoresis was carried out for 20 minutes. After 20 minutes, the power supply is turned off. The slides were gently lifted from the buffer and placed on a staining tray. The slides were carefully flooded with neutralizing buffer. After allowing the buffer to sit on the slide for 10 minutes the buffer was drained; the process is repeated two more times. To each slide, 150 μl of ethidium bromide stain (working solution) was added and the slide is covered with a fresh cover slip. Before visualization of the slides, the excess stain was blotted form the sides and back of the slide. The slide was observed under a fluorescent microscope (Olympus BX60 F-3) using a 40x objective linked to camera. Cells with damaged DNA appear like comets. Twenty-five cells were scored at random per slide to determine the percentage of comets formed. The damaged DNA or comets were photographed.

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Section III

Characterisation of Biliary PAH Metabolites of Estuarine Fish Oreochromis mossambicus by ESI-MS

INTRODUCTION:

PAHs in fish are not directly responsible for fish kills, but may induce a key step in the mechanism of malignant transformation through the formation of adducts between DNA and reactive electrophilic metabolites (Mathieu *et al.*, 1998). Although the effects of PAHs have not been extensively investigated to date, these compounds may affect neurotransmission mechanism in a similar fashion. PAHs in the gonads may be transmitted to the progeny from the parental gamete and this may enhance the process of mutagenesis and carcinogenesis in the off spring. Further, the reproductive systems can be affected to such an extent that they fail to develop eggs and spawn (Johnson *et al.*, 1993), cause precocious sexual maturation in young fish (Collier *et al.*, 1998) or result in the birth of deformed young (Manahan, 1990).

It is generally believed that adducts formation by the interaction of a chemical or metabolized chemical with cellular DNA plays an essential role in the initial step of mutations and neoplastic cell transformations that can lead to chemical carcinogenesis. Adducts can thus serve as molecular biomarkers suitable for use in risk assessment (Poirier and Beland, 1992, Ross *et al.*, 1996, Rindgen *et al.*, 1995, Wang *et al.*, 2000). Furthermore, the levels of DNA adducts can be used to document exposure to specific chemical hazard, to signal the onset of

adverse health effects. For these reasons, much effort has been directed to the identification of DNA adducts.

Fish is an important indicator of marine environmental health. A high incidence of liver tumors has been reported in several species of bottom dwelling fish collected from areas contaminated with these chemicals (Bauman *et al.*, 1987, Murchelano & Wolke, 1985, Myers *et al.*, 1991). Several studies have shown that bony fish can biotransform PAHs to metabolites, which are similar to those reported for mammalian species (Egass & Varnasi, 1982, Pangrekar *et al.*, 2003a, Sikka *et al.*, 1990, Swain & Melius, 1984, Varanasi *et al.*, 1986, Yuan *et al.*, 1999).

The coastal belt around India, particularly the west coast besides receiving anthropogenic chemical contaminants, are also subjected to natural changes in their chemical composition. For instance, the western continental shelf experiences the seasonal development of shallow suboxic zone characterized by intense denitrification (Naqvi *et al.*, 2000), resulting in the accumulation of nitrite (up to 16 μM) at shallow depths (30 m and less). At this concentration nitrite is non-toxic to fish, in contrast to the accumulation observed (2 mg or more) in aquaculture practice, leading to methemoglobinemia, a highly toxic condition in several species of fish (Russo & Thurston, 1991). It may also affect the dynamics of conjugative metabolism but not phase I

biotransformation in exposed fish (Goksoyr *et al.*, 1986). The effect of low concentrations of nitrite on the metabolism of xenobiotics such as PAHs in fish is not known.

Currently, only limited information is available on the metabolism of phenanthrene by fish. Goksoyr *et al.*, 1987, reported that the liver microsomes from control rainbow trout or cod metabolized phenanthrene predominantly at the K-region (9,10 position). This is in contrast to the reports by others on the metabolism of PAHs by fish showing that K-region of PAH is not the major site of metabolic attack by these organisms. Pangrekar *et al.*, 2003b have shown that brown bullhead (*Ameriurus nebulosus*) liver microsomes metabolizes phenanthrene predominantly in the benzo-ring 1, 2-dihydrodiol and to a lesser extent at the K-region (9,10 position), the 3, 4-dihydrodiol representing a minor proportion to total phenanthrene metabolite.

The research described here deals with, the short term effects of exposure of *Oreochromis mossambicus*, a species of tilapia, to non lethal concentrations of phenanthrene and nitrite by determining the types of metabolites including the DNA adducts produced by the ring oxidation and nitro reduction pathways. Several nitro PAHs are formed from their mother PAHs in air in the presence of nitrogen oxides (Ebert, 1998). To our knowledge there are no reports on the studies made to characterize *in vivo* formed phenanthrene metabolites in a

medium containing NO_2^- ions. PAH in fish can be metabolized rapidly to intermediates that either bind to liver DNA or form conjugates for ultimate transfer to bile (Varanasi, 1989; Collier & Varanasi, 1991; D'Adamo *et al.*, 1997; Livingstone, 1998; Broman *et al.*, 1990).

The strategy used here for the study of phenanthrene metabolism by *Oreochromis mossambicus*, a species of tilapia, involves the following three sets of experiments:

Set 1: The first set of studies deals exclusively with the metabolism of phenanthrene in *Oreochromis mossambicus*, a species of tilapia, by liver microsomes (Fish exposed to a water medium containing phenanthrene).

Set 2: In the second set a solution of the tricyclic aromatic hydrocarbon, i.e. phenanthrene dissolved in sunflower oil was injected into the fish and exposed to a medium containing nitrite ions (sodium nitrite ($NaNO_2$)).

Set 3: The third set deals with the metabolism of fish exposed to the aromatic hydrocarbon in the presence of nitrite ions in the aquatic medium (Fish exposed to a water medium containing Phenanthrene and nitrite ions).

Analysis of the bile for the metabolic products was done by Electrospray ionization mass spectrometry (ESI-MS) approach, which has proven to be a suitable analytical technique for identifying DNA adducts in biological systems. Besides the DNA adducts, other related metabolites possibly derived from the hydrocarbon have also been identified. The identification of molecular species is also supported by the literature reports.

RESULTS AND DISCUSSION:

Exposure to PAHs results in the induction of specific forms of cytochrome P450 1A1 that catalyzes aryl-hydrocarbons-hydroxylase (AHH), ethoxy-resorufin-O-deethylase (EROD) and 7-ethoxycoumarin-O-deethylase (ECOD) activity (Lech & Vodicnik, 1985; Veld van *et al.*, 1990; Collier *et al.*, 1992b; Pesonen, 1992; Di Giulio *et al.*, 1993). The net result of all these enzymatic reactions is the addition of an oxygen atom to the substrate; in most cases oxygen is further reduced to form a hydroxyl group (Masfaraud *et al.*, 1992; Sved *et al.*, 1992).

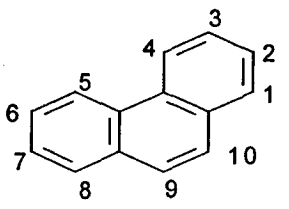
Contact with biological material can significantly affect the structure of chemical compound (Lech & Vodicnik, 1985; Stegeman, 1989; Linström-Seppa, 1990). In general, biotransformation of foreign chemicals serves two purposes to make them less toxic and/ or to

make them easier to excrete. Detoxification does not occur at any time. For example, many foreign compounds are metabolized to highly toxic products that are responsible for carcinogenesis and toxic effects (Ahokas, 1979).

In vitro experiments with benzo(a)pyrene (BaP) indicated that dihydrodiols, precursors of the main BaP metabolite bound to DNA, make up as much as 75% of the metabolites formed by cytochrome P-450 E (Stegeman & Kloepper-Sams,1987; Jimenez & Stegeman, 1990). Experiments have shown that a rise in monooxygenase activity due to pollutants can increase the amount of potentially reactive chemicals that can form adducts with DNA (Tuvikene, 1995).

In the present work phenanthrene has been studied for its metabolism using *Oreochromis mossambicus* for experimentation. Whenever necessary, the experiments have been repeated under identical conditions. Here reference is made to the third set of experiments.

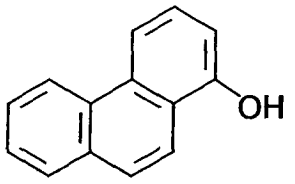
Set 1: Metabolism of phenanthrene 1 in *Oreochromis mossambicus*,



Phenanthrene

As mentioned above, in the first set of experiments fish *Oreochromis mossambicus*, was exposed only to phenanthrene as mentioned above.

Typical positive ESI-MS of bile metabolites extracted with methanol is shown in **Fig.1**. It showed molecular ion species at m/z 193, 239, 301, 413, 453 and 513. The ion at m/z 193 in the mass spectrum seems to originate from 1-hydroxyphenanthrene (M^+ =194). It is a bile metabolite occurring to the extent of (2-8 %) of total PAHs in three species of fish, i.e., common eels (*Anguilla anguilla*), European flounders (*Pleuronectes flesus*) and conger eels (*Conger conger*) from the Severn estuary (Ruddock *et al.*, 2002).



1-hydroxyphenanthrene

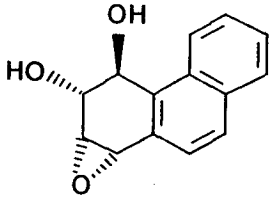
1

Pangrekar *et al.,* 2003b have reported phenols as the major metabolites (40.5%) formed by liver microsomes of brown bullhead (*Ameiurus nebulosus)*, a bottom dwelling fish species. This ion could also arise from the oxidation of phenanthrene at the K-region i.e., 9,10 position (Pangrekar *et al.*, 2003b). Photoxidation of phenanthrene to 9,10-epoxy-9,10-dihydrophenanthrene under stimulated environmental conditions is known (Patel *et al.*, 1978). The signal at m/z 239

reasonably corresponds to nitro hydroxy phenanthrene on the basis of its molecular weight.

In the MS/MS spectrum **(Fig.2)** the product ion of m/z 239 did not show any fragmentation except the fragment ions at m/z 38.9 and the base peak at 22.99 due to the potassium and sodium ions respectively. This could be attributed to the stability of the molecule at the low collision energy used (13eV). Increase in the collision energy to 32eV led to the spectrum illustrated in **Fig.3**. Even though no nitrite has been added to the medium, formation of nitro hydroxy phenanthrene is explained as being formed from the original PAH due to presence of nitrogen oxides in the air (Ebbert, 1988).

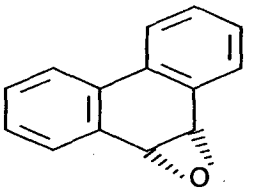
Metabolism of phenanthrene by liver microsomes of *O. mossambicus* under aerobic conditions besides the metabolites mentioned above also produces the ring oxidized metabolite *trans*-1, 2-dihydroxy-*anti*-3, 4-epoxy-1, 2, 3, 4-tetrahydro phenanthrene **(2)** as evident by the deprotonated molecular ion at m/z 227 in the negative ESI-MS **(Fig.4)** of the bile extract.



1, 2-dihydroxy-*anti*-3, 4-epoxy-1, 2, 3, 4-tetrahydrophenanthrene

2

Jacob *et al.*, 1982 have reported the metabolism of phenanthrene exclusively to 9,10-dihydrodiol by rat liver microsomes of untreated animals. Pretreated with various PAHs and related compounds led to additional oxidation at the 1, 2 and 3, 4 position. They also report secondary metabolites to dihydrodiol epoxides, detected as triol (Jacob *et al.*, 1982). Patel *et al.*, 1978 have also reported the photoxidation of phenanthrene in hexane aqueous phase, under simulated environmental conditions to yield 9, 10-epoxy-9,10-dihydrolphenanthrene **(3)**.



9, 10-epoxy-9, 10-dihydrodiolphenanthrene

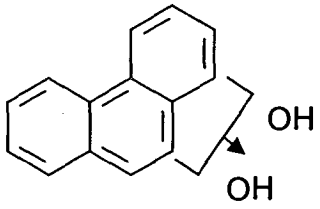
3

The above experiment was repeated under similar conditions and the observations made are as follows:

The positive ion ESI-MS of the methanolic extract of the fish bile **(Fig.5)** obtained by direct injection of the diluted solution into the instrument showed several molecular species, which included some of the impurities present in the bile as biological constituents of fish. Few of the selected precursor ions were subjected to ESI-MS/MS i.e. collision induced dissociation (CID). Thus, the CID spectrum of the molecular ion at m/z 233 **(Fig.5A)** under collision energy of 27eV

yielded daughter ions at m/z 215, 197, 173, 157, 102 and 99. Ions due to sodium and potassium were evident at m/z 22.98 and 38.95 respectively.

The sodiated molecular ion $[M+Na]^+$ at m/z 233 has been identified as 1,2 or 9,10-dihydroxyphenanthrene **(4)** based on the fragmentation observed. Formation of some of the fragment ions could be explained as shown in **scheme 1 (Table 1)**.

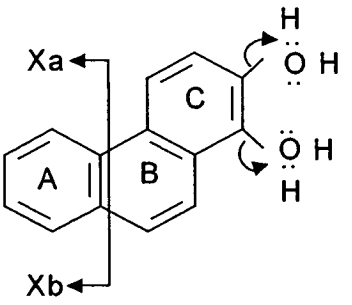


1, 2 or 3, 4 or 9,10-dihydroxyphenanthrene

4

Table-1: Fragmentation observed in the positive ESI-MS spectrum of **4**

Ions	m/z
$[M+Na]^+$	233
$[M+Na]^+$-H_2O	215
$[M+Na]^+$-$2H_2O$	197
[(M-XaXb)+ $Na]^+$	157
$[XaXb+Na]^+$	99

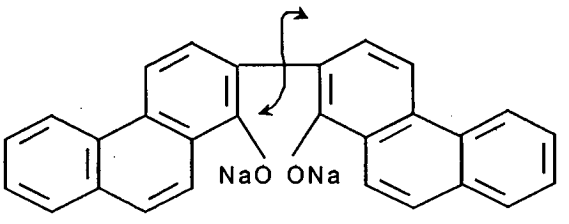


1,2 or 3, 4 or 9,10- dihydroxyphenanthrene

Scheme 1: Proposed fragmentation 1, 2 or 3, 4 or 9, 10-dihydroxyphenanthrene

The fragmentation observed in the mass spectrum **(Scheme 1)** is consistent with the –OH groups in benzo-ring, either A or C yielding 1, 2 -dihydroxy phenanthrene and hydroxylation at K region giving 9, 10-dihydroxy compound. This is supported by earlier observations that bottom dwelling fish species, *Ameriurus nebulosus*, metabolises phenantrene to phenols (40.5%) as the major metabolites (Pangrekar *et al.*, 2003).

The CID spectrum of ion at m/z 431 **(Fig.5B)** was attributed to a dimer probably obtained by the dimerization of the sodiated adduct ion as shown below.



5: Dimer

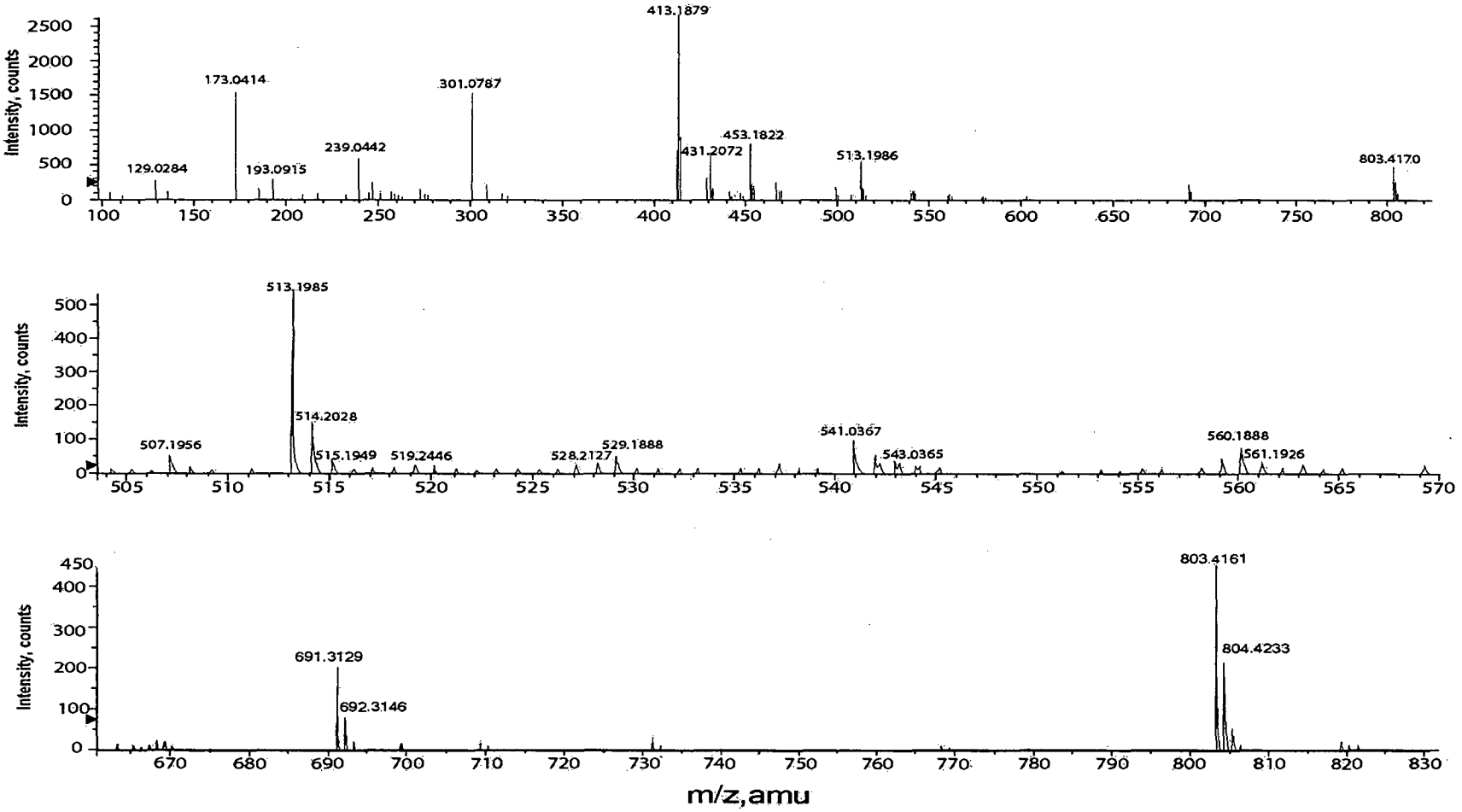


Fig.1: Positive ESI-MS of biliary metabolites of fish exposed to phenanthrene

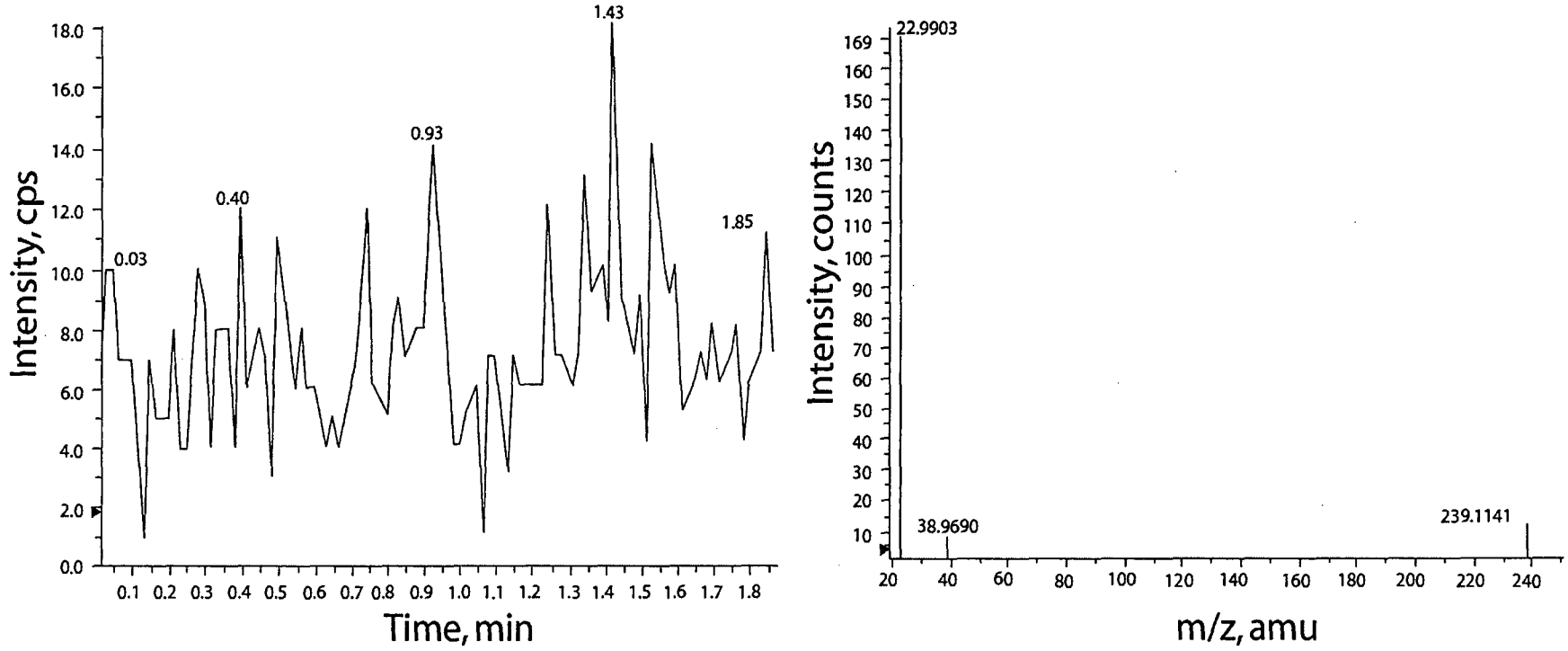


Fig. 2: Positive ESI MS/MS of the ion at m/z 239 at collision energy 13 eV

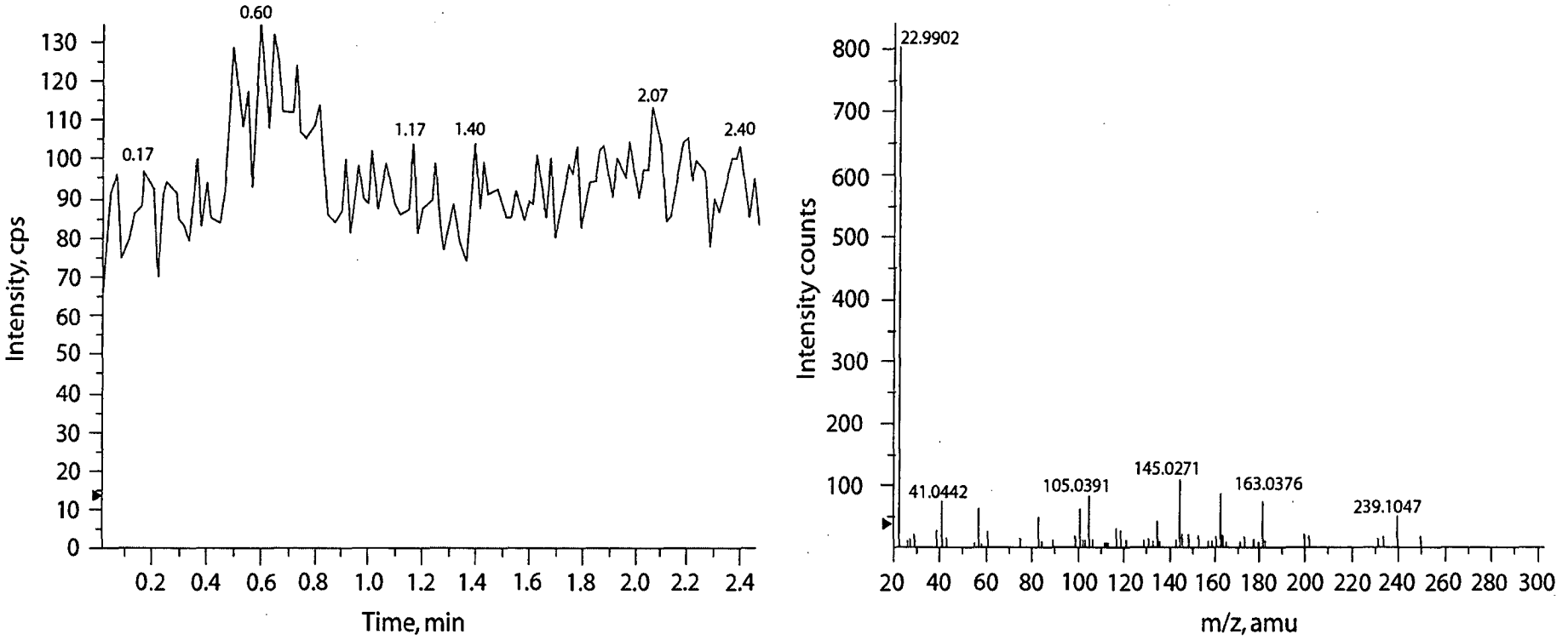


Fig.3: Positive mode ESI-MS/MS spectrum of nitro-hydroxy phenanthrene at collision energy 32 eV

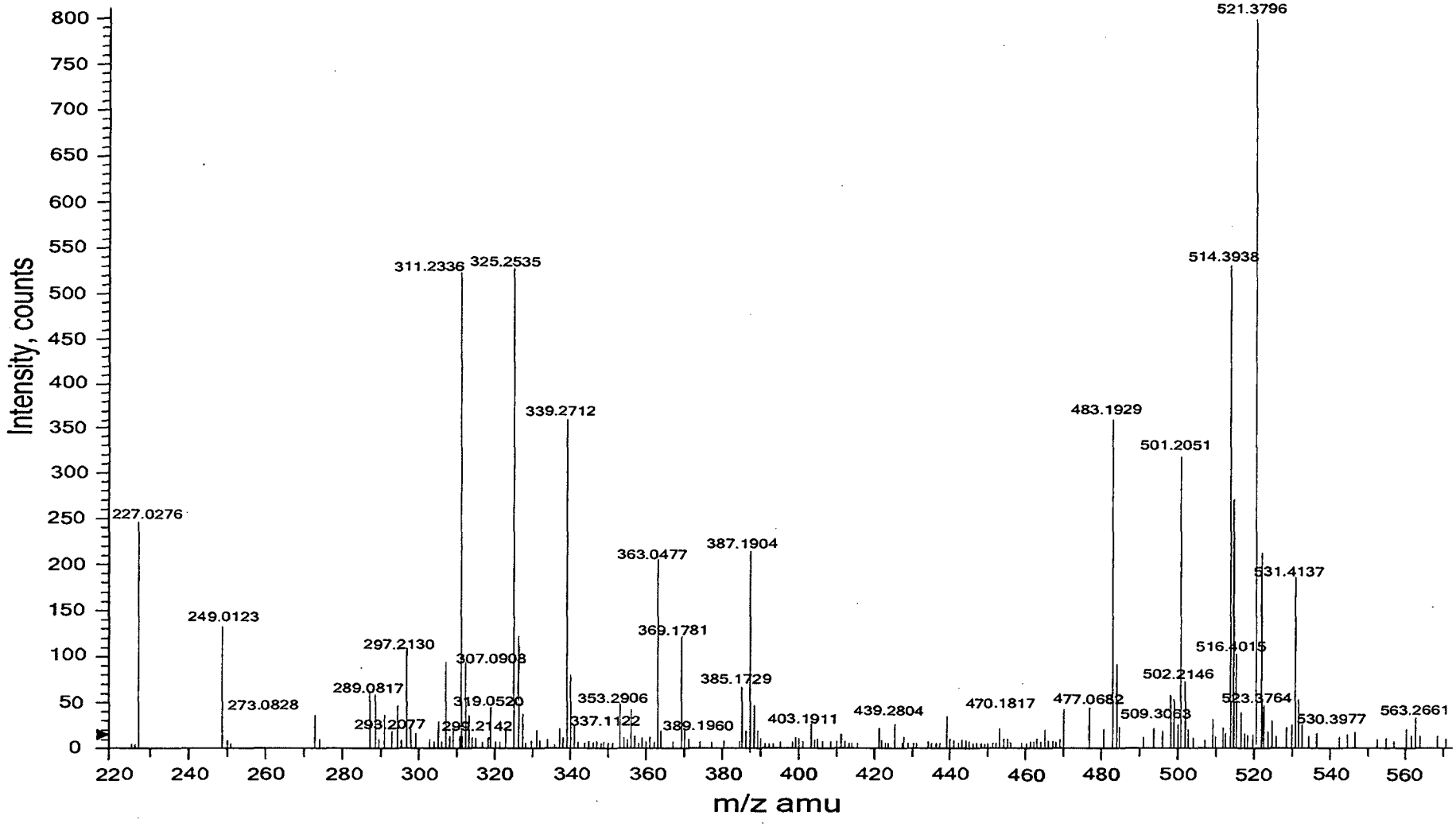


Fig.4: Negative ESI-MS of biliary metabolites of fish exposed to phenanthrene

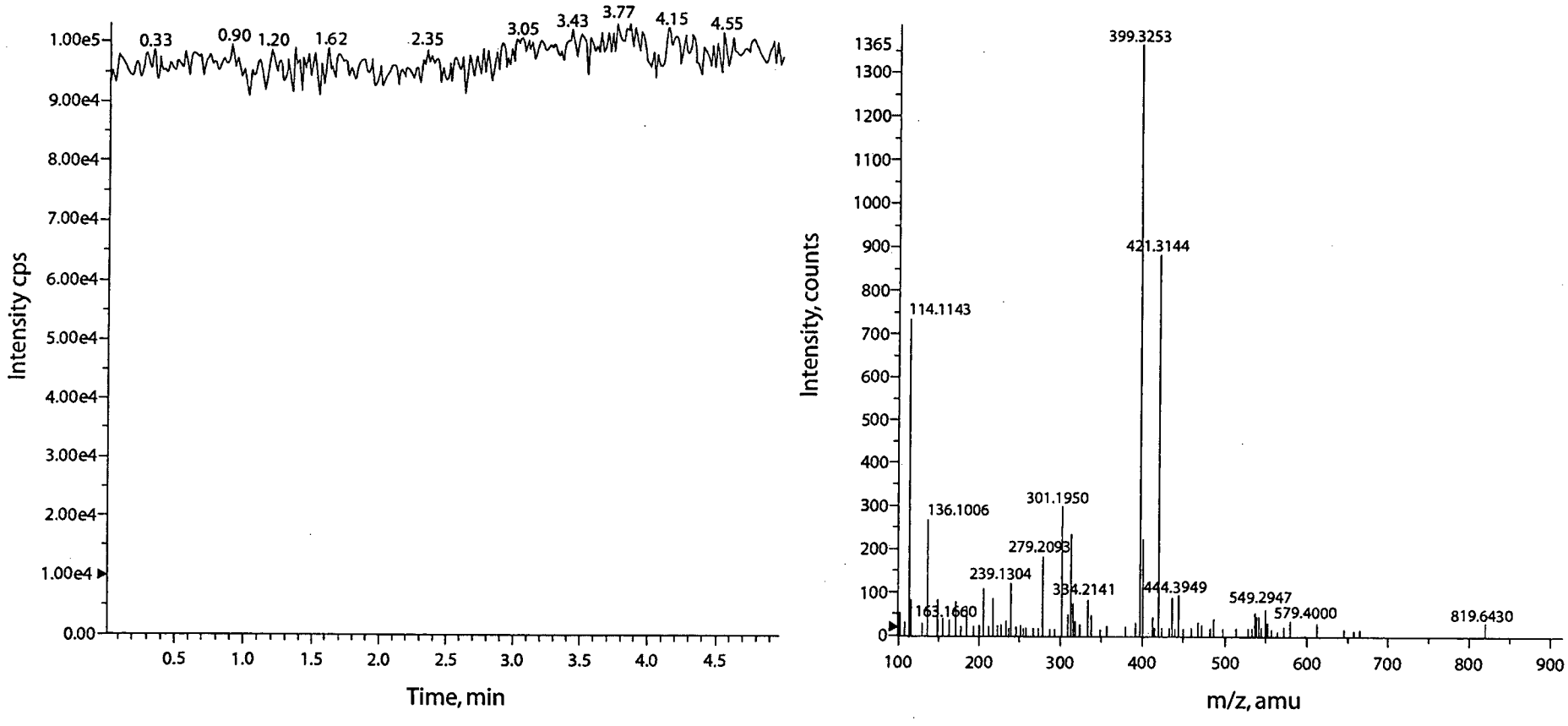


Fig.5: Positive ESI-MS of methanolic extract of bile metabolites

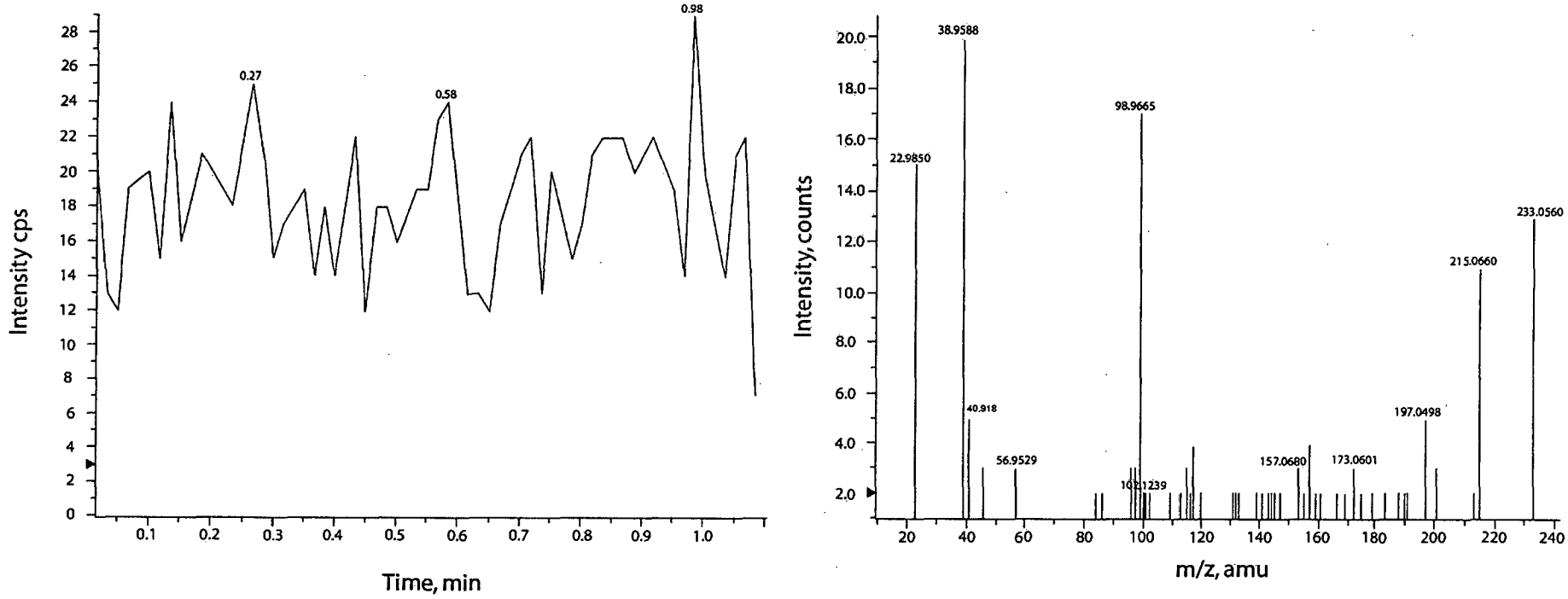


Fig.5A: Positive ESI-MS/MS of molecular ion species at m/z 233

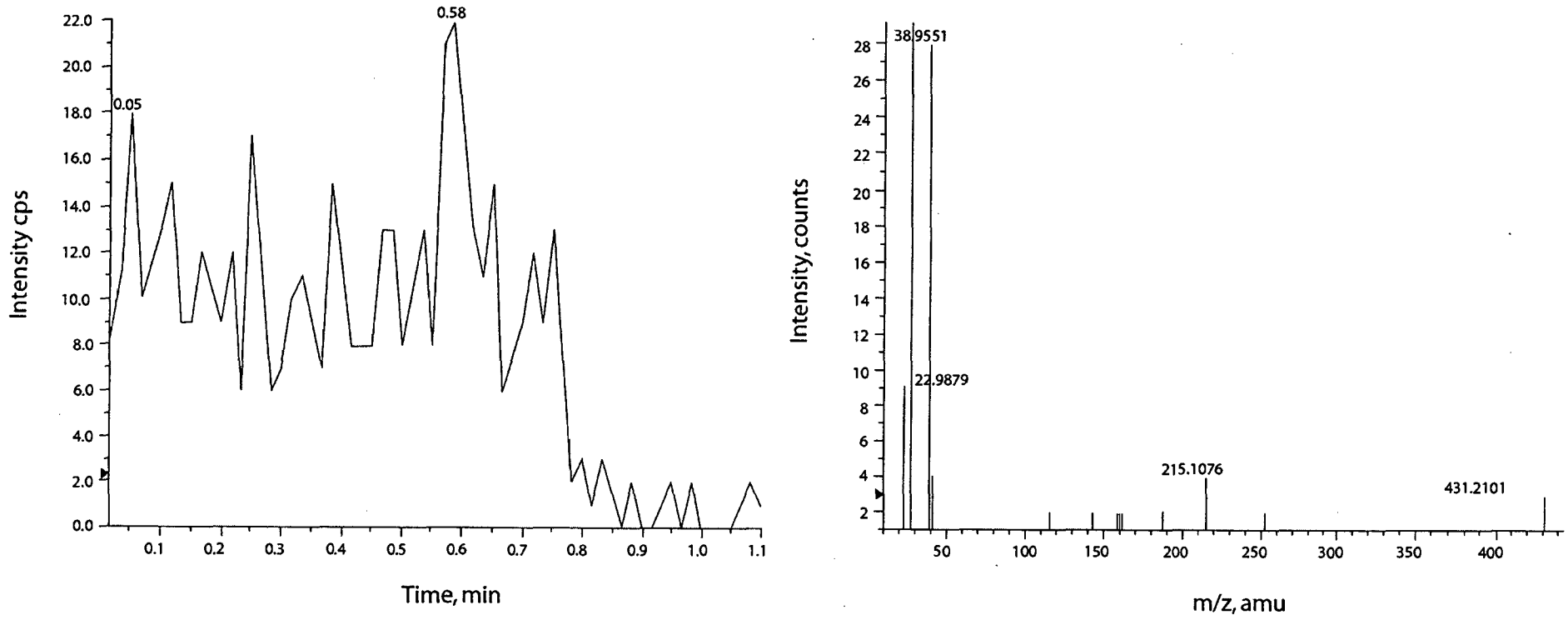


Fig.5B: Positive ESI-MS/MS of molecular ion species at m/z 431

Set 2: Metabolism of phenanthrene in sunflower oil injected to *Oreochromis mossambicus* and exposed to nitrite ions

The metabolism of nitro PAHs is even more complex than that of PAHs. It seems that there are at least five metabolic activation pathway through which mutation can be induced by nitro PAHs in bacterial and mammalian system and/or through DNA binding occurs. These are:

1. nitroreduction
2. nitroreduction followed by esterification (in particular esterification)
3. ring oxidation
4. ring oxidation and nitroreduction
5. ring oxidation and nitroreduction followed by esterification

For a particular nitro-PAH there may be several metabolic pathways often depending in the route of administration. Intestinal microflora seem to play an important role in nitroreduction of nitro-PAHs and in metabolism, by deconjugating metabolites thereby enabling enterohepatic recirculation (Kielhorn, 2003)

Oxidation of phenanthrene is higher when nitrite is present in water (Shailaja & Rodrigues, 2003). Arey (1998) and Atkinson and Arey (1994) have studied the gas phase reaction which occurs during the day time involving the OH radical initiated reaction of PAHs followed

by reactions with nitrogen dioxide (NO_2) leading to the formation of two isomers of nitro PAHs in low yields (5% less) but no 9-nitrophenanthrene is formed. In the dark (night time reactions) four nitro isomers including 9-nitrophenanthrene are reported to be found in trace yields due to the reaction with nitrate radical. In the present study both type of reactions are involved.

The ESI-MS of the biliary metabolites of fish to which phenanthrene (dissolved in sunflower oil) was injected and exposed to nitrite ions is shown in **Fig.6**. Some selected ions from this spectrum showing the presence of nitrite ions as evident by the presence of fragment at m/z 46 were subjected to MS/MS. Thus the parent ion at m/z 359 on CID at collision energy of 45eV **(Fig.6A)** yielded fragments of significant intensity at m/z 313, 255, 176, 59, 46, 38.9 and 22.9. The last three fragments were attributed to nitrite, potassium and sodium ions respectively. The fragment at m/z 313 results from the elimination of nitrite radical from the molecular ion (M^+-46). The ion at m/z 313 on further elimination of -$NHCOCH_3$ acetylamino group leads to ion at m/z 255. The ion at 59 was attributed to protonated acetylamino group. Fragmentation **(Table 2)** observed for molecular species with m/z 359 is well in agreement with the structure of metabolite, disodium salt of phenanthrene-3 or 6-nitro-3 or 6-acetylamino-7, 8-dihydrodiol and must have probably resulted from oxidation by monoxygenases, P450, 1A1 and 1B1, and microsomal epoxide hydrolase (EH) enzyme to

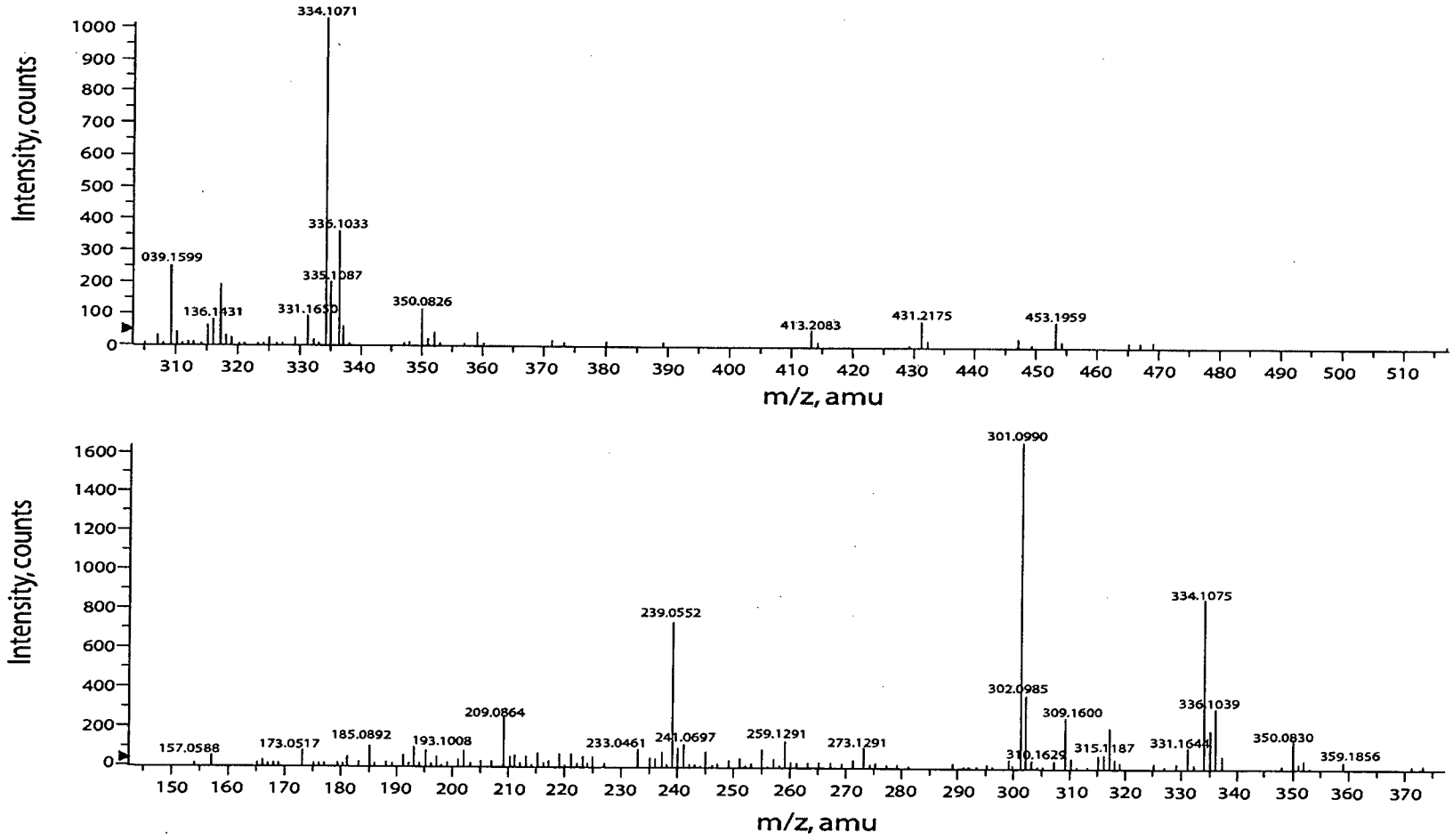


Fig.6: Positive ESI-MS of the biliary metabolites of fish (Set 2)

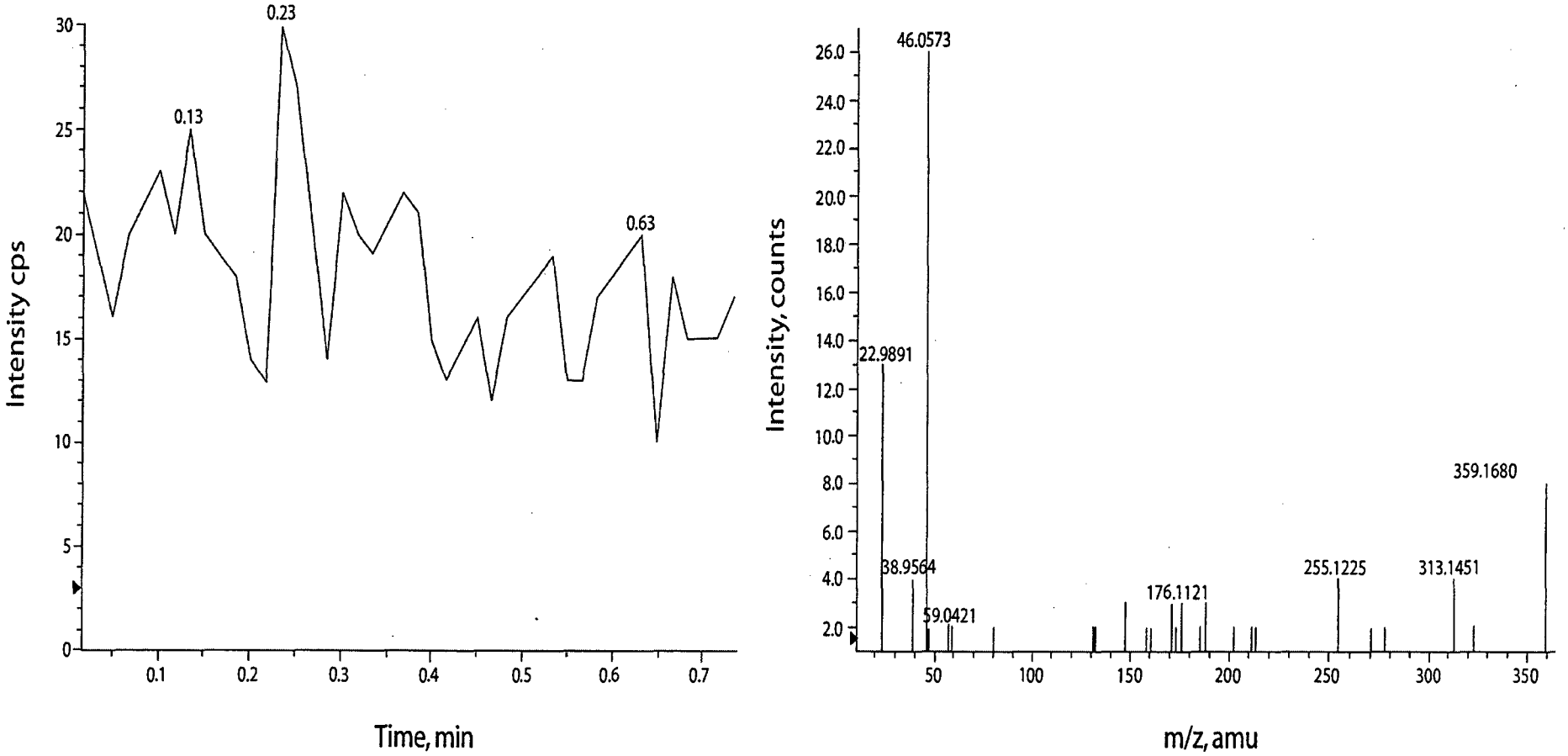
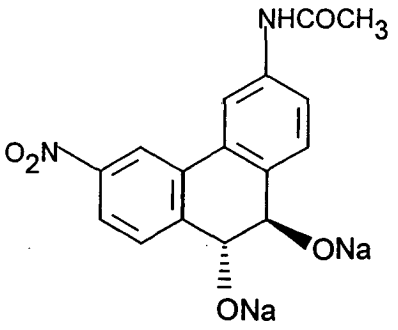


Fig.6A: Positive ESI-MS/MS of the molecular species at m/z 359

dihydrodiol followed by nitration, reduction and esterification. Since both the enzymes are stereospecific, four stereo isomers can be formed (Thakker *et al.*1985).

Table 2: Fragmentation in ESI-MS/MS spectra of 6

Ions	m/z
$[M+H]^+$	359
$[M+H-NO_2]^+$	313
$[313-NHCOCH_3]^+$	255
$[255-(2xONa)]^+$	176
$[NHCOCH_3+H]^+$	59



Disodium salt of phenanthrene-3 or
6-nitro-3 or 6-acetylamino-7, 8-dihydrodiol

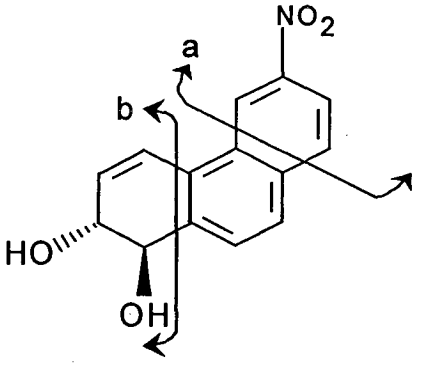
6

The CID spectrum of the molecular ion species at m/z 259 **(Fig.7)** in the positive ion mode is indicative of the formation of Phenanthrene-6-nitro-1, 2-dihydrodiol **(7)**. Probable genesis of the fragments is as shown in **Table 3.**

Table 3: Fragmentation of molecular ion species of m/z 259

Fragment ions	m/z
C-NO_2	58
a=$[C_4H_3NO_2+2H]^+$	99
$[M^+-(H_2O+NO_2)]^+$	195
b=$[M-(C_4O_2H_6+2H)]^+$	171
b=$C_4O_2H_6$	86

The product and mass spectrum of molecular ion species at m/z 259 is shown in **Fig.7 (Table 3)**. As observed ions at m/z 58 result from the loss of c-NO_2, whereas cleavage at a along with the loss of $2H^+$ leads to the formation of fragment at m/z 99. Simultaneous loss of water molecule along with the NO_2 group from the parent molecule yields fragment at m/z 195. Cleavage at 'b' corresponded to the fragment at m/z 86 and 171. Thus the molecule was identified as Phenanthrene-6-nitro-1, 2-dihydrodiol **(7)**.



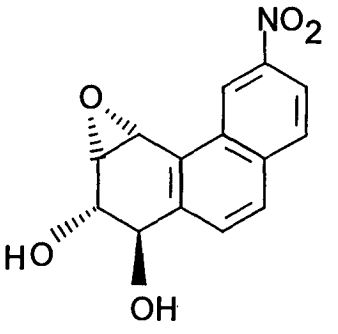
Phenanthrene-3-nitro-7, 8-dihydrodiol

7

Epoxidation of 3, 4 bond in phenanthrene-6-nitro-1, 2- dihydrodiol would lead to molecular species with m/z 273. The ESI-MS/MS of molecular species at m/z 273 **(Fig.8)** did confirm that it corresponded to Phenanthrene-6-nitro-1, 2-dihydrodiol-3, 4-epoxide **(8)**. The genesis of the various fragments in the product ion mass spectrum for the molecular species at m/z 273 obtained at collision energy of 40eV are as given in **Table 4**.

Table 4: Fragmentation of molecular species of m/z 273

Fragment	m/z
a = $C_6O_3H_6$	129 = (126 + $3H^+$)
C-NO_2	58.05
129-H_2O=111+$3H^+$	114
b= M^+ - [$C_4O_3H_6$+ H^+]	170= [273-(102+$2H^+$)]
C= $C_4H_3NO_2$+$2H^+$	99
[M^+-(NO_2+$2H_2O$)+Na	215=192+23



Phenanthrene-3-nitro-5, 6-epoxy-7,8-dihydrodiol

8

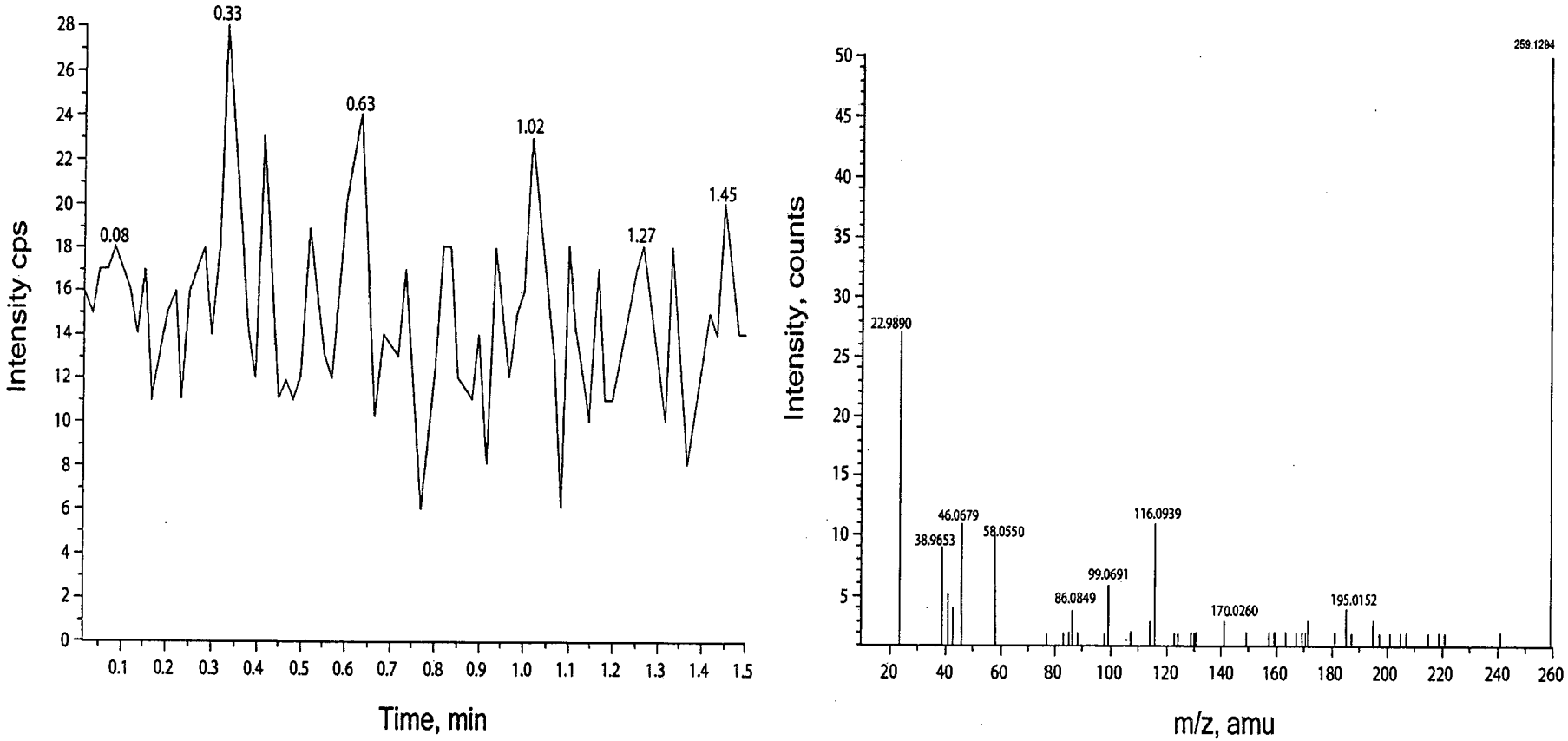


Fig.7: Positive ESI-MS/MS of the molecular ion species at m/z 259

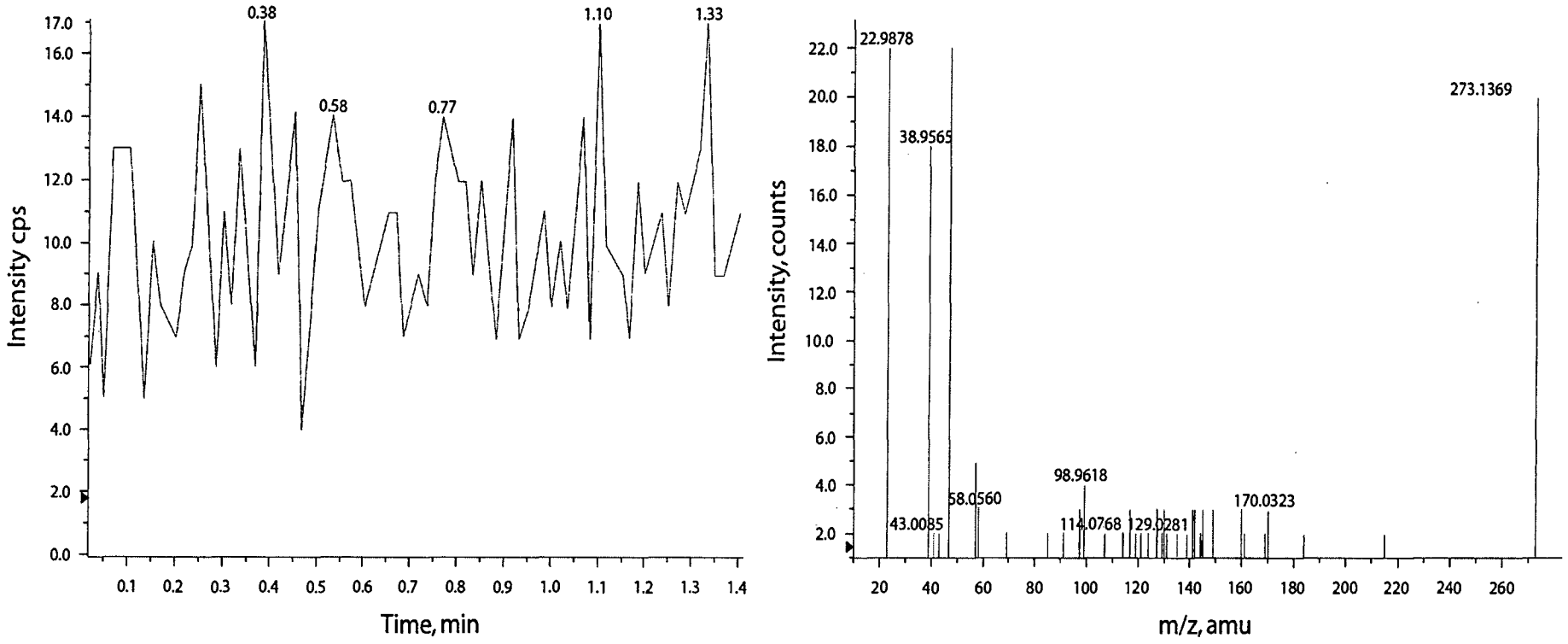


Fig.8: Positive ESI-MS/MS of the molecular ion species ion at m/z 273

Set 3: Phenanthrene metabolites formed when fish is exposed to the aromatic hydrocarbon with nitrite ion in the aquatic medium

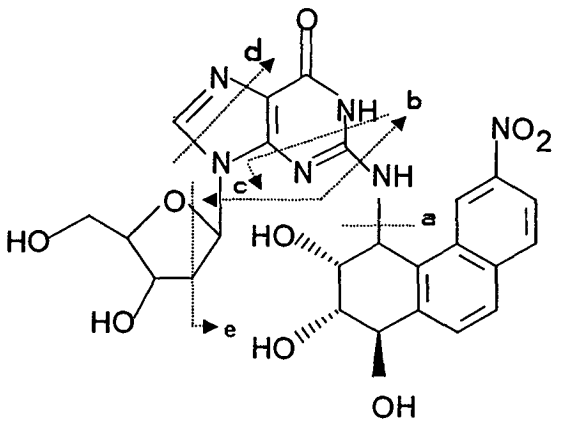
Based on the fragmentation observed in ESI-MS/MS, in the positive ion mode, and in analogy with the literature report the following metabolites, mainly DNA adducts have been identified in the bile of fish *O. mossambicus.*

The full scan ESI-MS in the positive ion mode of the methanolic extract of the fish (*O. mossambicus*) bile after the metabolism of phenanthrene in the presence of $NaNO_2$ is shown in **Fig.9**. As observed, the spectrum seems to be richer in high molecular weight molecular ions. These were analysed for adducts by LC/MS/SIM.

1) Identification of molecular ion peak at m/z 541

Thus the daughter ion spectrum **(Fig 9A)** of protonated molecular ion 541 with the collision energy set at 55 ev consisted of the protonated molecular ion and considerable number of fragments. Based on the fragmentation pattern the molecule was identified as N_1, N_2 or N_3 - deoxyguanosine nitrophenanthrene diol epoxide adduct **(9)**.

The genesis of the various fragments in the MS/MS spectrum **(Fig.9A)** could be explained as shown below.



9

Cleavage a ⟶ m/z 274

274-NO_2=228+H=229

229-H_2O=211+Na=239

274-3H_2O=219

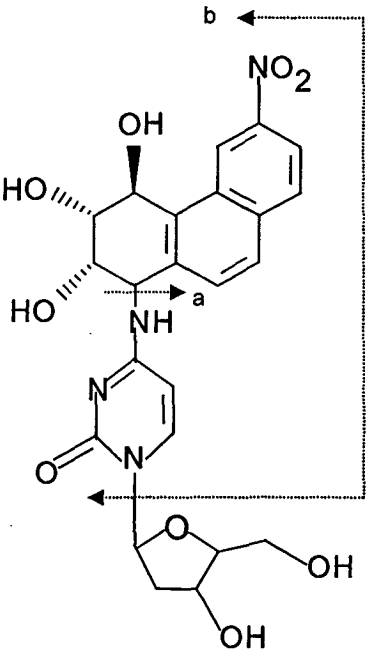
219-NO_2+2H=175

Deoxyribose (117) +deoxyguanine (134) + Na^+ =274

The major fragment at m/z 219 (base peak) results from the elimination of three molecules of water from nitrophenanthrene tetrahydrotriol moiety (m/z 274-a cleavage). Elimination of –NO_2 group from triol leads to protonated fragment at m/z 229, which on removal of one molecule of water gives sodiated ion at m/z 234. Cleavage b lead to fragment ion at m/z 133 corresponding to guanidine moiety. Simultaneous loss of deoxyribose and guanidine moieties from the molecule leads to the sodiated ion at m/z 274. Thus confirming the structure assigned to DNA adduct **(9)**.

2) Identification of molecular ion peak at m/z 499

Fragmentation observed in ESI-MS/MS of molecular ion species m/z 499 **(Fig.9B).** This molecular species on MS/MS yielded daughter ions at collision energy of 25eV as presented in **Fig 9B**. A characteristic fragmentation pattern of nitro phenanthrene-5, 6-diol-7, 8-epoxide N_3-deoxy cytidine is observed as illustrated in below.



3-nitrophenanthrene-5, 6-diol-7, 8-epoxide N_3-deoxycytidine adduct.

10

Proposed fragmentation observed for 3-nitrophenanthrene-5, 6-diol-7, 8-epoxide N_3-deoxycytidine adduct (m/z 499)

$[M+H-453]^+ = 46 \longrightarrow NO_2$ group

499-453 = 46

Cleavage a = m/z 274

$[274-2H_20]+$ = m/z 238+H= m/z 239

$[274-3H_20]+ = [220+H]^+$ = m/z 221

Cleavage $b=[(M+H)-(NO_2+d_R]+= [499-(46+117)]=[336+H]^+$= m/z 337

Elimination of 46 amu corresponding to $-NO_2$ group from the precursor ion at m/z 499 yielded fragment at m/z 453. The fragment ion at m/z 274 correspond to cleavage 'a' and when it loses two and three water molecules consecutively, protonated fragments at m/z 239 and 221 were formed. The protonated fragment at m/z 337 corresponds to the cleavage b with simultaneous removal of $-NO_2$ and deoxyribose from precursor ion.

3) Identification of molecular ion peak at m/z 513

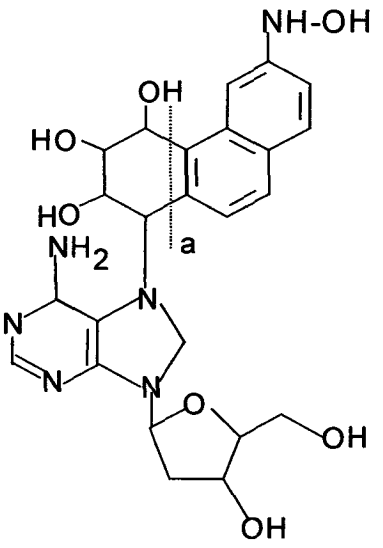
Proposed fragmentation for the molecular species with protonated ion at m/z 513 (Fig. 9C)

$[dA(135)+H]^+$ = m/z 136 dA=deoxyadenine; dR=deoxyribose

$[dA(135)+dR(117)+Na]^+ = 275$ (observed m/z 277)

$[dA(135)-NH_2(16)]+dR(117) = 236$ (observed m/z 235)

cleavage a = $[dA+dR+C_4H_7O_3]=355-3H_20$=m/z 301



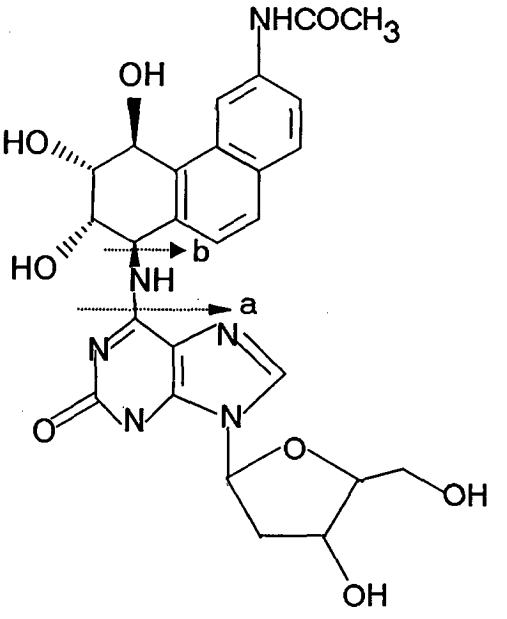
3-hydroxylamino phenanthrene-N^7 deoxyadenosine adduct

11

As evident from the ESI-MS spectrum **(Fig 9C)** protonated deoxyadenine moiety gives the fragment ion at m/z 136. Simultaneous loss of deoxyadenine and deoxyribose moieties results in the sodiated ion of dR and dA with the elimination of $-NH_2$ group fragment m/z 235 is formed.Removal of three molecules after the cleavage at a gave the base peak at m/z 301.Thus the molecular species with protonated precursor ion m/z 513 was identified as 3-hydroxylamino phenanthrene diol epoxide-N7-deoxyadenosine adduct **(11)**.

4) Identification of molecular ion peak at m/z 537

The fragment observed in the ESI-MS/MS **(Fig.9D)** of precursor ion at m/z 537 could be rationalized as follows.



Phenanthrene-3-N-acetylamino-5, 6-diol-7, 8-epoxy-N_6-deoxyadenosine **12**

Besides yielding the sodiated ion at m/z 255, cleavage at a leads to the protonated base peak at m/z 301. Removal of acetyl amino and imino group from the resulting fragment resulted in the formation of fragmentation at m/z 227. Cleavage b gives the sodiated ion at m/z 308. Based on the fragmentation pathway adduct with pseudomolecular ion at m/z 537 was identified as phenanthrene-3-N-acetylamino-5, 6-diol-7, 8-epoxy-N_6-deoxyadenosine **(12)**.

In the current study, phenanthrene metabolites including DNA adducts formed from metabolism of phenanthrene in the presence of NO_2^- ions has been determined on the basis of the molecular species observed in the ESI-MS of extract of fish bile. The greatest advantage of this technique is that it permits the detection of fmol quantities of metabolites including DNA adducts in biological systems. Based on the metabolites identified the following pathway, as given in the **scheme 2** below, has been envisaged for phenanthrene metabolism.

It is concluded that based on all the metabolites identified in the third set of experiments it may be concluded that chances of formation of nitro DNA adduct is more when nitrite is in the medium. It is also evident that nitroreduction and acetylation is involved in the process of DNA adduct formation.

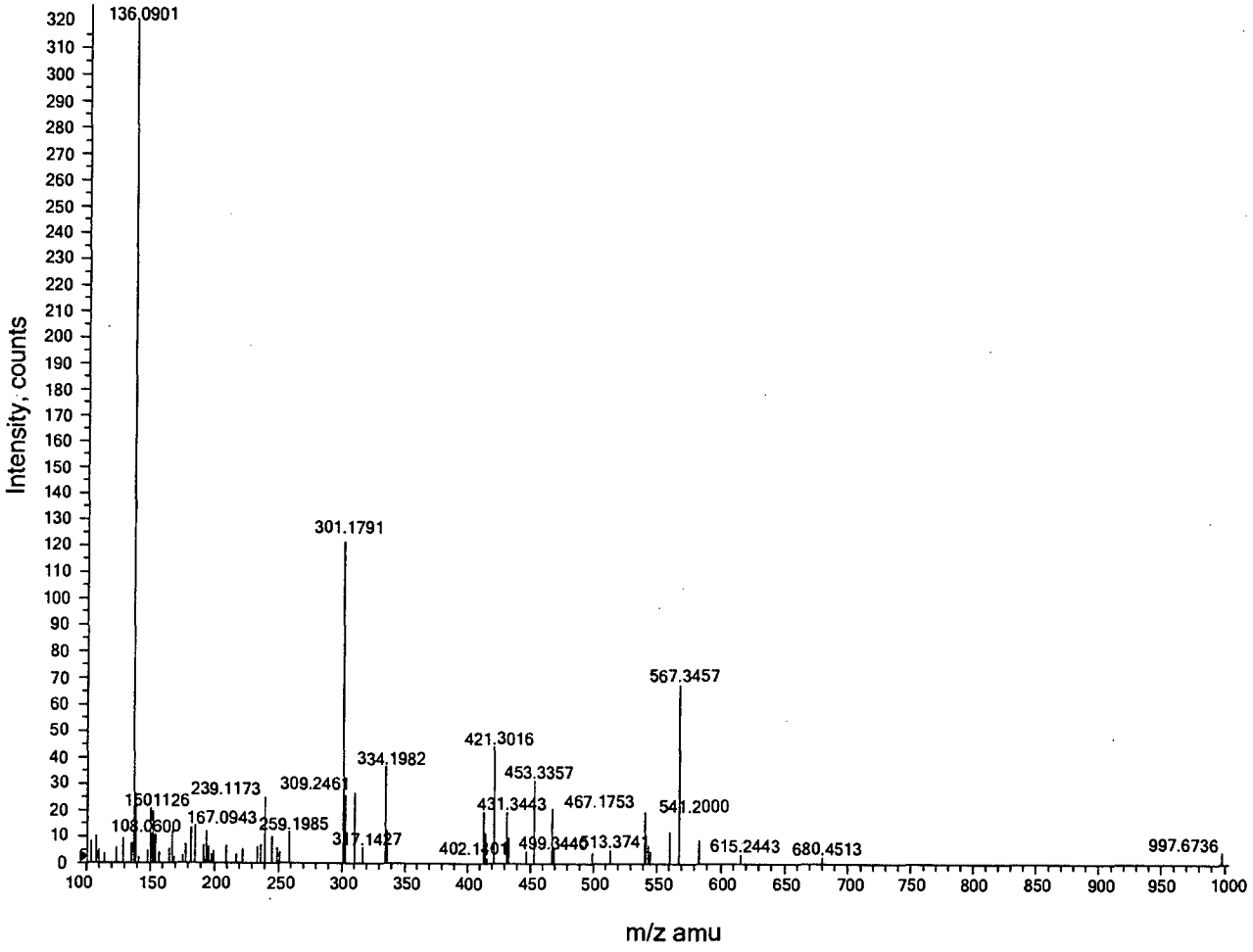


Fig.9: The full scan ESI-MS in the positive ion mode (set 3)

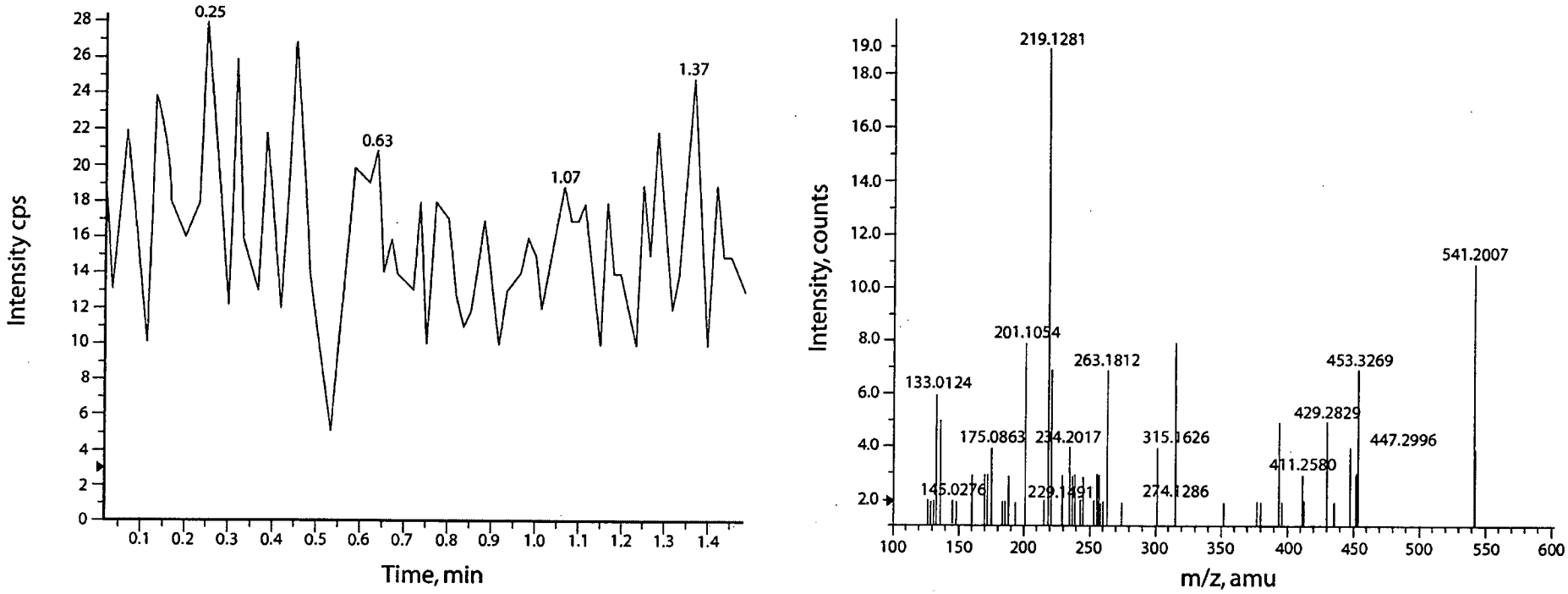


Fig.9A: Positive ESI MS/MS of molecular ion species at m/z 541

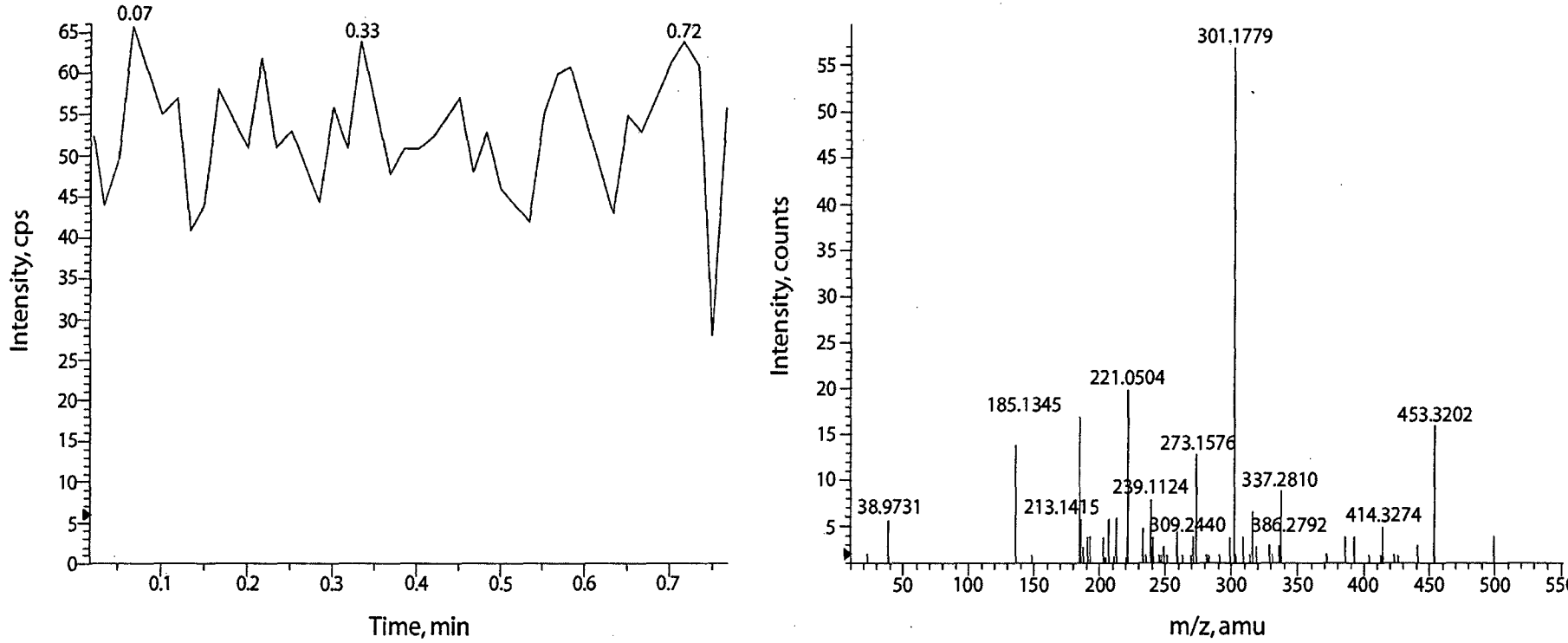


Fig.9B: ESI MS/MS of molecular ion species at m/z 499

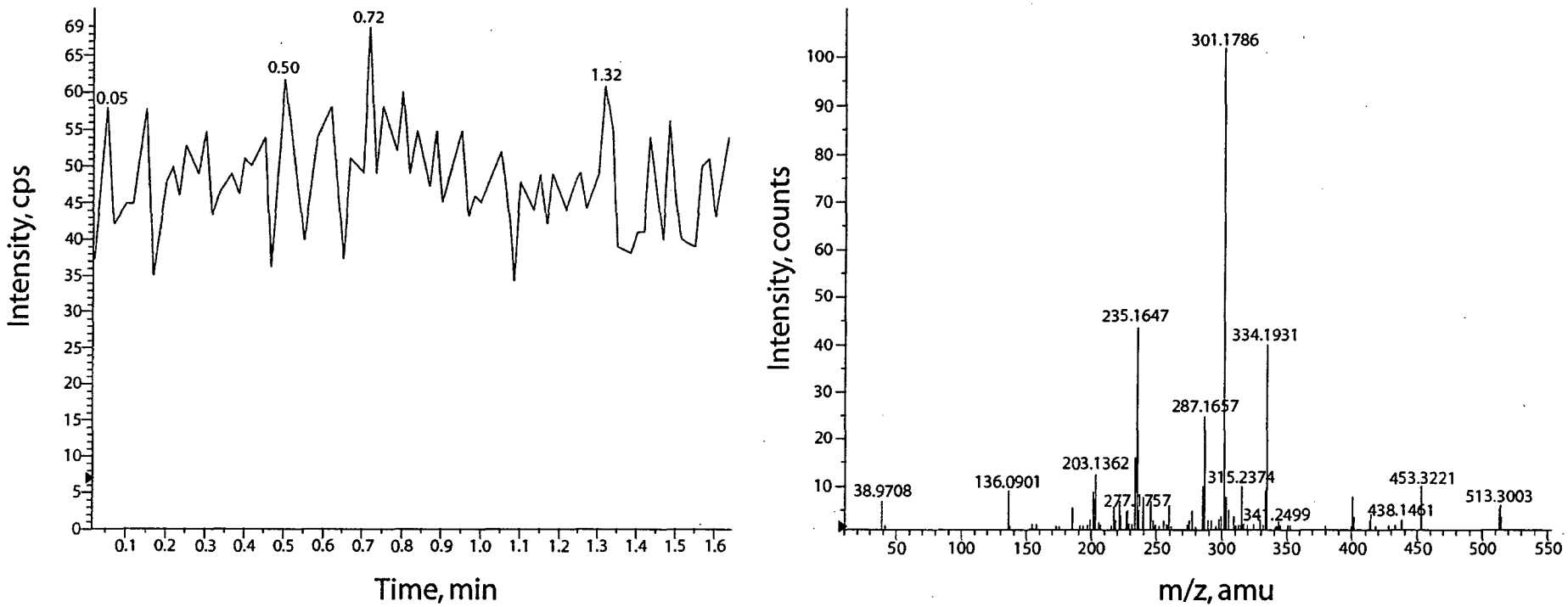


Fig.9C: ESI MS/MS of molecular ion species at m/z 513.

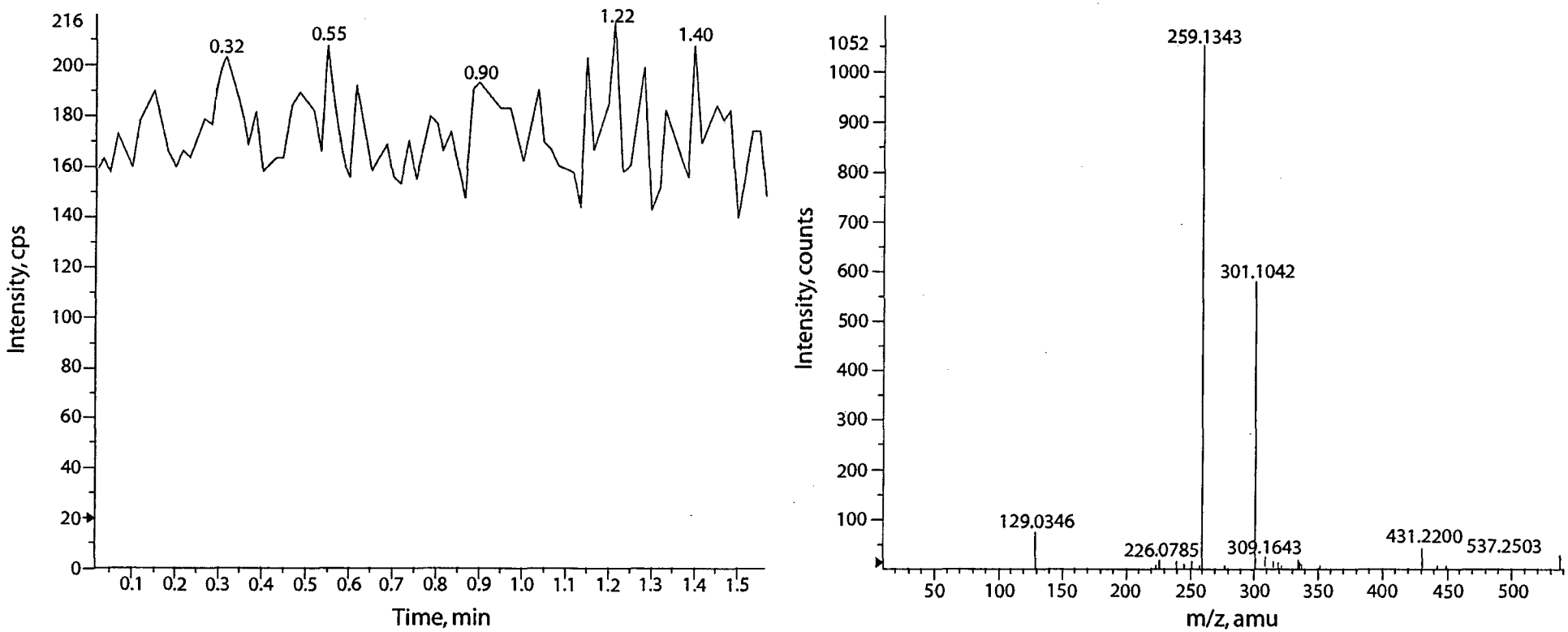
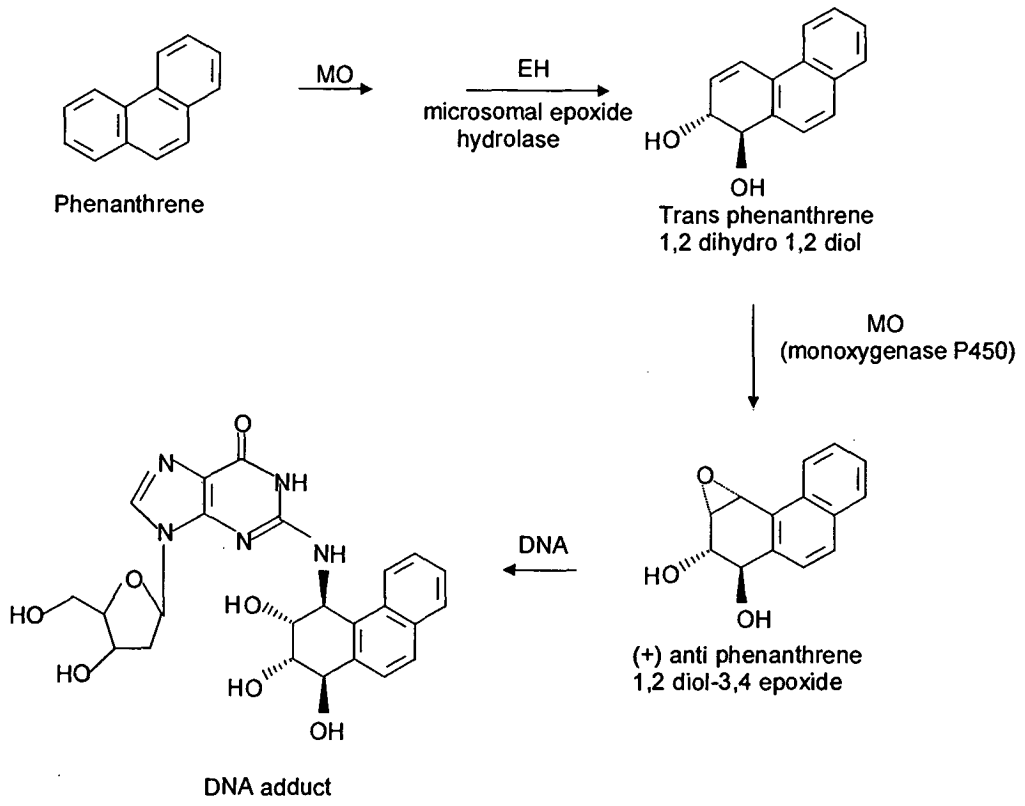


Fig.9D: ESI MS/MS of molecular ion species at m/z 537.



Scheme 2: Metabolism of phenanthrene

In analogy with the literature reports (De-Jin *et al.*, 1997,Wang *et al.*, 2003) and the metabolites identified, pathways involved in the metabolism of phenanthrene in the presence of a NO_2^- have been envisaged. Thus the diol epoxides are formed by the action of monoxygenase cytochrome P450 and microsomal epoxide hydrolase (EH). Since the enzymes are stereospecific, four stereo isomers can be found.

It might be noted that the values shown in the +ve mode ESI/MS is short by 1 amu the discrepancy observed could be due to calibration.

EXPERIMENTAL

Exposure Protocol:

Oreochromis mossambicus (6.8 to 18.6g) obtained from a local estuarine fish farm were acclimatized in the laboratory for a period of 7days in stored tap water amended with reagent grade NaCl (final salt concentration $15gL^{-1}$). The exposure experiments (9-12 fish per tank) were conducted for 15 d in 30 L glass aquaria with continuous aeration. A Control tank with 6 fish received equivalent amounts of dimethylformamide (DMF) and nitrite. The test medium consisted of pure phenanthrene (Sigma, USA) dissolved in dimethylformamide to give a final concentration of 2 mgl^{-1} with and without the simultaneous addition of an aqueos solution of sodium nitrite (final concentration 1 µM). The control and the test medium was renewed everyday. Commercial feed was provided *ad libitum* during the period of the experiment.

The fish were sacrificed after 15 days of exposure. The gall bladder was detached from the liver or the bile was collected with a syringe. The bile samples were stored at –20°c and stored until analysis. Before analysis the bile samples were diluted 100 times (10 µl + 990 µl) with distilled water/methanol 50:50 v/v and used as such for ESI-MS analysis without deconjugation.

ESI mass spectrometry:

ESI-MS experiments were performed on a QTOF XL mass spectrometer, Applied Biosystems Instrument (Canada). The instrument was operated in the positive as well as negative ion mode. All the analysis were performed using ion spray source with the following settings: ion spray voltage 5500 V, nebuliser gas (N_2) 20 (arbitrary units) and curtain gas (N_2) 20 (arbitrary units). The declustering potential (DP) and the collision energy were optimized around 15 and 3 V respectively during infusion experiment and 15 and 40 V respectively for MS/MS experiments.

The sample was directly injected at a constant flow rate of 10 ml/min into the electrospray source using integrated syringe pump. Data acquistion and processing were carried out using mass Lynx NT version 3.5 supplied with the instrument. The MS survey range was m/z 0-950 (+ve mode) and m/z 220-680 (-ve mode) with scan duration of 1.0 s. Each mass spectrum was recorded over a period of 1 s and mass spectra were accumulated over a period of 5 minutes for both single analyzer profiles and CID experiments. For each of the ion species examined the lock mass in each product ion mass spectrum was the observed monoisotopic mass/charge (m/z) ratio of the precursor ion. The bile samples after extraction with methanol: water (1:1) were analysed by direct ESI-MS, mainly in the positive ion mode.

Negative ion mode is taken whenever necessary. These conditions were chosen since certain compounds have less tendency to form $[M+H]^+$ protonated quasimolecular ion by addition of protons but, on the other hand, deprotonated with production of $[M-H]^+$ ions is more likely to occur. Wang *et al.*, 2003 observed greatest ion intensities under proton transfer conditions when 0.1% CH_3COOH in acetonitrile is used as solvent. This is largely because protonation in acetonotrile occurs from the major protonated species in the solvent namely $[CH_3CNH]^+$ which is a modest Bronsted acid. In the present study MeOH:H_2O (1:1) has been used.

Here mention is made of the fact that the instrument was not calibrated each time. Hence, sometimes discrepancies have been observed in the values for protonated molecular ions to the extent of 1 or 2 amu.

The above experiment could not be repeated for characterization of biliary chrysene metabolites due to unavailability of pure chrysene (Sigma, USA)

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Section IV

Isolation of Two Saponins from the Mangrove Lumnitzera racemosa and its Application in the Bioremediation of PAH

INTRODUCTION:

The removal of polycyclic aromatic hydrocarbon (PAH) contaminants present a challenge to scientists and engineers because these compounds have a low aqueous solubility and low vapour pressure. One way to enhance PAH's solubility in the aqueous phase is to add surfactants at sufficiently high concentration to form micelles and solubilize the PAH (Prak & Pritchard, 2002). Surfactants have been found to be effective in solubilizing PAH contaminants from soil particles (Yeom *et al.*, 1995; Zheng & Obbard, 2000)

Although several PAHs are among the most hazardous constituents of soil pollution, we are far from understanding fully the factors governing their bioelimination *in situ*. Of crucial importance here is the bioavailability of these compounds, which are poorly soluble in water and readily absorbed to the surfaces of soil solids. Moreover, microbial degradation of PAH in soils might be hindered or repressed by a predominance of other dissolved organics (carbohydrates, carboxylic acids etc) (Thurman, 1985).

Pump-and-treat is too slow to clean up hydrophobic organic compounds (HOCs) with reasonable periods of time (Guha *et al.*, 1998; Finkel *et al.*, 1999). Surfactant flushing has been used for removing these compounds with low water solubility, since surfactants can enhance the clean up of soils and groundwater contaminated with

HOCs by mobilizing and partitioning them into the hydrophobic cores of surfactant's micelles (Chan Lan Chun, 2002).

To enhance bioavailability and accordingly biodegradation of hydrophobic, high molecular weight PAHs, the use of biologically or chemically produced surfactants has been investigated. Laboratory studies have demonstrated that some combination of non-ionic surfactants and bacterial strains (pure cultures as well as consortion) stimulate the biodegradation of PAHs in liquid or soil systems (Guerin & Jones, 1988; Aronstein *et al.* 1991; Lantz *et al* 1995; Tiehm., 1994). We felt that phytogenic surfactants, such as saponins could perhaps play a similar role.

Saponins are an important group of plant secondary metabolites that are widespread throughout the plant kingdom (Osbourn, 1996; Price *et al.*, 1987; Hostettman *et al.*, 1991; Fenwick *et al*; 1992; Hostettman & Marston, 1995). The name saponin is derived from *sapo*, the latin word for soap, since these molecules have surfactant properties and give stable soap like foams in aqueous solution. Chemically, the term saponin has become accepted to define a group of structurally diverse molecules that consists of glycosylated steroids, steroidal alkaloids and triterpenoids. The secondary metabolites often occur in plant as complex mixtures and saponin content and composition may vary markedly depending on the genetic background of the plant material, the tissue type, the age and physiological state of the plant and

environmental factors (Osbourn, 1996; Price *et al*; 1987; Hostettman *et al*; 1991; Fenwick *et al*; 1992; Hostettman & Marston; 1995, Roddick, 1974). Although, saponins, which are complex, compounds composed of sugar and steriod or triterpeniod moieties are widely distributed in plants, they are uncommon animal constituents. In 1943, Yamanouchi first isolated a saponin of animal origin from a sea cucumber and named it holothurin. Later in 1952, Nigrelli & Zahl obtained a saponin also named holothurin from the Bahamian sea cucumber, *Actinopyga agassizi*. The toxic principle of starfish is a saponin called asterosaponin (Hashimoto & Yasumoto, 1960, Yasumoto *et al.*, 1964, Yasumoto & Hashimoto, 1965, Yasumoto & Hashimoto, 1967). Saponins are well known toxic components of Echinoderms like feather stars or sea lilies, starfish, brittle stars, sea urchins, heart urchins, sand dollars and sea cucumbers (Hashimoto, 1979). A variety of saponins of varied sugar and aglycone composition which have been isolated from sea cucumbers and starfish exhibit a board spectrum of physiological activity. Chemically, saponins derived from sea cucumbers are triterpenoid saponins, whereas those from starfish are steroidal saponins (Scheuer, 1971 & Ikegami, 1973).

Saponins have been variously attributed with a diverse range of properties, some of which include both beneficial and detrimental effects on human health, piscicidal, insecticidal and molluscicidal activities, allelopathic action, antinutritional effects, sweetness and bitterness and as phyto protectants that defend plants against attack

by microbes and herbivores (Tschesche 1971, Schondeck & Schlosser ,1976; Osbourn, 1996; Price *et al.*, 1987; Hostettman *et al.*,1991; Fenwick *et al.*, 1992; Hostettman & Marston, 1995; Osbourn, 1996).

Some saponins possess a wide variety of activities like anti-carcinogenic properties (Hostettman & Marston, 1995), anti-inflammatory, anti-allergic, anti viral activities with effects on the physiology of the cardiac and blood systems (Lacaille – Dubois & Wagner, 1996) and immunomodulatory and cytostatic activities (Quetin- Le clereq *et al.*, 1992). Numerous plants have been shown to produce pesticidal compounds (Ciccia *et al.*, 2000) as a chemical defence mechanism against predators or infection. There are a limited number of reports regarding the use of saponins as larvicidal agents. Zarroug *et al* (1990) have reported the use of the saponin rich plants *Balanites aeryptiaca* as a mosquito larvicide. Besides their beneficial effect on human health and protection of plants they are also known to be good emulsifiers, a property that is made use of in food, cosmetics and bioremediation of oils.

Recent studies indicate that certain saponins can form stable 'surface complexes' at various oil-in-water interfaces together with other monomeric emulsifiers and stabilize the emulsion in an improved manner. The spilling of oil in the sea is a major problem that can destroy coastline. The petroleum industry has traditionally been the

major users of biosurfactants, in oil removal applications. They offer several advantages over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability (Desai & Banet, 1997)

During the course of investigations on the biological activity of marine organisms, including mangrove plants, the crude methanolic extract of *Lumnitzera racemosa*, a plant belonging to family Combretaceae, was found to be bactericidal against a number of pathogenic bacteria. The activity on fractionation was located in n-butanol fraction and was found to be due to the presence of flavonoids. While purifying the active bactericidal flavonoids, a fraction rich in saponins was obtained and since saponins are a group of phytochemicals having strong surfactant properties (Rao, 1996) we thought of investigating, the purified saponins from this mangrove, with respect to bioavailability of PAHs. The protective effect of this saponin against the DNA damage caused by PAHs has also been studied. This section has been subdivided into the following sub-sections:

A. Isolation and identification of saponins from *Lumnitzera racemosa* using spectroscopic techniques.

B. Efficiency of saponin in the remediation of PAHs as a function of EROD activity in fish *Oreochromis mossambicus*

C. Assessment of pollution by PAHs in fishes and evaluation of its bioremediation by plant derived saponins, using Comet assay.

RESULTS:

A. Isolation and identification of saponins from *Lumnitzera racemosa* using spectroscopic techniques.

The aerial parts, leaves and twigs are of special relevance because they can provide a larger amount of plant material with minimum damage to the plant as they are renewable.

Sephadex LH-20 filtration of the active n-butanol fraction followed by silica gel chromatography yielded saponin **1** and **2** as colourless compounds.

Compound **1,** the less polar one with a R_f value of 0.42 gave positive Libermann- Buchard test indicative of a triterpenoid system. The infra red spectrum **(Fig.1)** of **1** showed absorption at 3394 cm^{-1} for hydroxyl groups and absorption at 1710 for the –COOH ketonic group. The ^1H NMR spectrum **(Fig. 2)** in C_6D_5N revealed the presence of seven tertiary methyl groups at δ 0.79, 0.98, 1.045, 1.077, 1.131, 1.32 and 1.57 as singlets and a doublet at δ 1.63 (3H,d, J=6Hz) assigned to the secondary methyl of rhamnose. In addition, there were doublets at δ 3.3 and δ 3.6 (J=6.9Hz) suggesting the presence of 13, 28-epoxide moiety as observed for epoxy oleanane compounds. This was supported by the presence in its ^{13}C NMR **(Fig.3)** of oxygenated quarternary carbon signal at δ 86.4. Multiplets due to H-3 and H-16 were evident at δ 3.2 and 4.1 respectively (signal for H-16 overlapped due to severe spectral crowding in the region δ 4-5 characteristic of

oligosaccharides). In its ^{1}HNMR spectrum the anomeric proton signals for sugar units in the molecule overlapped with the broad –OH signal at δ 5.79. Methanolysis afforded the aglycone primulagenin A identified on the basis of comparison of its ^{13}C NMR data **(Fig.4,Table 1)** with the literature values (Ohtani *et al.*, 1993) and the sugars rhamnose, glucose, galactose and glucuronic acid. The sugars were identified by TLC comparison with the standard sugars. TLC solvent system (Bu-OH: Ac-OH: Et_2O: H_2O; 9:6:3:1). The interglycosidic linkage as well as the position of the attachment of the carbohydrate moiety to the aglycone was established on the basis of ^{13}CNMR data. The assignments of carbon resonances due to sugar moieties of **1** was done by comparison with those reported for the saccharide chain in triterpenoid saponins and by considering glycosidation effects. The glycosylation shift of C-3 carbon from δ 78.1 in primulagenin A to 89.9 in the saponin indicated that carbohydrate moiety is attached to C-3 of aglycone. Anomeric carbon resonances at 103.7, 102.3 and 101.2 demonstrated that all the monosaccharides units are substituted at C-1.

FABMS **(Fig.5)** in the negative mode with the molecular ion $[M-H]^-$ at m/z 1249 was indicative of molecular weight of 1250. FABMS was used for establishing sugar sequence analysis. The mass spectrum showed base peak at m/z 1249 $[M-H]^-$ and fragments at m/z 1103 [(M-H) - dhex]$^-$; m/z 1073 [(M -H)-GlcA)]$^-$; m/z 957[(M -H) - 2dhex]$^-$; and at m/z 795.6 [(M -H) – (2dhex+ hex)]$^-$. Simultaneous losses of

fragments 146 (deoxyhexose) and 176 (glucuronic acid) was indicative of two terminal sugars suggestive of two sites of attachment of a branched side chain. The fragment at m/z 793 corresponds to the loss of 456 amu indicative of $[M-H]^-$ of aglycone to be 457. Though the number of sugar units in the NMR spectrum were not clear due to superimposibility of signals FABMS clearly indicated that the saponin is a pentasaccharide. The fragmentation observed is suggestive of the following structure for the saponin **1**

dhex — dhex — hex — hex — aglycone
|
GlcA

Acid hydrolysis of saponin yielded primulagenin A, (saponins with protoprimulagenin A as the aglycone yield primulagenin A triterpenoid on hydrolysis due to the opening of oxido ring) besides the sugars mentioned above. Hence, the saponin was assigned structure **1.**

Rha — Rha — Galactose — Gluc— protoprimulagenin A
|
GlcA

1

Thus, based on the spectral data it was evident that **1** is a new saponin identified as 3-o-rhamnopyranosyl–rhamnopyranosyl–galactopyranosyl (glucuronopyranosyl)- glucopyranoside-13, 28-epoxy oleanane.

NMR of saponin **1** is complex with many signals overlapping in the region for sugars. Hence we could not assign all the carbon chemical

shifts for all the constituent sugars of carbohydrate moiety. But it is clear from its FABMS that it is a pentasaccharide. Higher resolution NMR would be more appropriate.

Though the material is TLC pure, FABMS is indicative of the presence of additional saponin having the same molecular mass i.e. deprotonated molecular ion at m/z 1249 but the fragmentation pattern is slightly different and is indicative of different sugar sequence.

The following fragments were observed for the second component: fragments at m/z 1103 $[(M-H) - dhex]^-$; m/z 1073 $[(M-H)-Glc\ A)]^-$; m/z 941 $[(M-H) - (dhex + hex)]^-$ and at m/z 795.6 $[(M-H) - (2dhex+ hex)]^-$. Hence the compound present as trace impurities should have the following structure **1A**:

dhex— hex — dhex — hex — Aglycone
I
Glc A

Rha — Galactose — Rha — Gluc — protoprimulagenin A
I
GlcA

1A

Compound **2,** the more polar one had a molecular formula of $C_{60}H_{98}O_{27}$ deduced from the analysis of ^{13}C NMR **(Fig.6)** and high resolution ESI-MS data, with the sodiated molecular ion $[M+Na]^+$ at m/z 1273 **(Fig.7)** indicative of molecular weight of 1250. ESI-MS in

combination with MS/MS technique has been used for sugar sequence analysis.

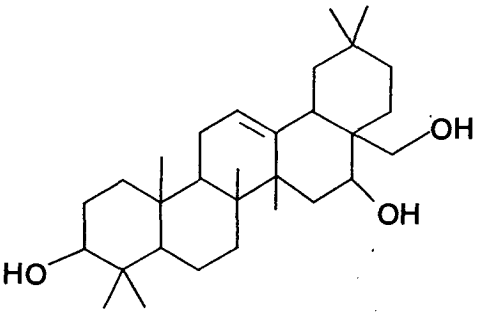
The collision induced dissociation of the sodiated molecular ion at m/z 1273, at 80 eV energy and declustering potential of 15.0eV yielded daughter ions that were separated in time of flight (TOF) mass analyzer. The resulting MS/MS spectrum **(Fig.8)** showed fragment ions at m/z 1127 $[M+Na\text{-}dhex]^+$, m/z 981$[M+Na\text{-}2\ dhex]^+$, m/z 819 $[M+Na\text{-}2\ dhex\text{-}hex]^+$, m/z 643 [M +Na-2 dhex-hex-glucuronic acid]$^+$. In principle, fragment ion of m/z 643 should result in two bond cleavage that yield less abundant product ions from an energy point of view. The mass difference between m/z 1273 and the m/z 815 is 458 Da corresponding to the mass of aglycone. The fragment ion at m/z 643 also indicates attachment of aglycone with a hexose (458+162). MS/MS fragmentation observed is suggestive of the following gross structure for the saponin **2**

dhex — dhex — hex — hex — aglycone
|
GlcA

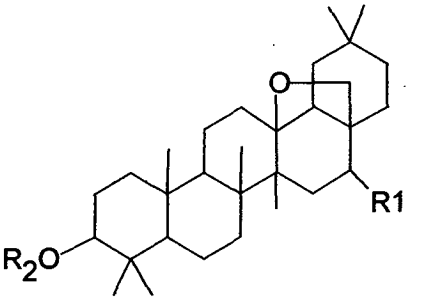
Acid hydrolysis of saponin **2** yielded the same aglycone, primulagenin A, as the one obtained from compound **1** indicative of both the compounds differ only in the carbohydrate moiety (saponins with protoprimulagenin A as the aglycone yield primulagenin A as the aglycone on hydrolysis due to the opening of oxido ring). Sugars glucose, rhamnose and glucuronic acid in the proportion of 2:2:1 were also obtained. The sugars were identified on the basis of comparison

of R_f values of hydrolysed product with the standard sugars. Hence, the saponin **2** was assigned the following structure:

Rha — Rha — gluc — gluc — protoprimulagenin A
|
Glc A



PRIMULAGENIN A



SAPONIN 1

R1=—OH

R2=—Gluc—Galac—Rha—Rha
|
Glc A

Saponin 1A:

R1=—OH

R2=—Gluc—Rha—Galac—Rha
|
GlcA

Saponin 2:

R1=—OH

R2=—Gluc—Gluc—Rha—Rha
|
GlcA

Table 1: ^{13} C NMR assignments of saponins 1 and 2 and the hydrolysed aglycone

CARBON NO.	PRIMULAGENIN A δ_C	SAPONIN 1 δ_C	SUGARS δ_C	SAPONIN 2 δ_C	SUGARS δ_C
1	39.8	39.2	101.2	39.2	103.9
2	27.2	26.6	79.4	26.8	80.1
3	78.1	89.9	79.1	89.4	76.0
4	39.8	39.8	70.1	39.7	72.3
5	55.2	55.6	75.8	55.1	76.7
6	19.4	18.2	62.0	18.6	176.2
7	33.5	32.9		31.4	102.1
8	40.0	42.4	103.6	43.8	78.3
9	46.6	50.5	74.2	50.8	75.4
10	37.1	36.8	78.5	37.3	70.8
11	24.5	19.2	71.2	19.3	78.3
12	121.8	34.5	78.5	33.7	60.9
13	145.3	86.4	63.5	85.8	99.1
14	42.6	44.6		43.8	74.4
15	35.0	37.0	102.7	36.4	69.6
16	73.2	78.0	72.7	78.1	76.0
17	41.3	44.6	72.2	43.8	77.1
18	42.0	51.6	73.3	51.7	62.1
19	48.3	39.0	69.7	38.9	101.0
20	31.5	18.6	36.8	36.4	70.5
21	38.0	31.9		31.9	77.1
22	29.8	36.6		36.7	73.7
23	27.2	27.8		27.5	68.9
24	14.7	16.4		16.1	18.3
25	14.8	16.5		16.3	100.3
26	16.0	18.5		18.3	70.8
27	26.0	19.6		19.1	72.2
28	71.1	78.2		77.7	74.4
29	32.0	33.8		33.7	63.5
30	26.0	24.8		24.6	18.6

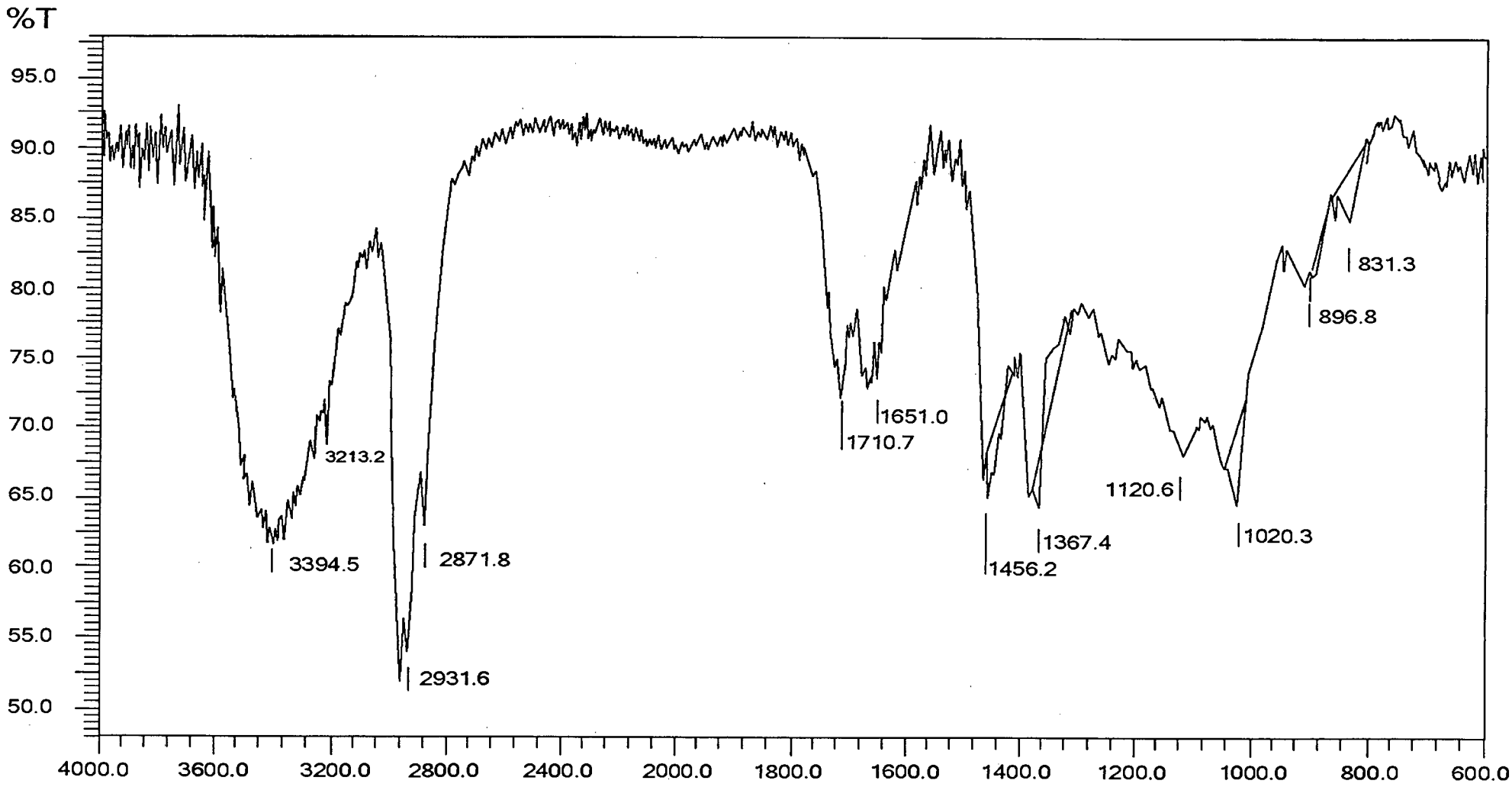


Fig.1: Infra Red spectrum of saponin 1

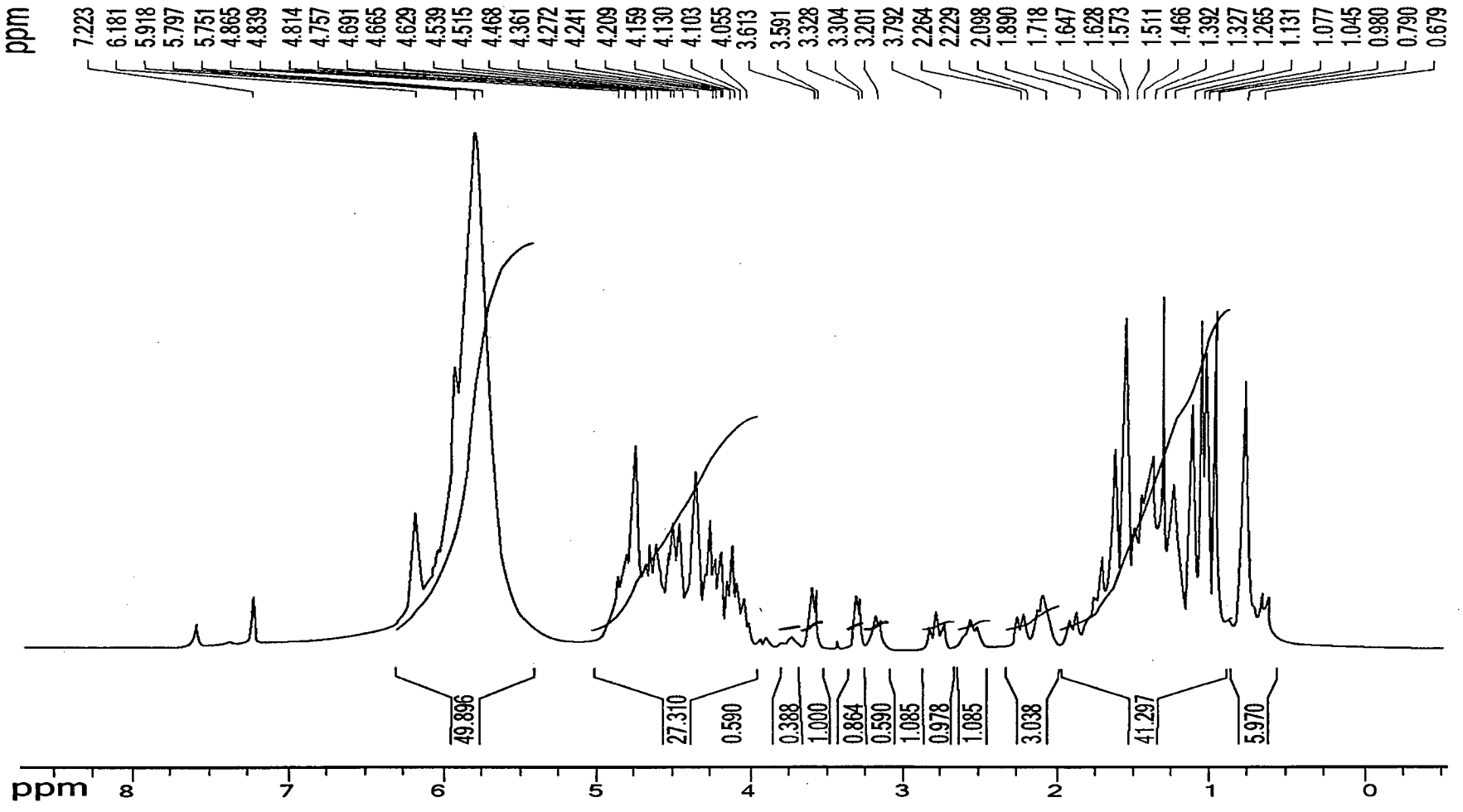


Fig.2: ^{1}H NMR spectrum of saponin 1

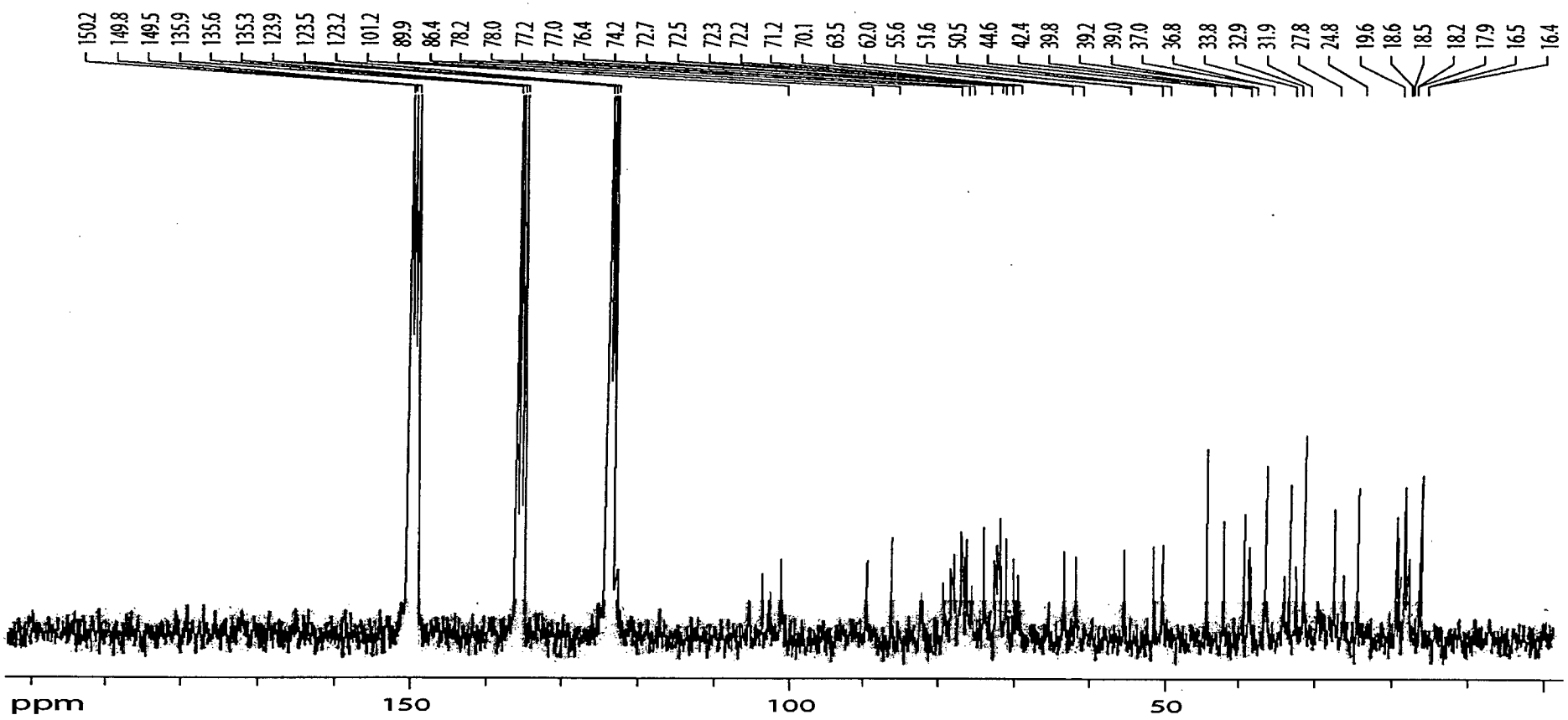


Fig.3: ^{13}C NMR spectrum of saponin 1

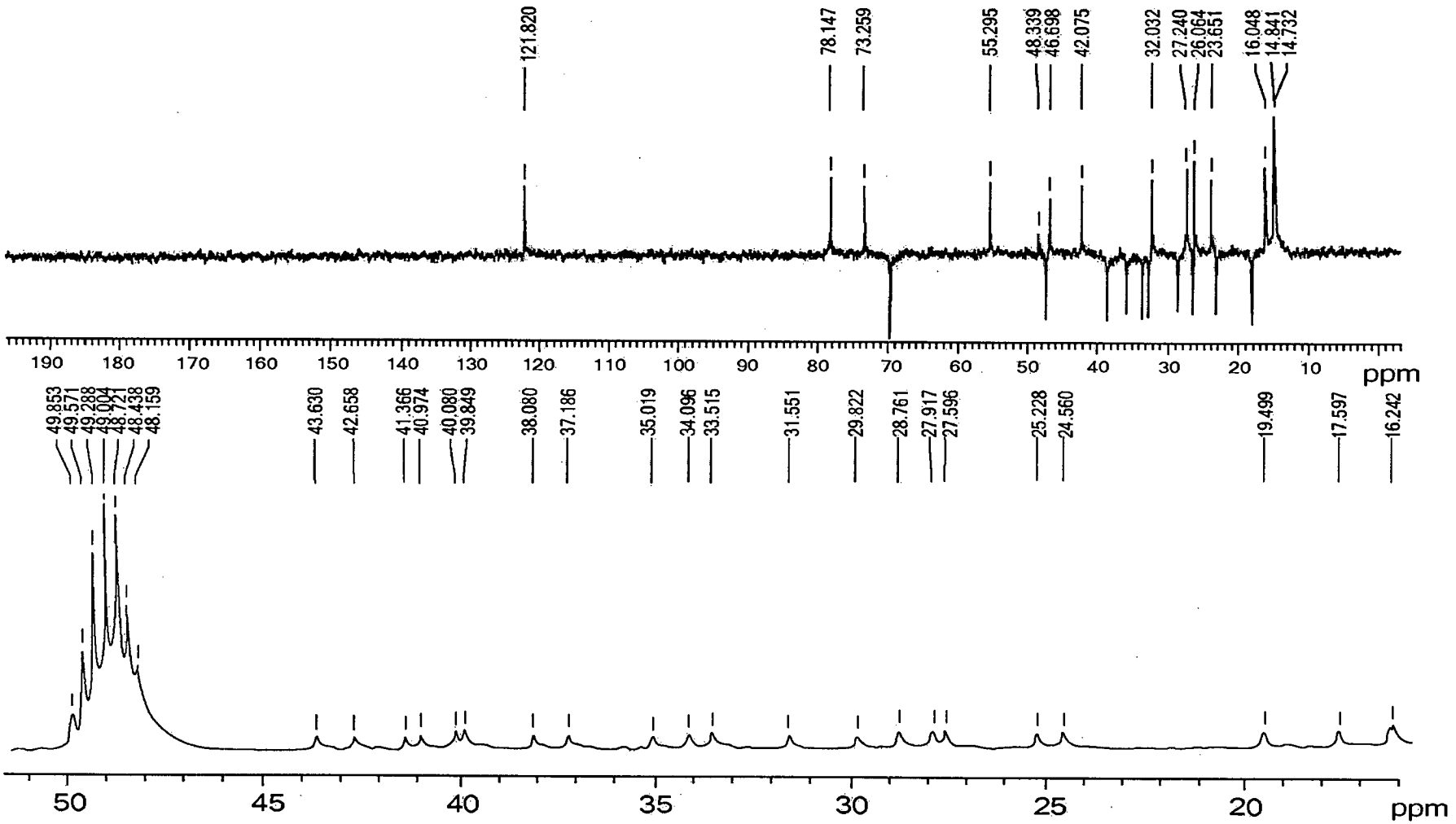


Fig.4: ^{13}C NMR spectrum of Primulagenin A

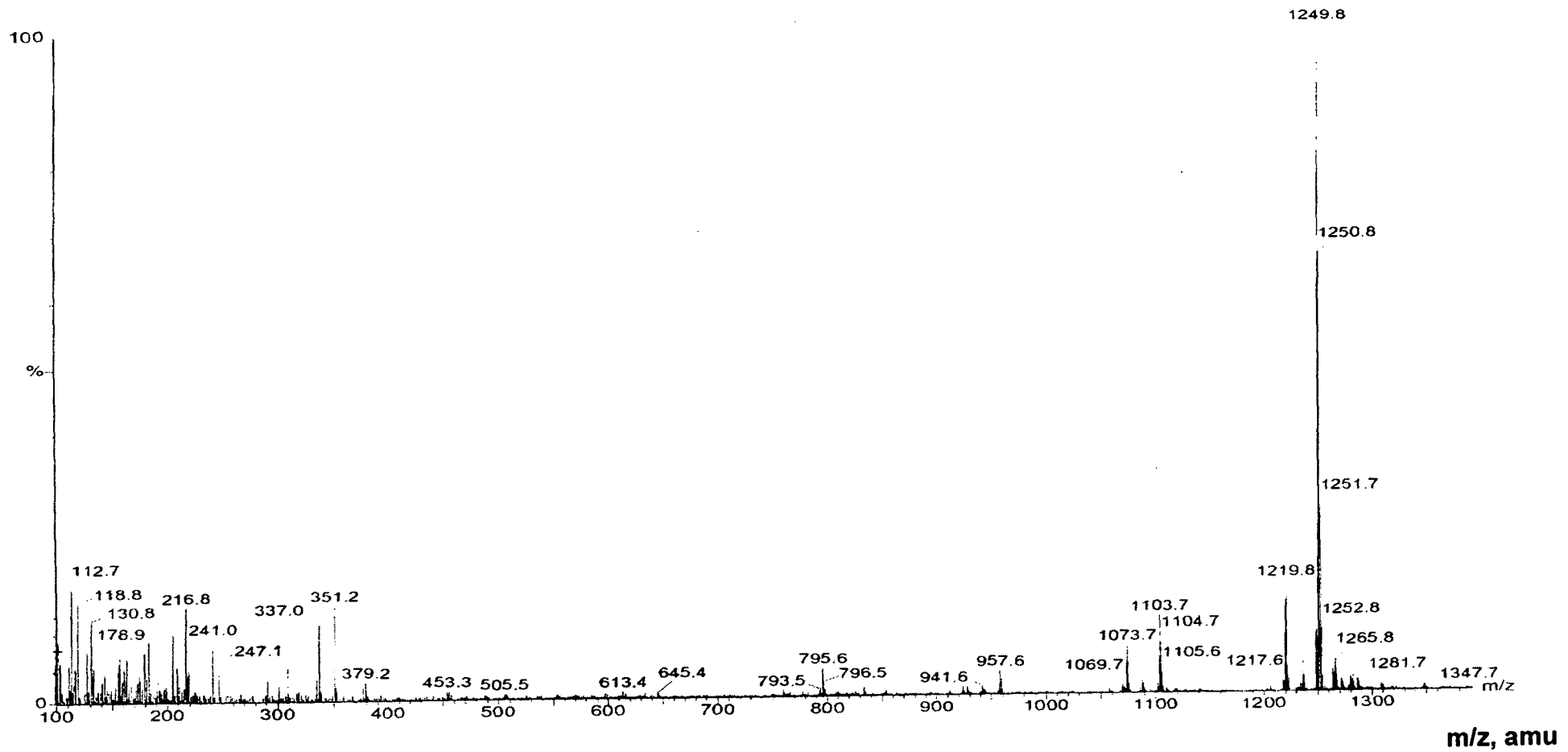


Fig.5: FABMS (negative ion mode) of saponin 1

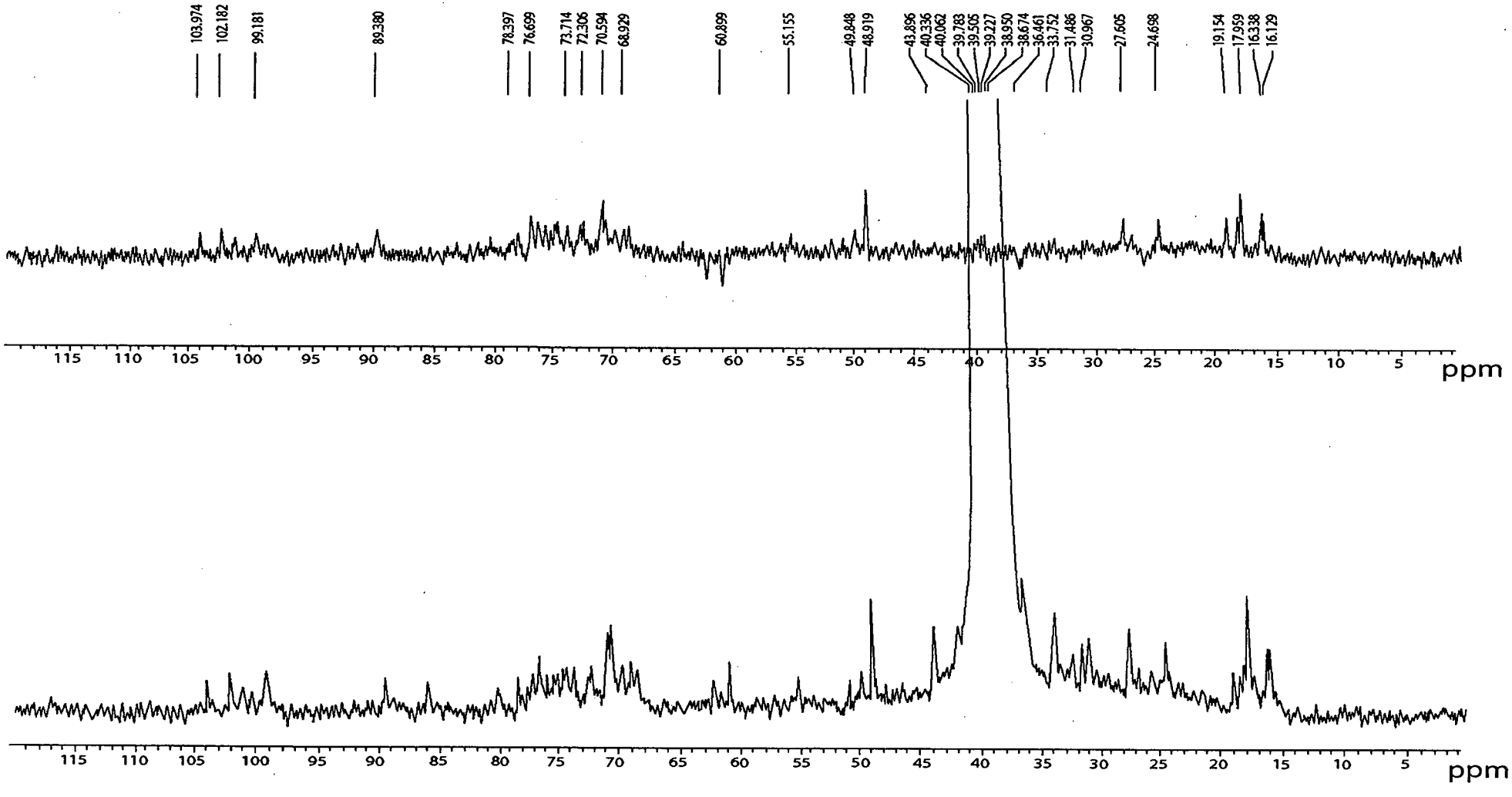


Fig.6: ^{13}C NMR spectrum of saponin 2

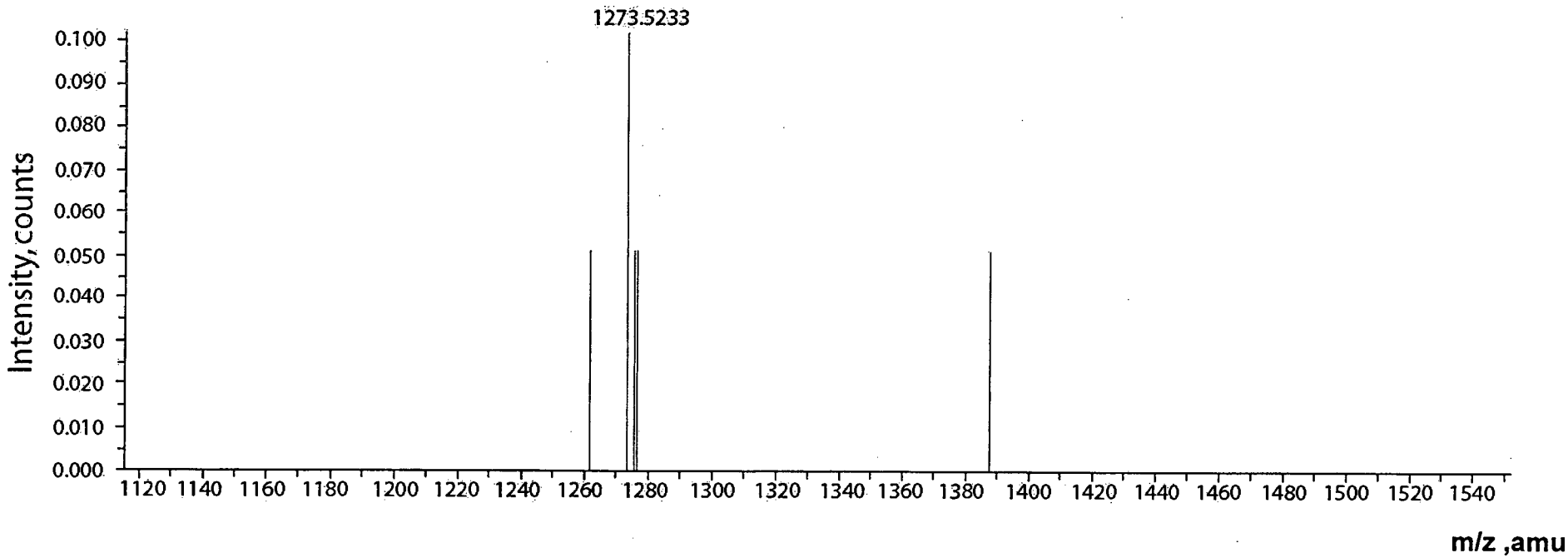


Fig.7: ESI-MS of saponin 2

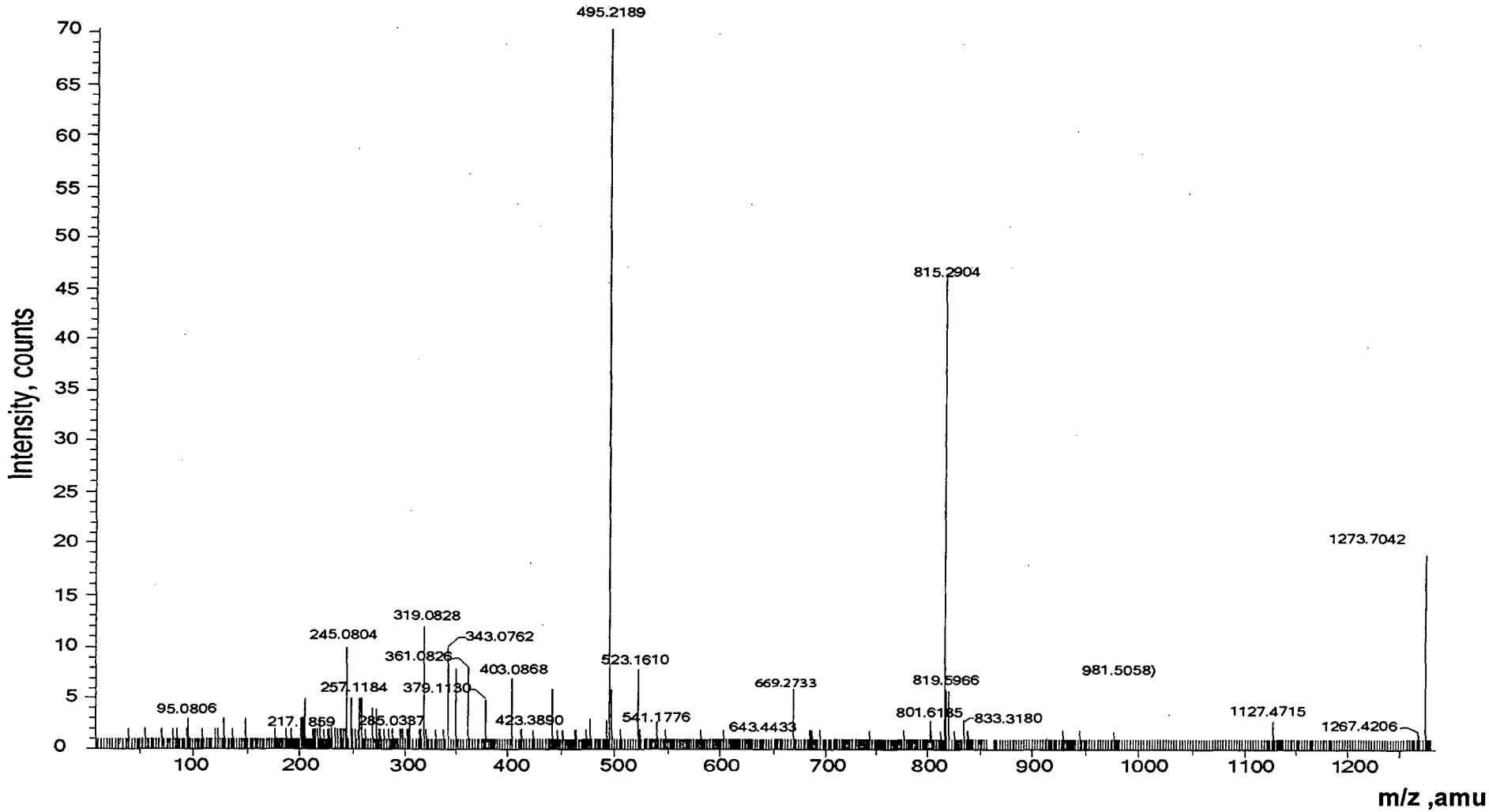


Fig.8: ESI-MS/MS of saponin 2

B. Efficiency of saponin in the remediation of PAHs as a function of EROD activity in fish *Oreochromis mossambicus*

EROD ACTIVITY:

During the past two decades, the use of biological responses (Biomarkers) has become relevant in toxicological assessments since it allows the early detection of adverse effects of contaminants on particular test species at the sub-lethal level (Passino, 1984; Goksoyr & Forlin, 1992; Livingstone; 1993; Peakall & Shugart; 1993) and the study of metabolic enzymes seems to be particularly promising (Payne *et al.*, 1987).

The EROD assay measures the induction of cytochrome P-450 dependant monooxygenase (MFO) system in fish livers. Exposure to xenobiotic compounds results in induction or increased synthesis of particular cytochrome P-450 enzyme. Of the cytochrome P-450 enzymes, the cytochrome P-450 1A sub-family is especially sensitive to induction by a range of organic contaminants including petroleum hydrocarbons, PCBs, dioxins, furans, organochlorine pesticides and PAHs (Payne *et al.*, 1987; Goksoyr & Forlin, 1992, Holdway *et al.*, 1995; Denison & Heath – Pagliuso, 1998). It has been widely recognized as a biological indicator of exposure to anthropogenic organic contaminants with a planar configuration such as PAHs, PCBs, several pesticides and

of PAHs as a function of EROD activity in fish *Oreochromis mossambicus* has been investigated using the experimental protocol as depicted under experimental section. The results obtained are given in **Table 2**

C. Assessment of pollution by PAHs in fishes and its bioremediation by saponins, using Comet assay.

In the marine environment, deleterious effects are caused to organisms due to the release of a number of industrial, agricultural and commercial chemicals (Livingstone, 2001). Many of these chemicals may exhibit genotoxic activities, revealing genetic disorders, diseases and mortality to exposed organisms (Nehls & Segner, 2001). DNA adducts and DNA strand breaks are potentially mutagenic lesions that have been proposed as genotoxicity biomarkers for the biomonitoring of the marine environment (Mitchelmore & Chipman, 1998; Lyons *et al.*, 1999). Fish are important indicators of marine environmental health (Allen *et al.*, 2002). The impacts on fish are studied by measuring the changes at the cellular or biochemical level (Hugget *et al.*, 1992). DNA damage is a sublethal indicator that can be caused by a variety of environmental contaminants including metals and PAHs (Shugart, 1998). Our previous studies showed that the genotoxicity of PAH was enhanced in the presence of nitrite in the medium. We found that the presence of nitrite in the environment produced

genotoxic nitro-PAH metabolites. Therefore we tested 2 saponins (1 & 2) against genotoxicity caused by phenanthrene and chrysene in presence of nitrite by single cell gel electrophoresis or Comet assay (Singh *et al.*, 1988; Fairbairn *et al.*, 1995) (As explained in **Chapter 2, Section III**). The results are shown in **Figs C & D**.

DISCUSSION:

Many plant derived chemicals including those generated from root turnover, stimulate microorganisms to biodegrade xenobiotics (Miya & Firestone, 2001; Donnelly *et al.*, 1994; Haby & Crowley, 1996, Fletcher & Hegde, 1995; Isidorov and Jdanova, 2002). In particular, salicylate which induces systemic acquired resistance (SAR) in plants (Meyer *et al*; 1999) has been linked to the microbial degradation of Naphthalene, a polycylic aromatic hydrocarbon (Yen & Gunsalus, 1982; Meer *et al.*, 1992). Chen and Aitken (1999) demonstrated the ability of salicylate to greatly enhance the rate of removal of other PAHs such as fluoranthene, pyrene, benz(a)anthracene, chrysene and benz (a)pyrene.

Biochemical indicators such as EROD activity serve to link contamination and biological effects and therefore provide unique information on ecosystem health (Bucheli & Fent; 1995). Induction of EROD activity has been used to infer effects such as cancer

related liver lesions in fish (Myers *et al*; 1998) and reproductive impairment in fish (Johnson *et al*; 1988). Cavanagh *et al.*, 2000, measured EROD activity in pikey bream (*Acanthopagrus berda*) in northern Queensland and provided evidence that this bio indicator is a useful tool in assessing relative contamination between sites.

Table 2: Effect of saponin 1 on the EROD activity of fish exposed to PAH

Sr. No.	Tank	Wt. of liver (g)	Wt of liver in 0.2 ml of liver (g)	Amt. of Resorufin (n moles) produced over 10 mins	Amt. of Resorufin (n moles) produced per mins	Amt. of Resorufin (n moles) produced per g of liver	Mean LSI %
1.	Control	0.2291	0.0070	12.5289	1.2529	177.7343	
2.	Control	0.3453	0.0106	11.4724	1.1472	107.9794	
					MEAN	**142.8568**	**1.8554**
3	PAH	0.2901	0.0089	49.6477	4.9648	556.2051	
4.	PAH	0.1973	0.0061	22.2700	2.2270	366.8398	
					MEAN	**461.5224**	**2.1223**
5.	PAH + SAPONIN	0.5604	0.0172	43.8018	4.3802	254.0254	
6.	PAH + SAPONIN	0.4525	0.0139	33.5405	3.3541	240.8989	
					MEAN	**247.4621**	**1.4560**

Table **2** represents the EROD activity and its corresponding liver somatic index in fish *Oreochromis mossambicus* exposed to PAH and PAH-saponin **1** mixture. EROD activity in fish exposed to PAH mixture increased by 30.95 % more than the control group. But in

fish exposed to PAH mixture in presence of saponin **1** EROD activity decreased by 53.6186% compared to fish exposed to PAH mixture only. Liver somatic index decreased by 68.60 % in fish exposed to PAH mixture in presence of saponin 1 compared to fish exposed to PAH mixture only. HPLC analysis of biliary metabolites of fish exposed to individual PAHs (Phenanthrene and Chrysene) in presence of saponin 1 showed absence or decrease of certain metabolites actually formed in the presence of Phenanthrene and chrysene. **Fig. A** represents the High-performance liquid chromatography (HPLC) chromatograms of experiment 1 wherein fish have been exposed to phenanthrene and phenanthrene + saponin 1 mixture simultaneously. **Fig. B** represents the HPLC chromatograms of experiment 2 wherein fish have been exposed to Chrysene and chrysene + saponin 1 mixture.

Fig. A1 and **B1** represents HPLC chromatograms of biliary metabolites of fish from the control tank. **Fig. A2** and. **B2** represents HPLC chromatograms of biliary metabolites of fish exposed to Phenanthrene and Chrysene respectively. The most common biliary PAH metabolites reported in literature are the hydroxy –PAHs (Ruddock *et al.*, 2003). **Figs A3** and **B3** represent HPLC chromatograms of biliary metabolites of fish exposed to Phenanthrene+ Saponin 1 mixture and Chrysene + Saponin 1 mixture respectively. There is evidence of marked decrease or absence of the PAH metabolites from Figs. A3 and B3.

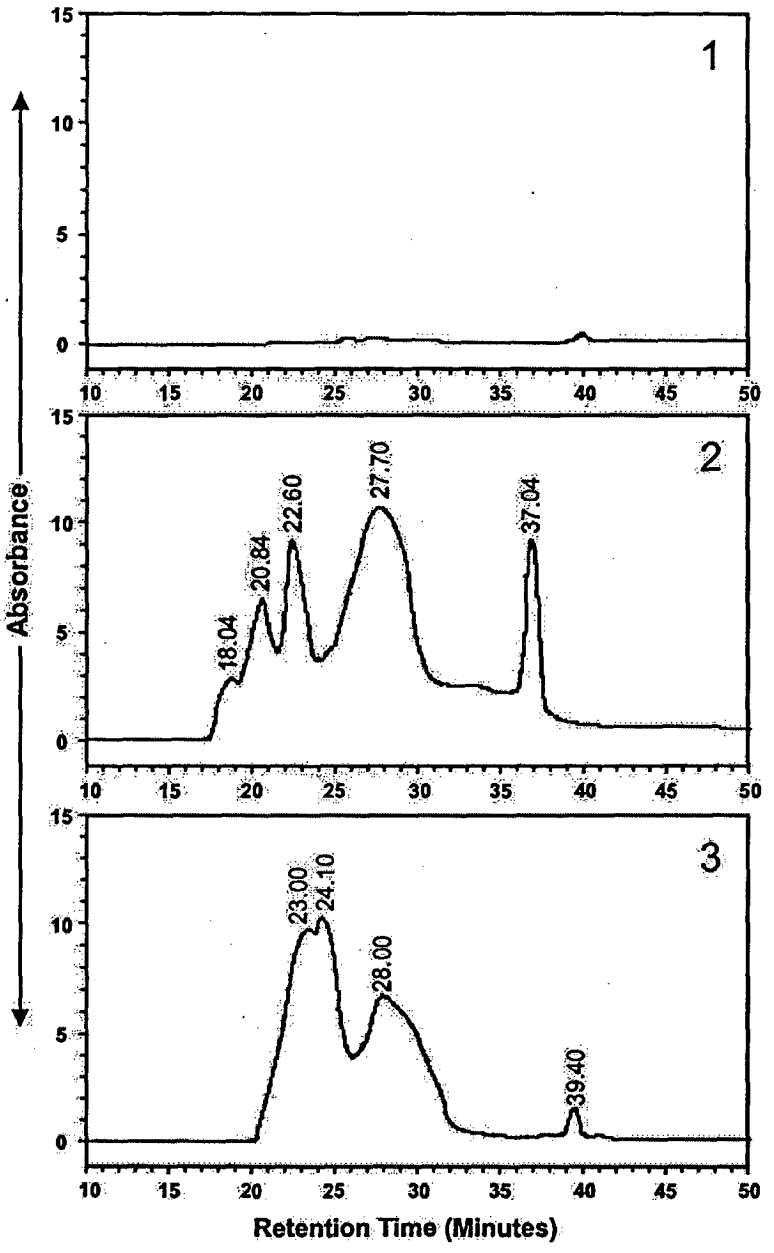


Fig.A: HPLC chromatograms of biliary metabolites of fish *Oreochromis mossambicus* exposed to phenanthrene (A2) and phenanthrene plus saponin 1 (A3)

High concentrations of surfactants have been used in pump-and-treat technologies for non biological remediation of contaminated sites (Abdul & Gibson, 1991; Fountain *et al.*, 1991). Plant secondary metabolites are often effective at very low concentrations and inherent in their natural origins is that they are environmentally friendly (Singer *et al.*, 2003), Therefore these compounds can find their way in the industrial manufacture of surfactants as there would be far fewer concerns about introducing them in the environment. The above study is a preliminary study carried out to investigate the surfactant properties of the saponin 1 with respect to PAHs and its bioavailability and to see whether saponins can be used to tackle environmental pollution and thus protect the biota.

The results of comet assay are presented in **Table 2** and **Fig. C & D**. DNA strand breaks was observed in nearly 84.52 %(± 1.68) in the cells of the fish exposed to Phenanthrene and nitrite and 72 % (± 5.65) in fish exposed to chrysene and nitrite as compared to in the control (Fish exposed to nitrite only). DNA strand break was not observed in the control. Although fish exposed to phenanthrene and nitrite in presence of saponin 1 did not show any significant decrease in the number of comets scored (64.15%, ±0.22), but in case of liver cells exposed to chrysene and nitrite in presence of

saponin 1, no DNA strand breaks were observed. Saponin 2 showed interesting results in both the cases. No DNA damage was observed in liver cells exposed to phenanthrene and nitrite as well as Chrysene and nitrite. Liver somatic Index (%) and DNA damage (%) data is presented in **Table 3** and in the column chart below **(Fig C & D)**. Liver somatic index of the fish remained unaffected upon treatment with saponin 1 and 2 in case of fish exposed to chrysene and nitrite. In case of fish exposed Phenanthrene and nitrite, only saponin 2 showed promising results.

TABLE 3: DNA damage and liver somatic index in fish treated with saponins

Experiment 1	**Cells with DNA damage (%)**	**Liver somatic Index (%)**
Control	0	23.66 (± 0.9)
Fish exposed to Phenanthrene and Nitrite	84.52 (± 1.68)	10.715 (± 0.3)
Fish exposed to Phenanthrene+saponin 1 mixture and Nitrite	64.155 (± 0.22)	7.68 (± 0.38)
Fish exposed to Phenanthrene+saponin 2 mixture and Nitrite	0	22.99 (± 0.39)
Experiment 2		
Control	0	29.133 (+ 0.66)
Fish exposed to Chrysene and Nitrite	72 (± 5.65)	47.26 (± 0.86)
Fish exposed to Chrysene+saponin 1 mixture and Nitrite	0	30.427 (± 0.54)
Fish exposed to Chrysene+saponin 2 mixture and Nitrite	0	29.215 (± 0.74)

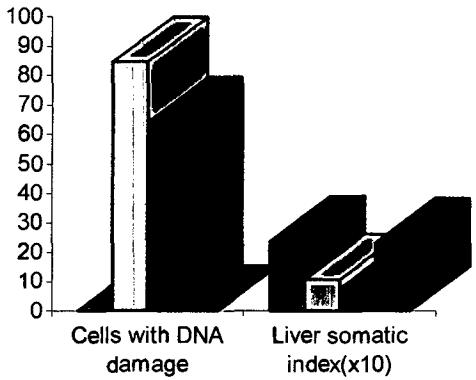


Fig C: Biological effects of exposure of *O. mossambicus* (n=12) to Phenanthrene and Nitrite

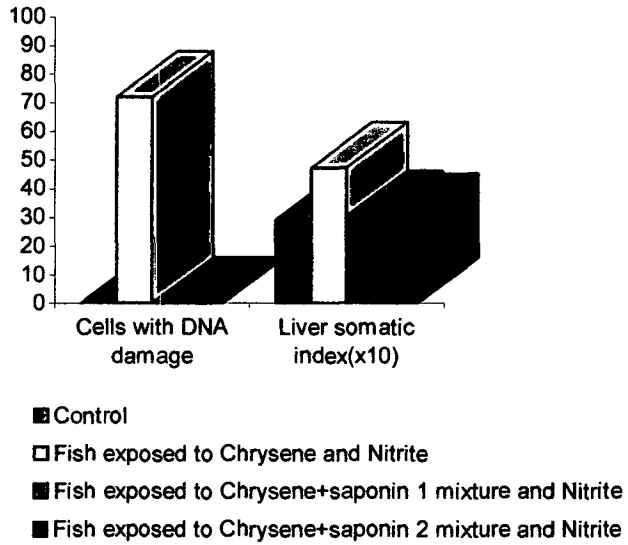


Fig D: Biological effects of exposure of *O. mossambicus* (n=12) to Chrysene and Nitrite

The above results of Comet Assay were confirmed by carrying out *in vitro* tests with a standard carcinogen, crystal violet. This was done by exposing the liver to 0.625% (conc) of crystal violet for 30 mins both in presence of Saponin 1 and 2. A control was simultaneously run with crystal violet alone. Liver cells exposed to crystal violet showed type 1 DNA damage **(Fig.F5)** whereas DNA damage was not observed at all in cells exposed to crystal violet both in presence of Saponin 1 and Saponin 2. The results of comet assay are represented by fluorescence photomicrograph images (Magnification = 40x) of hepatocytes of *O. mossambicus* showing damaged DNA in fish exposed to PAH and nitrite as compared to the undamaged in control. **(Fig E1-E4 & F1-F4)**

Secondary plant metabolites have an important role in developing the myriad of organic pollutant-degrading enzymes found in nature. The link between secondary plant metabolites and enzymatic diversity has yet to be exploited, with potential applications in fields as varied as pest management, bioremediation and fine chemical production (Singer *et al.*, 2003). The above initial investigation offers further scope for research in the field of pollution management.

Fig. E1

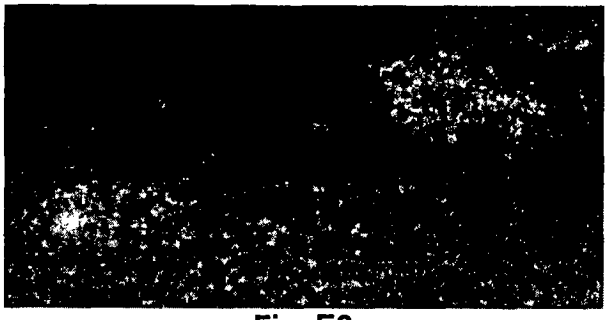


Fig. E2

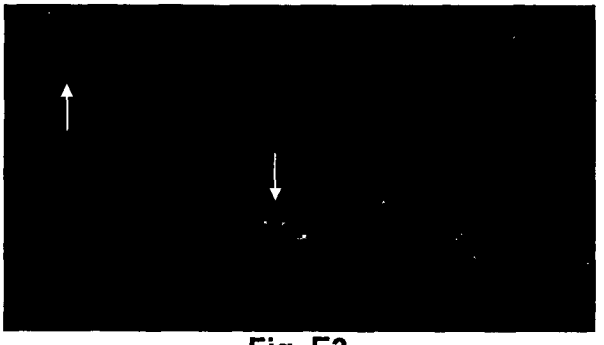


Fig. E3

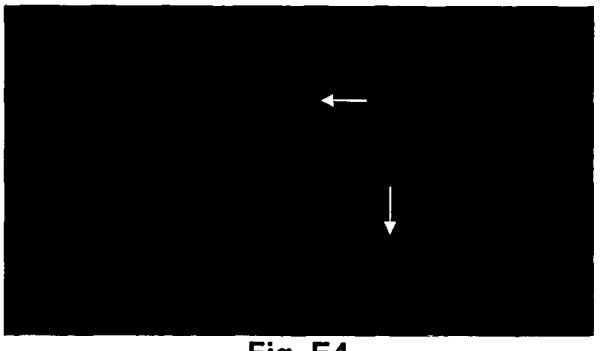


Fig. E4

Comet Assay images of hepatocytes of *O. mossambicus* showing damaged DNA (comets) in (E2) i.e. fish exposed to phenanthrene and nitrite as compared to the undamaged in control (E1). Fig.E3 and E4 show the protected/ undamaged DNA (indicated by arrows) in fish treated with saponin 1 and saponin 2 respectively.
(Magnification = 40x)

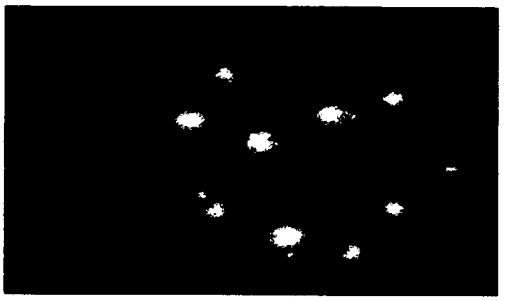


Fig.F1

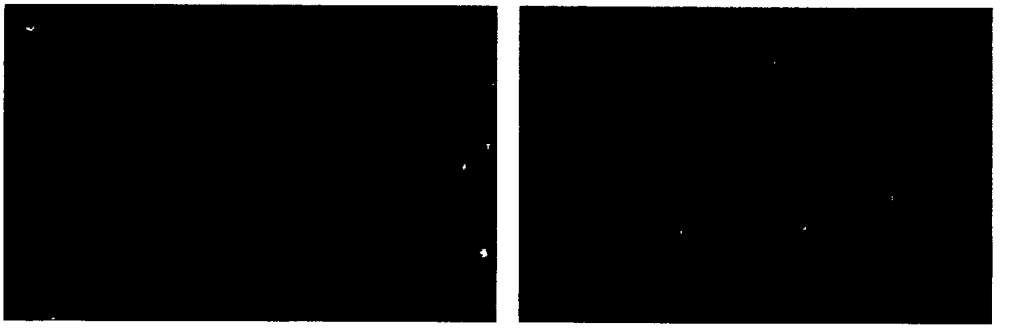


Fig.F2

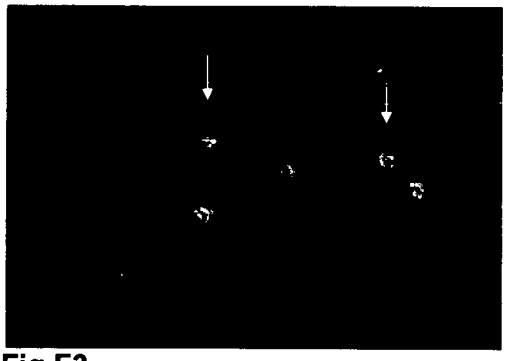


Fig.F3

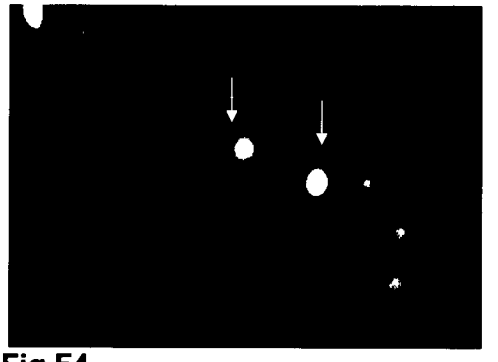


Fig.F4

Fig.F5

Comet Assay images of hepatocytes of *O. mossambicus* showing damaged DNA (comets) in (F2) i.e. fish exposed to chrysene and nitrite as compared to the undamaged in control (F1). Fig.F3 and F4 show the protected/ undamaged DNA (indicated by arrows) in fish treated with saponin 1 and saponin 2 respectively (indicated by arrows). Fig.F5 shows type 1 DNA damage on exposure to crystal violet (standard carcinogen). (Magnification = 40x)

EXPERIMENTAL

A. Isolation and identification of saponins from *Lumnitzera racemosa* using spectroscopic techniques.

Instrumental analysis:

NMR data were recorded in pyridine, on a Bruker Avance 300 MHz instrument, using TMS (tetramethylsilane) as internal standard. Chemical shifts are given in δ values. ESI/MS experiments were performed on a Q-TOFXL MS/MS Applied Biosystems Instrument. The instrument was operated in the positive ion mode. All the analysis was performed using ion spray source with the following setting: ion spray voltage 5500V, nebuliser gas (N_2) 20 (arbitrary units) and curtain gas (N_2) 20 (arbitrary units). The declustering potential and the collision energy was optimised around 60 and 3V respectively during infusion experiment and 15V and 80V respectively for MS/MS experiments.

The sample was directly injected at a constant flow rate of 10 µl/min into the electrospray source using integrated syringe pump. Data acquisition and processing were carried out using mass Lynx NT version 3.5 supplied with the instrument. The MS scanning range was 50-1100 with scan duration of 1.0 sec. Each mass spectrum was recorded over a period of 1 sec. and mass spectrum were accumulated over a period of at least 5 minutes for both single analyses profile and CID experiments.

Preparation of the crude extract and its fractionation:

About 2kg (weight after extraction) of fresh plant material (*Lumnitzera racemosa*) located in Ratnagiri, Maharastra, west coast of India was harvested, leaves and stems were cut into small pieces and extracted in methanol for about a week. The solvent was removed to yield the crude extract as a brown gummy mass. It was taken in 10% aqueous methanol and partitioned successively with petroleum ether (60°C-80°C) followed by chloroform and n-butanol yielding respective fractions on removal of the solvents. The residue was taken as water soluble.

Isolation and purification of the saponins:

The n-butanol fraction, exhibiting the antibacterial activity, after concentration was resuspended in methanol and loaded onto a column of Sephadex LH-20 equilibrated with methanol. Elution of the column with the same solvent led to a fraction rich in a compound **1** with R_f value of 0.28 in butanol: water: acetic acid (5:4:1). It was purified by repeated chromatography over silica gel with methanol: chloroform as the gradient eluent. It was identified on the basis of spectral data and chemical degradation.

TLC was performed on Merck precoated Kieselgel 60 F_{254} and spots were detected by exposure to iodine as yellowish brown

spots or as purplish spots by spraying with methanolic sulphuric acid.

Acid hydrolysis of the saponins:

Compound **1** (10 mg) in methanol was refluxed in 1N HCl (1ml) for 2 hrs on a Water bath. At the end of that period, methanol was removed under vaccum and the residue extracted with chloroform. The aglycone obtained on the removal of chloroform was purified by repeated chromatography over silica gel to yield aglycone, primulagenin A (2mg). The aqueous residue contained sugars. They were spotted on TLC along with the standards. TLC solvent system :(Bu-OH: Ac-OH: ET_2O: H_2O: 9:6:3:1).

B. Efficiency of saponin in the remediation of PAHs as a function of EROD activity in fish *Oreochromis mossambicus*

Exposure protocol:

Oreochromis mossambicus (8.6 to 11g) obtained from a local estuarine fish farm were acclimatized in the laboratory for 7d in stored tap water .The exposure experiments (6-8 fish per tank) were conducted for 10 days in 30-L glass aquaria with continuous aeration. Control tanks with 6 fish each received equivalent amounts of only DMF. The test medium was renewed every day. Commercial feed was provided *ad libitum* during the period of the experiment. Three tanks were maintained; one under control and

the other two under test. 1 ml of dimethyl formamide (DMF) was added to the control tank and 1 ml of known concentration (1ppm) of Phenanthrene was added to the second tank. To the third tank a mixture of 0.5 ml of saponin (30 mg/L concentration) + 0.5 ml Phenanthrene (0.5 ppm) was added. The experiment was repeated with Chrysene. The above experiment was carried for 10 days. At the end of that period the fish were harvested and dissected to study the EROD activity.

EROD activity measurement:

Reagents:

1. Sodium Phosphate dibasic anhydrous (Na_2HPO_4) (0.1 M)
   pH=7.4
2. Potassium dihydrogen orthophosphate (KH_2PO_4) (0.1M)
3. Potassium Chloride (KCl) (0.1M)
4. EDTA ($C_{10}H_6N_2O_8$) (1μM)
5. Tris – HCl buffer (0.1M)
6. Ethoxyresorufin (2μM)
7. NADPH (0.25 μM)

Preparation of Buffers:

Tris-HCl buffer: To 0.1 M Tris HCl solution, 0.1N HCl is added to adjust the pH to 7.5

Na_2HPO_4 buffer: To 0.1 M Na_2HPO_4 , KH_2PO_4 solution is slowly added to adjust the pH to 7.4

To 250 ml of the above solution is added to 250 ml of distilled water containing 0.1 M KCl and 1μM EDTA solution. Buffer is stored under refrigeration.

Procedure:

EROD activity was determined in the post-mitochondrial supernatant prepared according to Gunther *et al.* (1997). About 0.20 to 0.70 g of liver from each fish was homogenized in phosphate buffered saline within on hour of collection using a homogeniser. As the liver samples were too small to be processed individually for enzyme activity, they were pooled before homogenization The homogenates were centrifuged for 15 mins at 13,000 g at 1°C and the supernatant was evaluated spectrophotometrically according to the method of Klotz *et al.*, 1984.

Enzyme reaction:

Enzyme assay was conducted using NADPH as the electron donor and resorufin (85 nm) as internal standard. 0.2 ml of the above supernatant is treated with 1.0 ml Tris-HCl buffer, 0.1 ml substrate (Ethoxyresorufin) and 0.1 ml NADPH. The reaction mixture is incubated at room temperature for 10 minutes. After 10 minutes the

reaction was stopped by adding 2.5 ml methanol and 0.05 ml resorufin (internal standard). The activity denoted by an increase in absorbance at 572 nm (Klotz *et al.*, 1984) was measured in a Shimadzu spectrophotometer (model UV-1201V) and reported in terms of nmol resorufin formed min^{-1} g^{-1} liver.

HPLC analysis of biliary PAH metabolites

The gall bladder was detached from the liver and the bile was collected with a syringe. The bile samples were stored at –20°c until analysis. Before analysis the bile samples were diluted 100 times (10µl + 990µl) with distilled water/methanol 50:50. High-performance liquid chromatography (HPLC) separation with fluorescence detection was done using a Thermo Quest instrument. Bile samples for HPLC separation were used without preliminary deconjugation. PAH metabolites were separated on a reverse-phase C18 column (ChromSpher 5) using methanol / water (50:50 v/v) as the solvent.

Hepatic biomarkers

At the conclusion of each experiment, livers were excised, weighed and the liver somatic index (LSI) determined as percentage ratio of liver weight to body weight.

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Miscellaneous Studies

Leishmanicidal and Anti-Microbial Activities of Benzoxazolinone-2-one and its Synthesized Analogs

INTRODUCTION:

Since the first report on the hypnotic properties of 2-benzoxazolinone (Lespagnol *et al.*, 1944; Lespagnol & Lefebvre, 1945), a number of derivatives has been tested for various activities including anti-convulsant (Bywater *et al.*, 1945), antipyretic (Bonte *et al.*, 1974), analgesic (Bonte *et al.*, 1974;Renard *et al.*, 1980;Erodğan *et al.*, 1989;Pilli *et al.*, 1993;Erol & Demirdamar, 1994;Palaska *et al.*, 1995;Gö Khan *et al.*, 1996), cardiotonic (Vaccher *et al.*, 1996, Bonte *et al.*, 1990), anti ulcer (Katsura *et al.*, 1991), anti-neoplastic (Advani & Sam) or anti -bacterial, anti-microbial and anti-fungal effects (Strutz *et al.*, 1956;Varma and kapoor, 1979; Erol *et al.*, 1989; Erodğan & Yulug, 1989; Erdogan *et al.*, 1989; Erol *et al.*, 1989).

Acanthus illicifolius L. (Acanthaceae) is a mangrove plant found in the estuaries of the rivers Mandovi and Zuari in Goa, India. The plant is useful in the treatment of asthma, rheumatic pain (Chopra *et al.*, 1956) and possesses analgesic and anti-inflammatory activities (Agshikar *et al.*, 1979). During a systematic chemical examination of the plant, benzoxazoline-2-one (BOA) was isolated as one of the constituents of the leaves (Murthy *et al.*, 1984). Drugs like sodium stibogluconate and pentamidine though toxic are still used clinically for the treatment of *Leismania donovani* infection because of the non-availability of other non toxic and effective drugs. Therefore, it was considered of interest

to screen BOA for its effect on *Leishmania donovani* promastigotes in an *in vitro* culture system. It was found that BOA possessed strong leishmanicidal activity compared to the activity of pentamidine (Kapil *et al.*, 1994).

In order to increase the potency of the molecule (BOA) for its leishmanicidal properties it was thought desirable to prepare different derivatives of BOA for testing. These have been synthesized by condensation of urea or thiourea with respective substituted o-amino phenols and identified on the basis of spectral data. Unfortunately, none of the synthesized six analogs of BOA had the potency exhibited by the parent molecule. These molecules have also been tested for their antimicrobial potency.

RESULTS AND DISCUSSION:

All the seven compounds were synthesized using the procedure given under experimental section by reacting appropriate o-amino-phenol derivative with urea in pyridine as solvent. The IR **(Fig 1a-7a)**, ^{1}HNMR **(Fig.1b-7b)** and ^{13}CNMR **(Fig 1c-7c)** spectrum of the compounds are given to evidence the structure of the compound. Spectral data of 2-benzoxazolinone derivatives are shown in **Table 1 & 2.** In the IR spectrum of the compounds the bands for the lactam

group are evident between 1636-1792 cm^{-1}. The –NH proton appeared as an intense band at 3038-3272 cm^{-1}. ^{1}HNMR spectrum in $CDCl_3$ displayed characteristic broad singlet at δ 9.9 due to –NH proton and a multiplet between δ 6.7-7.3 due to aromatic protons. Methyl substituents are evident between δ 1.9-2.35 for substitution in the ring and at δ 3.5 for N-methyl group.

Leishmanicidal and antimicrobial activity results are given in **Table 3, 4, 5 & 6** respectively. Antimicrobial activity was evaluated against bacterial and fungal strains including three multidrug resistant bacterial strains. Streptomycin and Nystatin were used as standard antibacterial and antifungal agents respectively. Leishmanicidal activity exhibited by benzoxazoline-2-one was as good as the standard pentamidine, a drug at present being used clinically. Introduction of substituent in the benzene ring results in marked decrease in the activity. Conversion of lactam C=O to thione and –NH proton to N-CH_3 also drastically reduces the activity. Thus the unsubstituted benzene and the lactam group seem to be essential for the activity. All the molecules were almost ineffective as the anti-microbial agents.

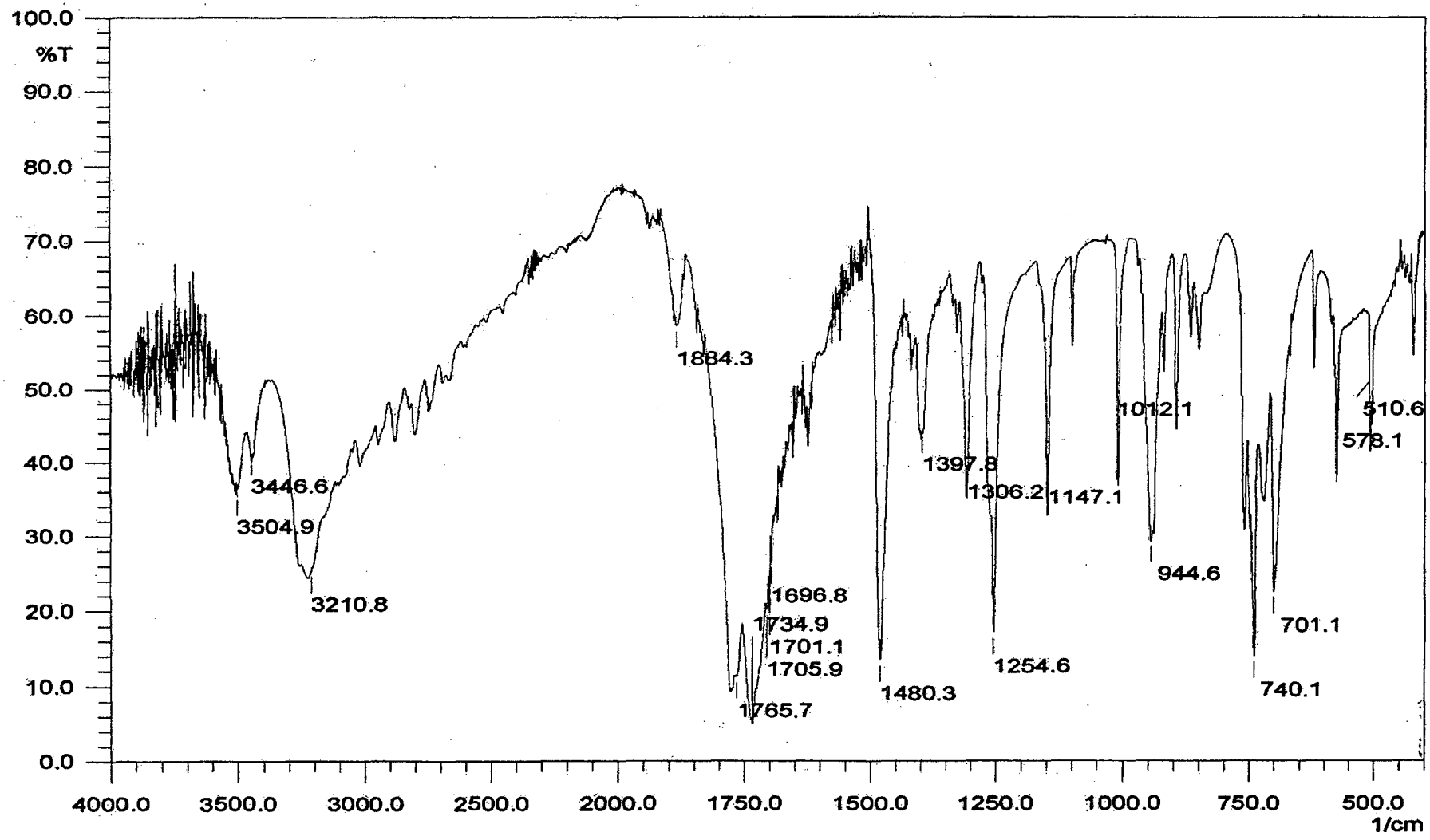


Fig.1a: IR spectrum of Benzoxazoline-2-one

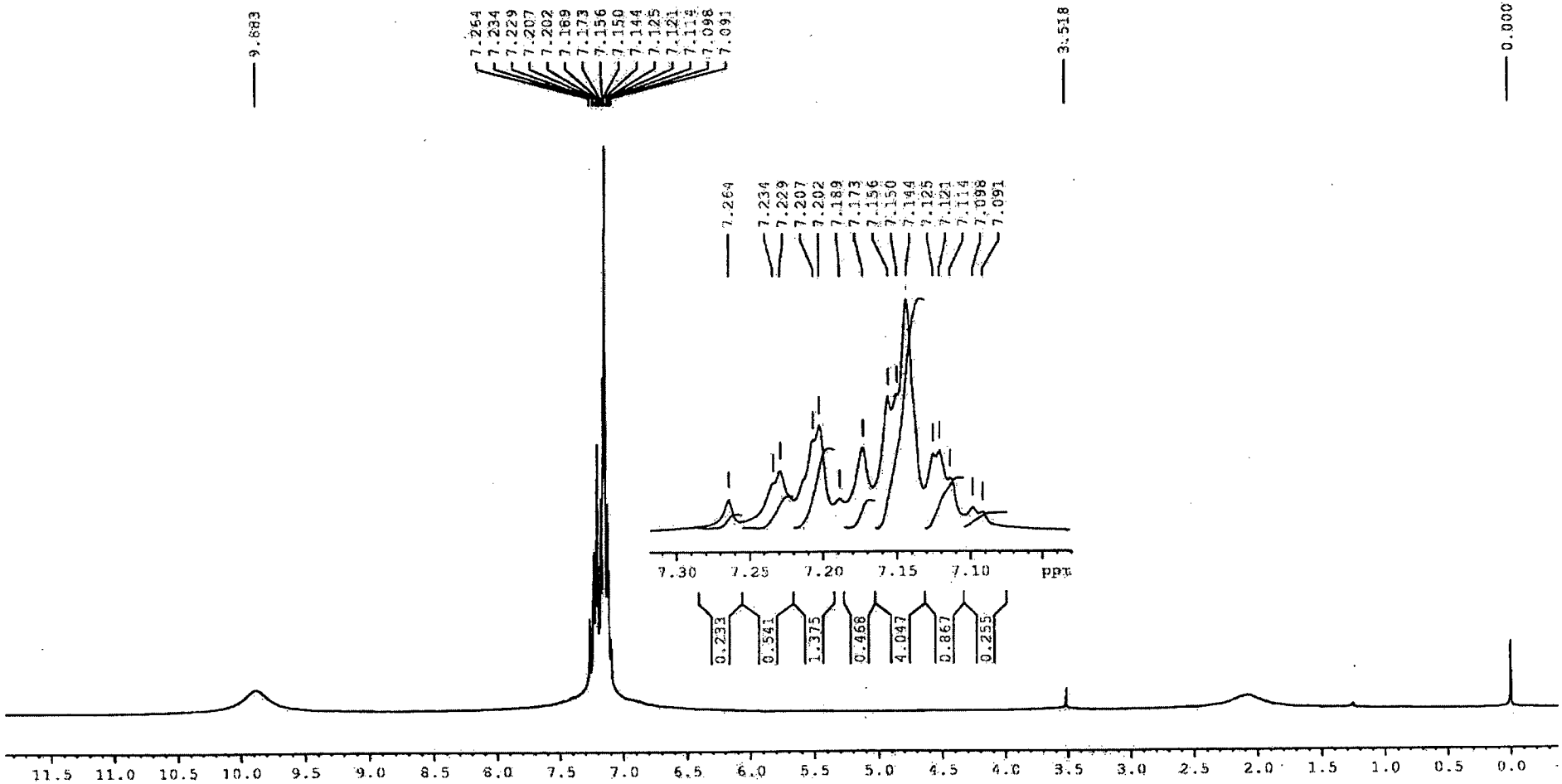


Fig.1b: ^{1}H NMR spectrum of Benzoxazoline-2-one

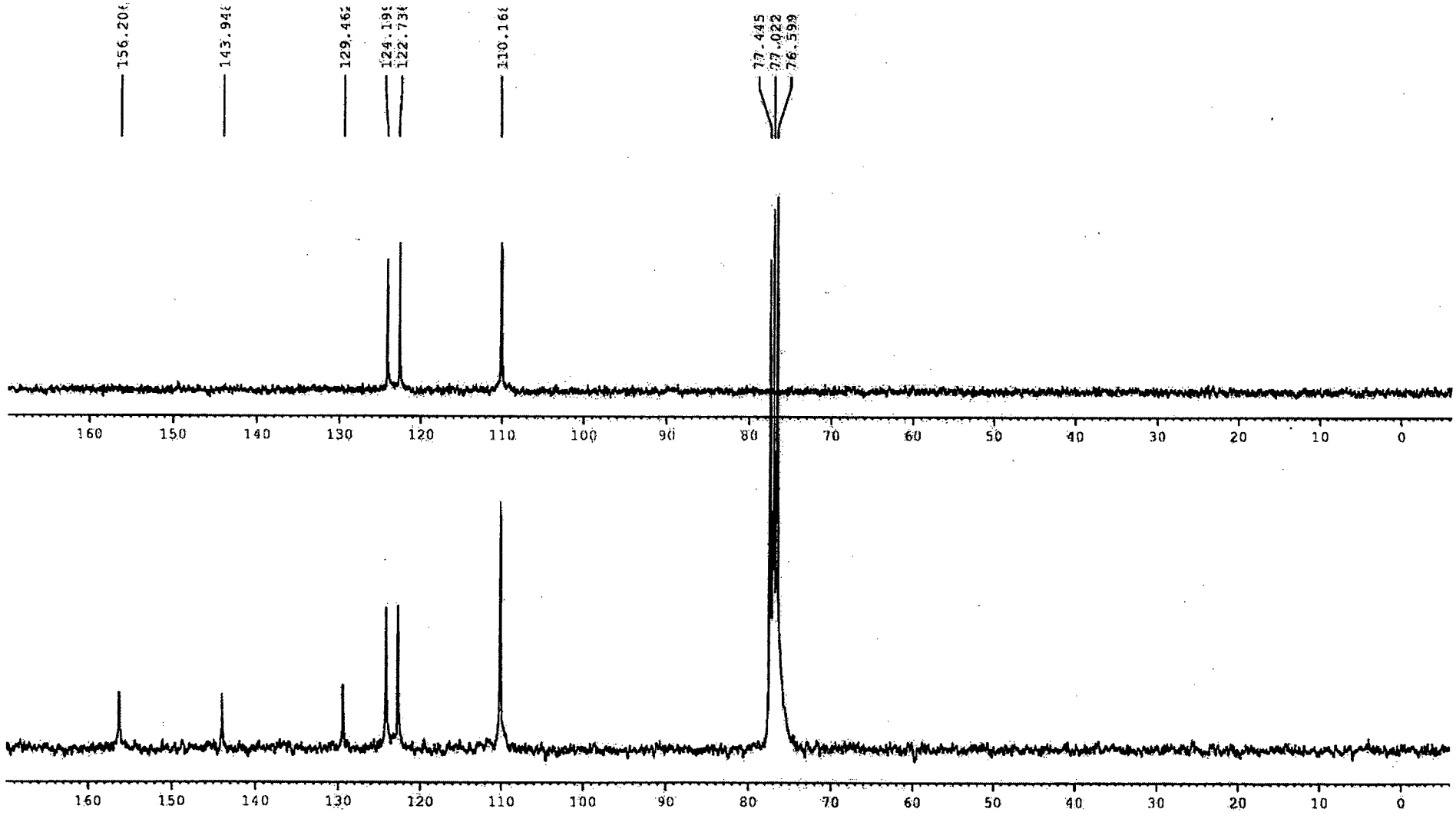


Fig.1c: ^{13}C NMR & DEPT spectra of Benzoxazoline-2-one

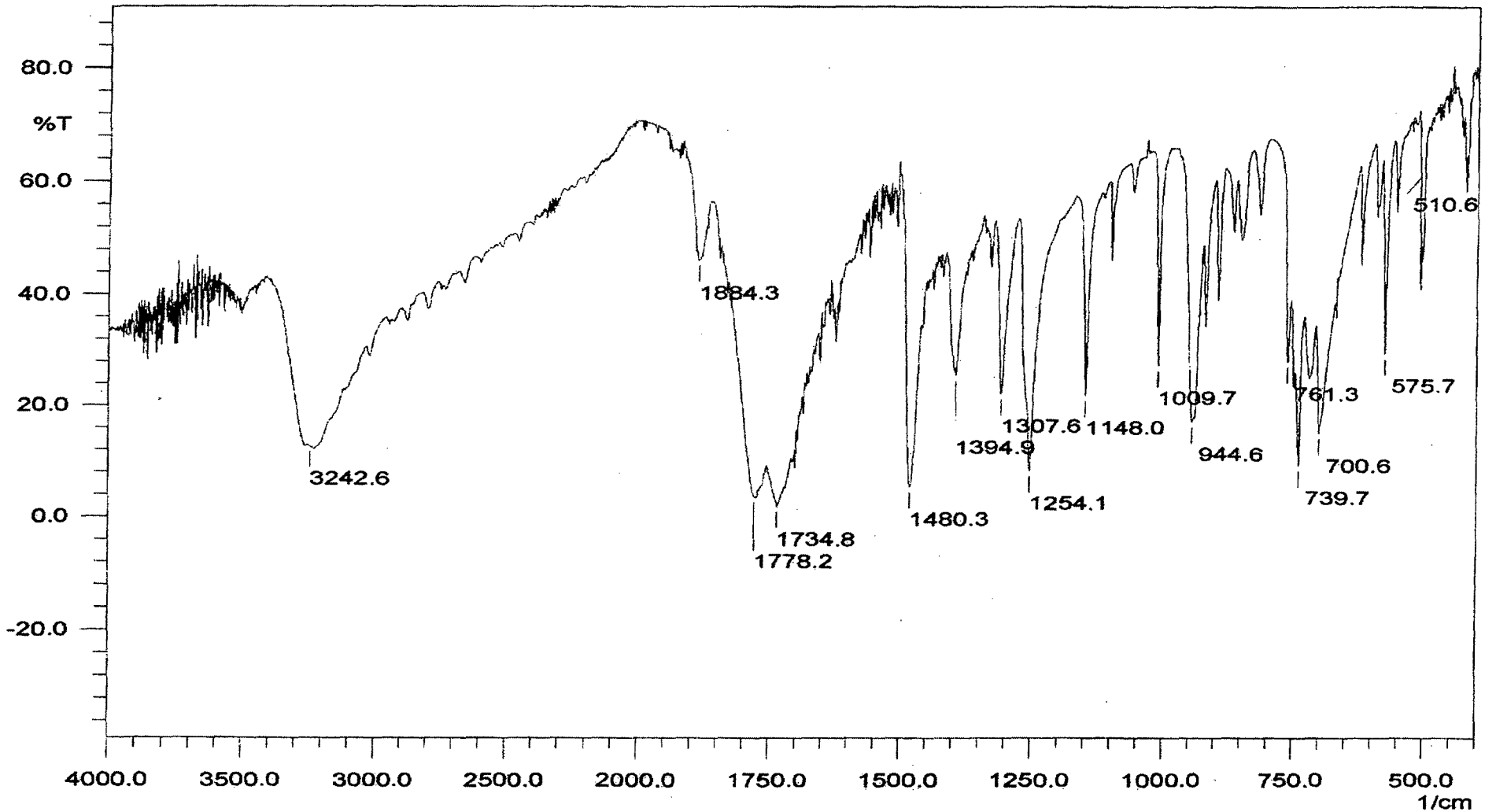


Fig.2a: IR spectrum of 6-chlorobenzoxazoline-2-one

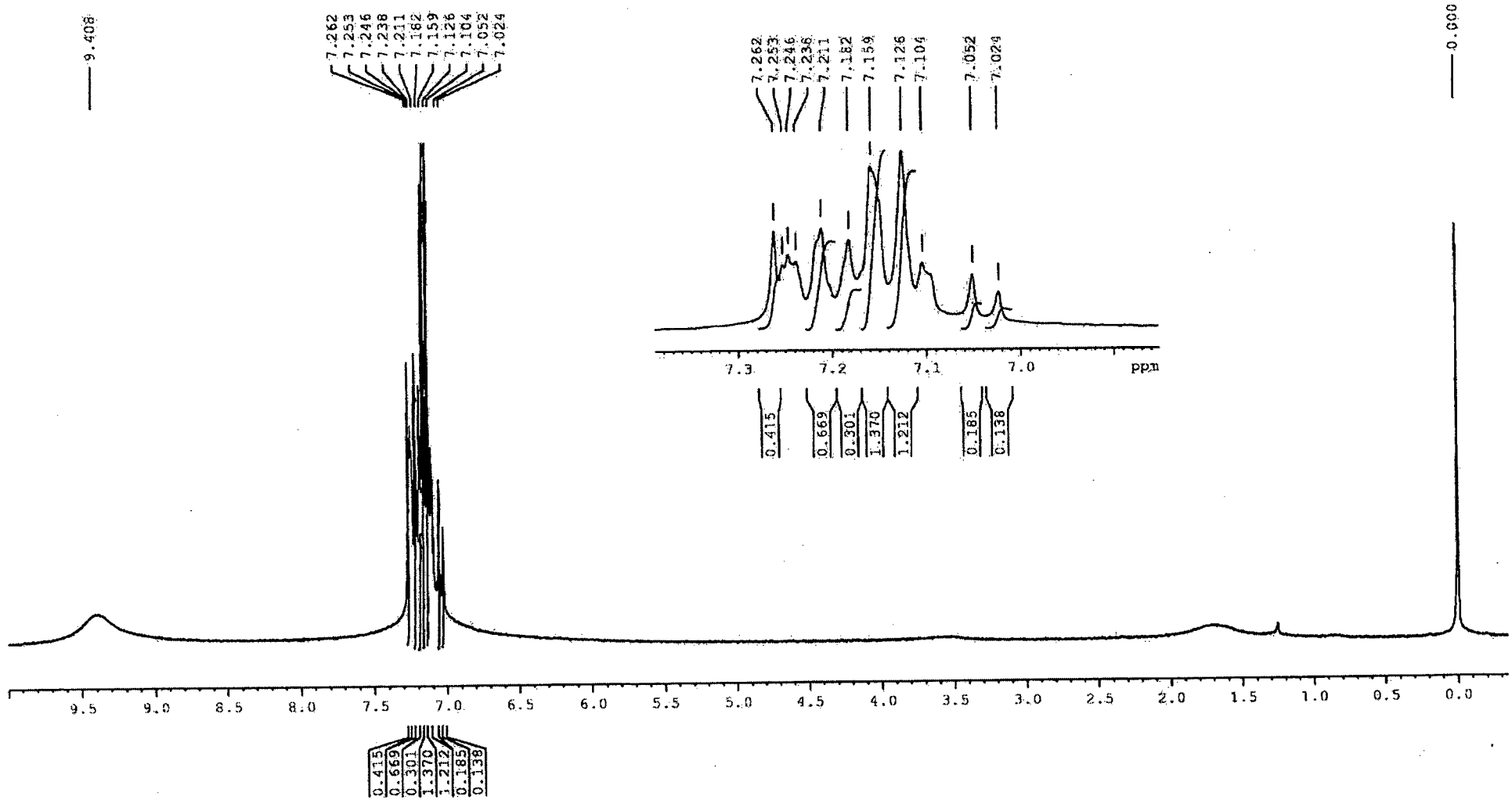


Fig.2b: ^{1}H NMR spectrum of 6-chlorobenzoxazoline-2-one

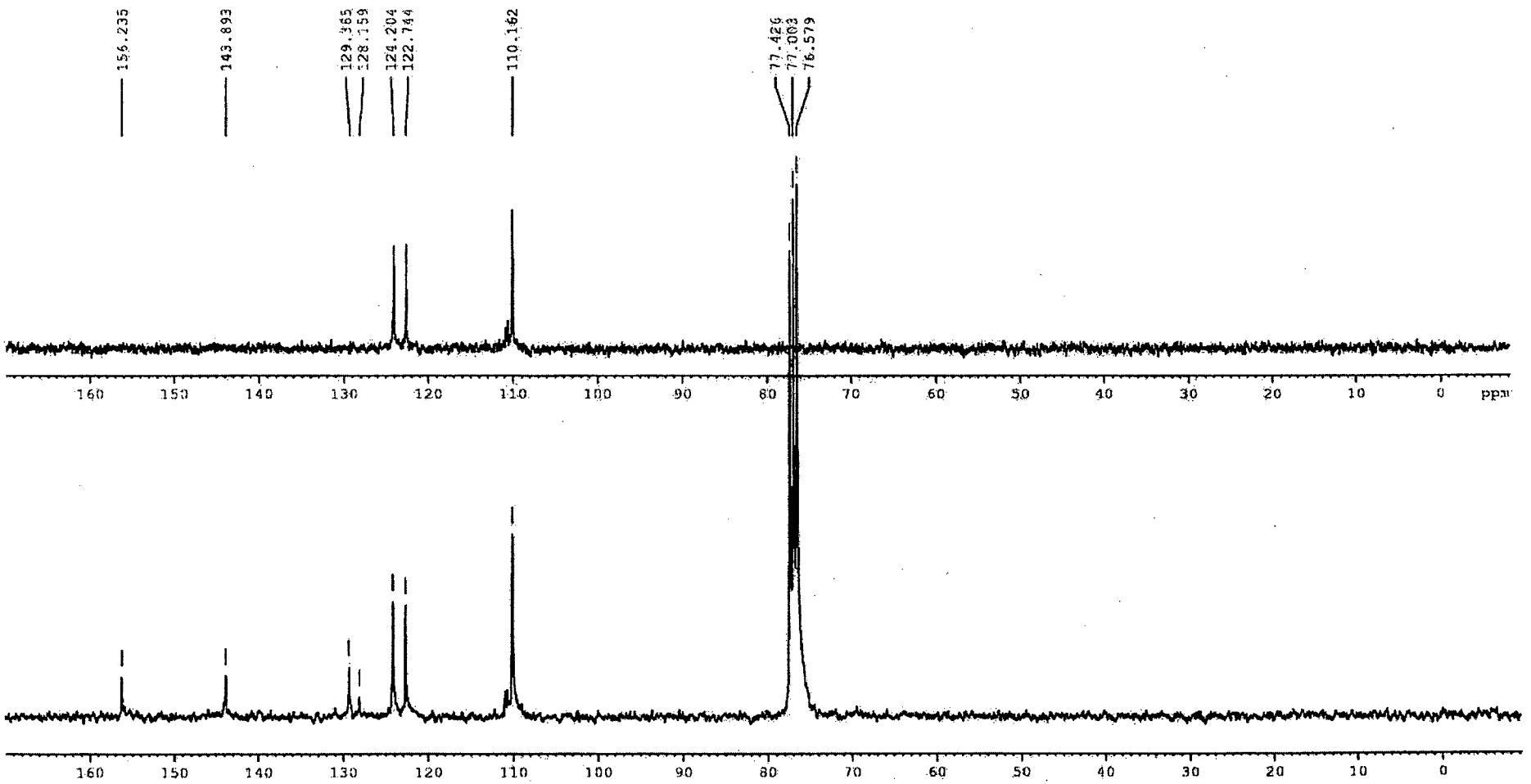


Fig.2c: ^{13}C NMR & DEPT spectra of 6-chlorobenzoxazoline-2-one

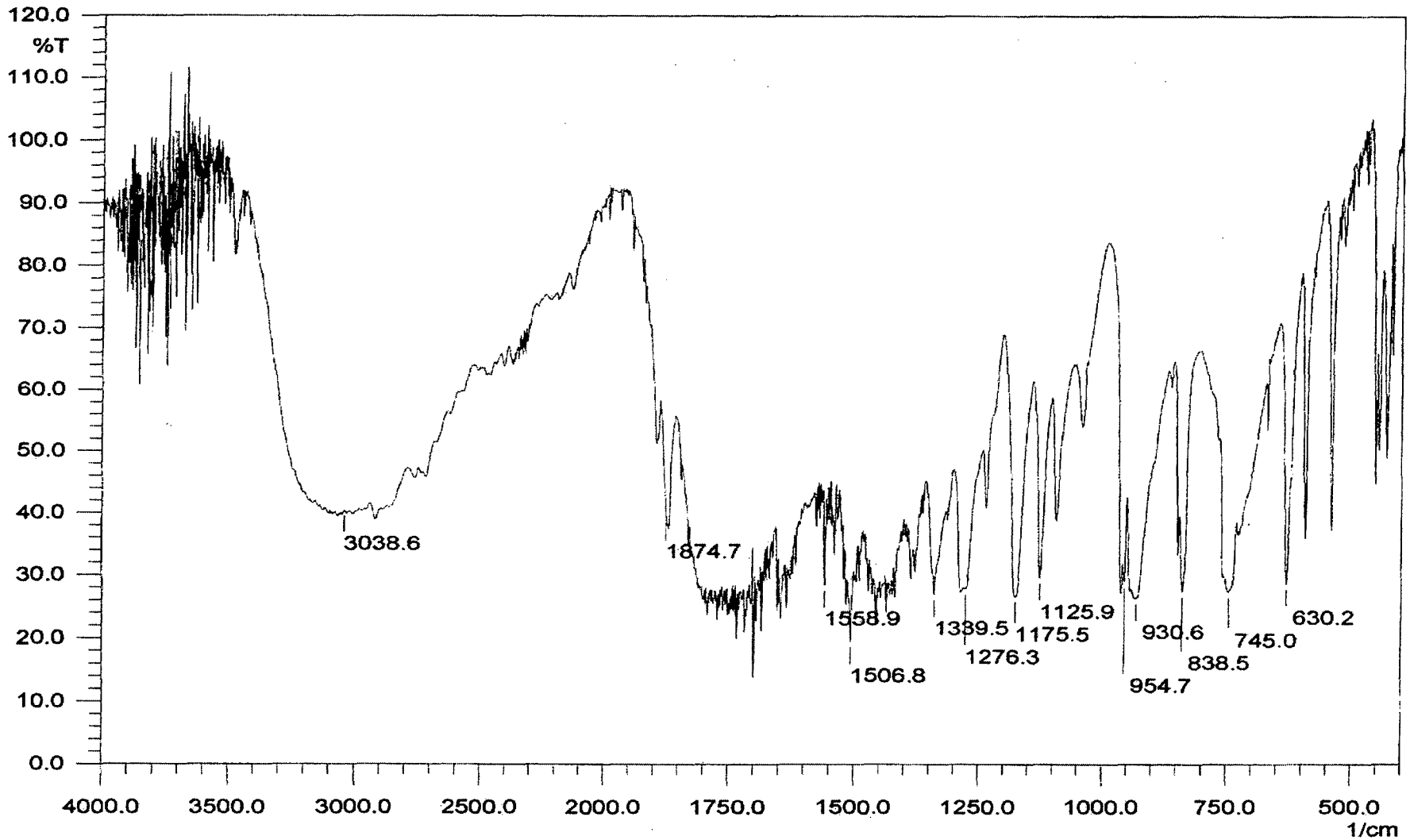


Fig.3a: IR spectrum of 5, 7-dimethylbenzoxazoline-2-one

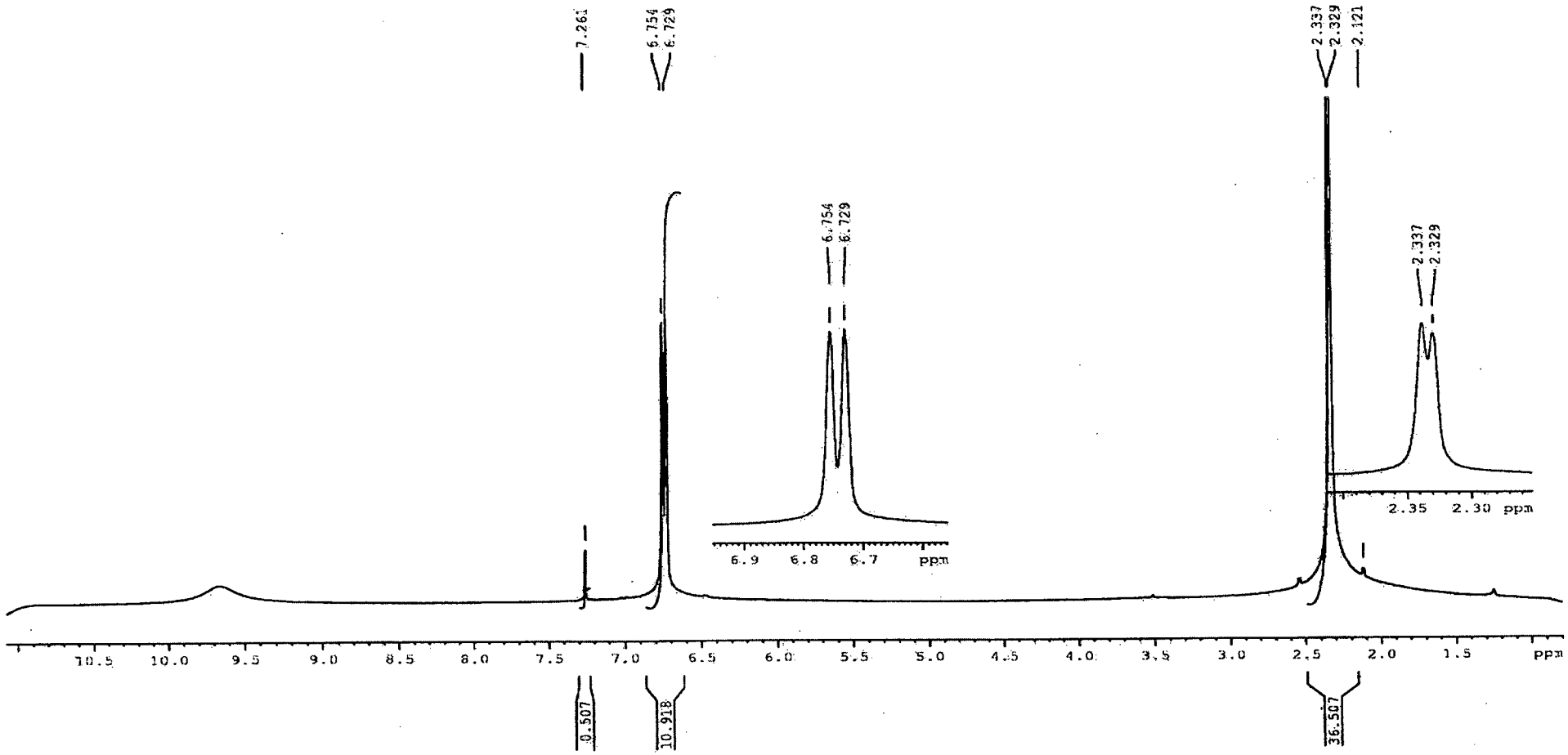


Fig.3b: ^{1}H NMR spectrum of 5, 7-dimethyl benzoxazoline-2-one

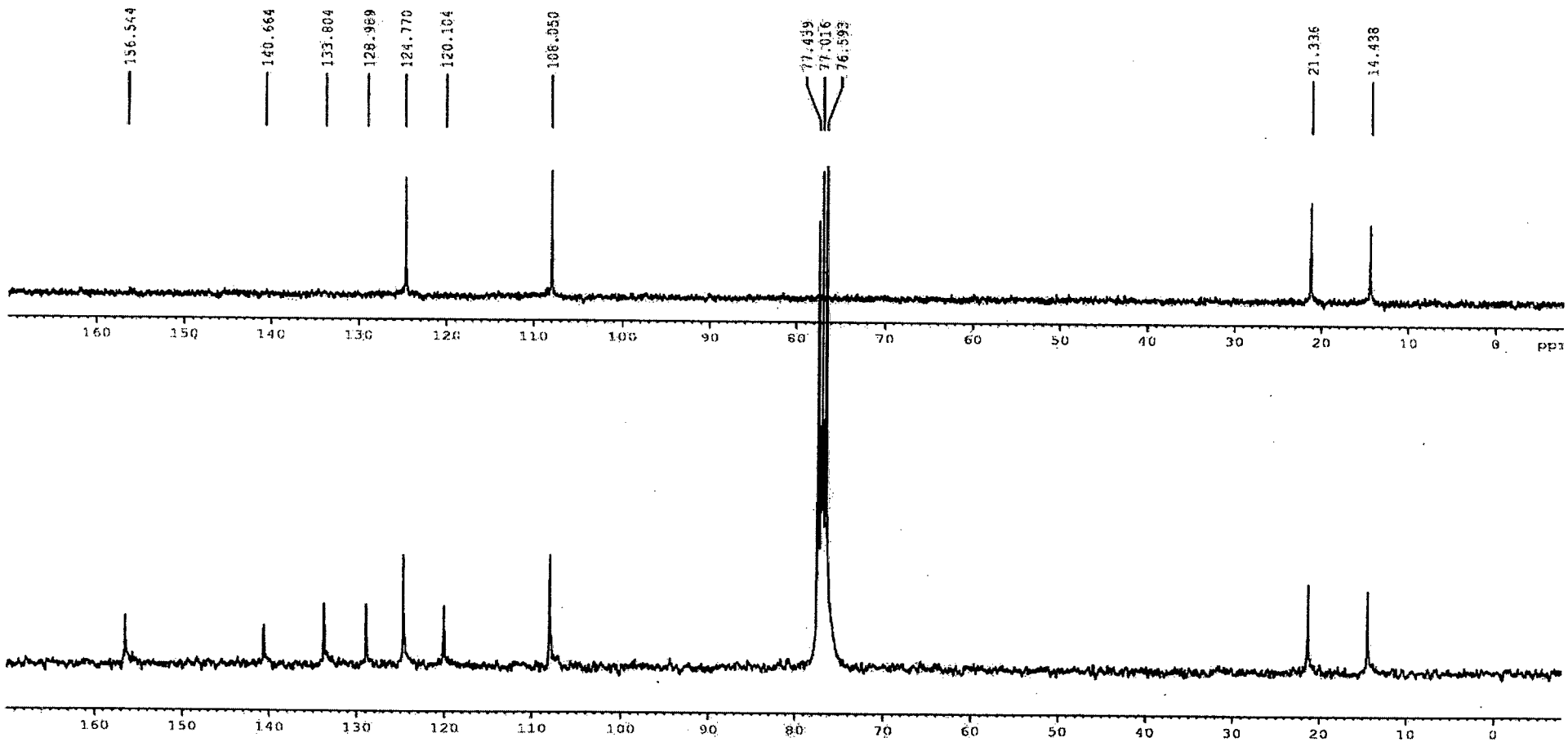


Fig.3c: ^{13}C NMR spectrum of 5, 7-dimethyl benzoxazoline-2-one

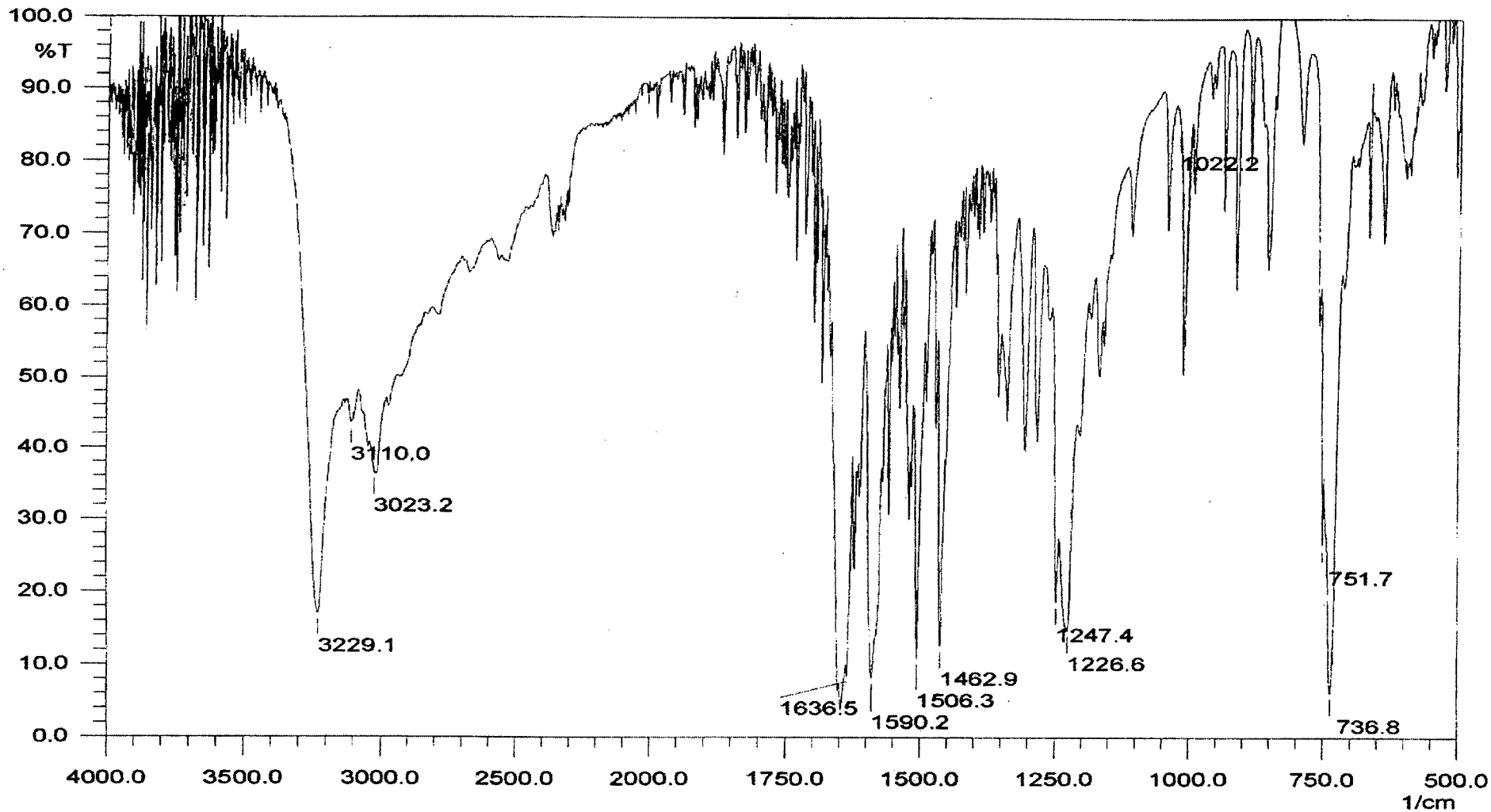


Fig.4a: IR spectrum of Benzoxazoline-2-thione

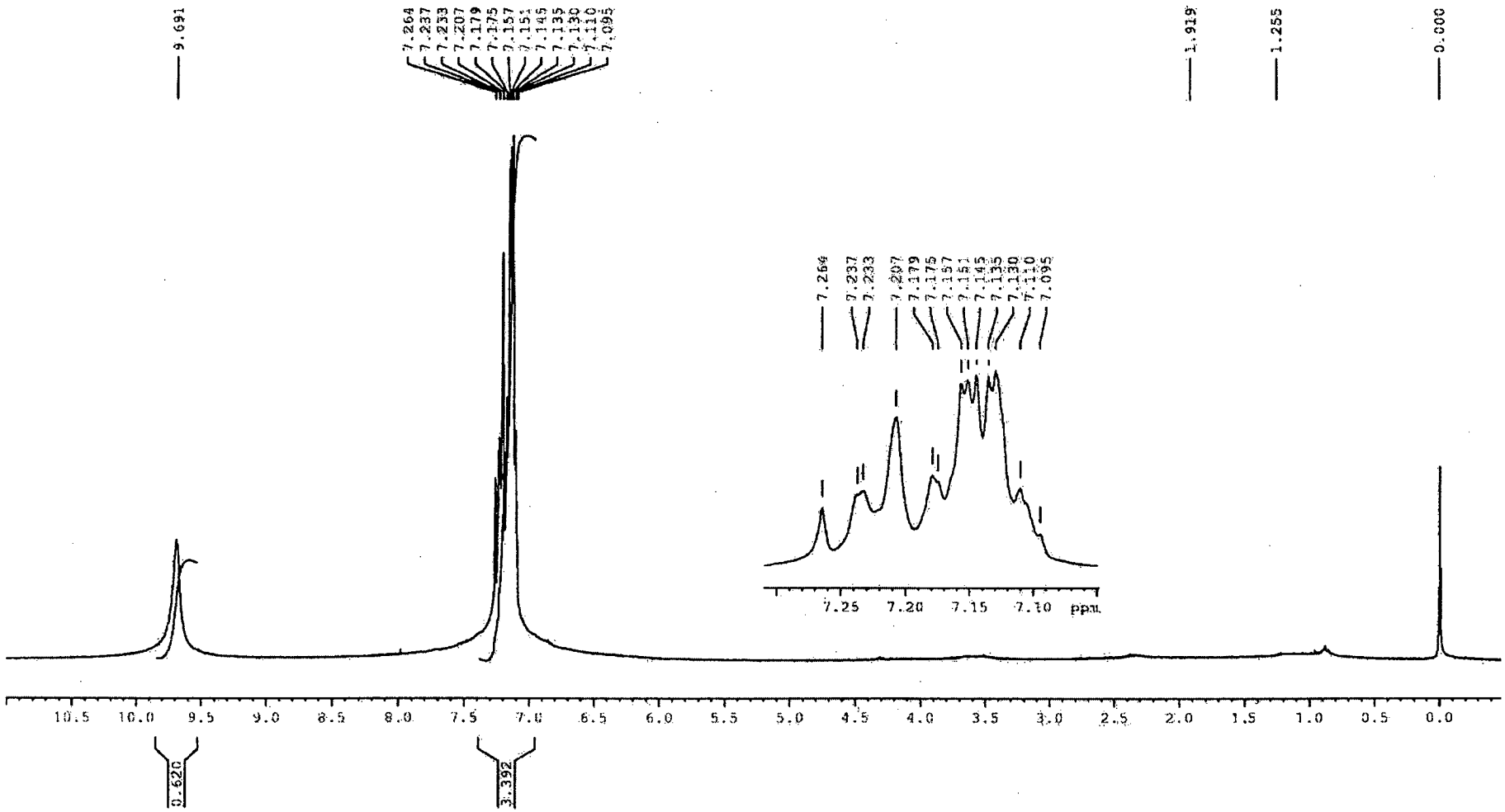


Fig.4b: ^{1}H NMR spectrum of benzoxazoline-2-thione

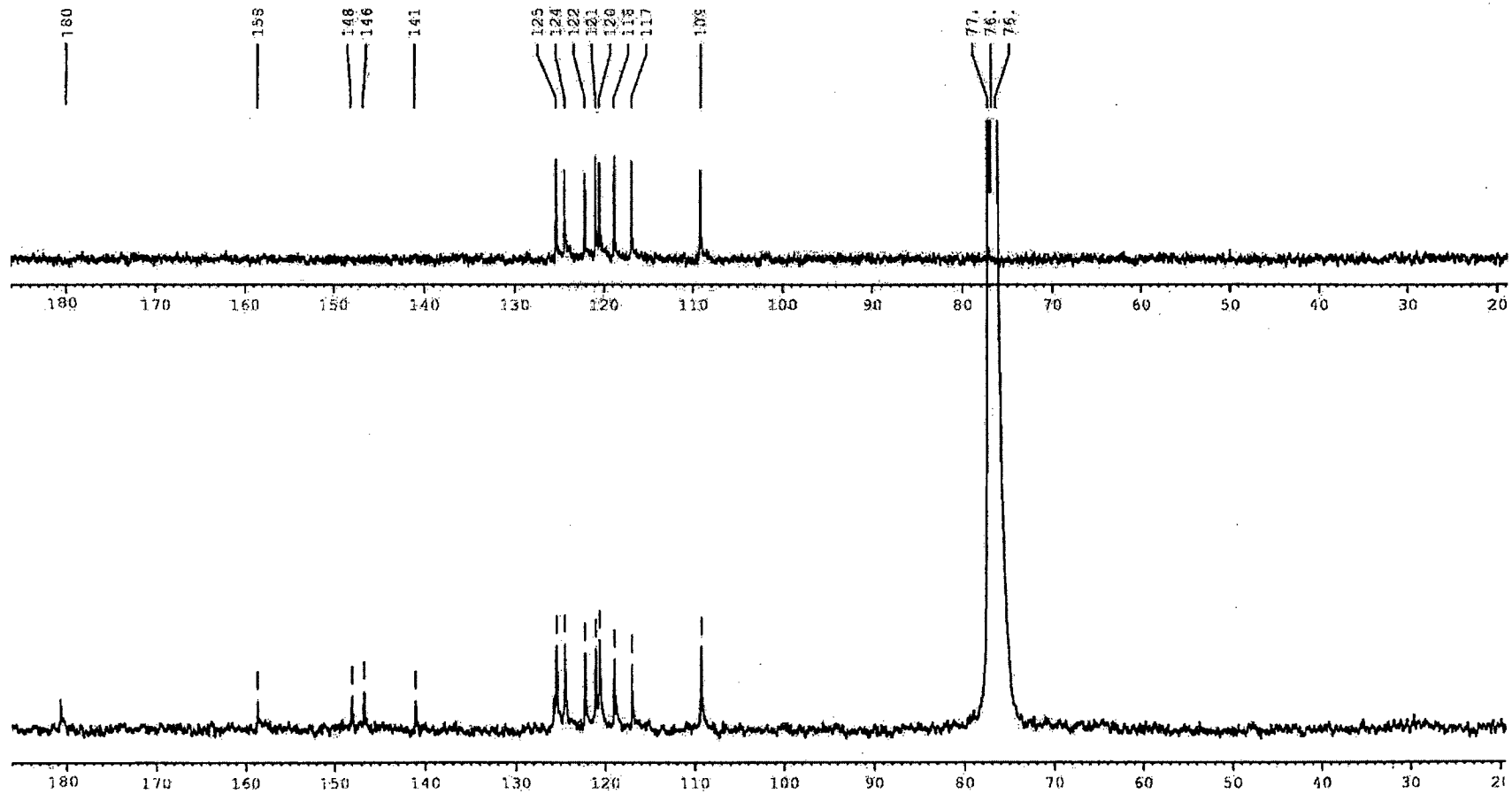


Fig.4c: ^{13}C NMR & DEPT spectra of benzoxazoline-2-thione

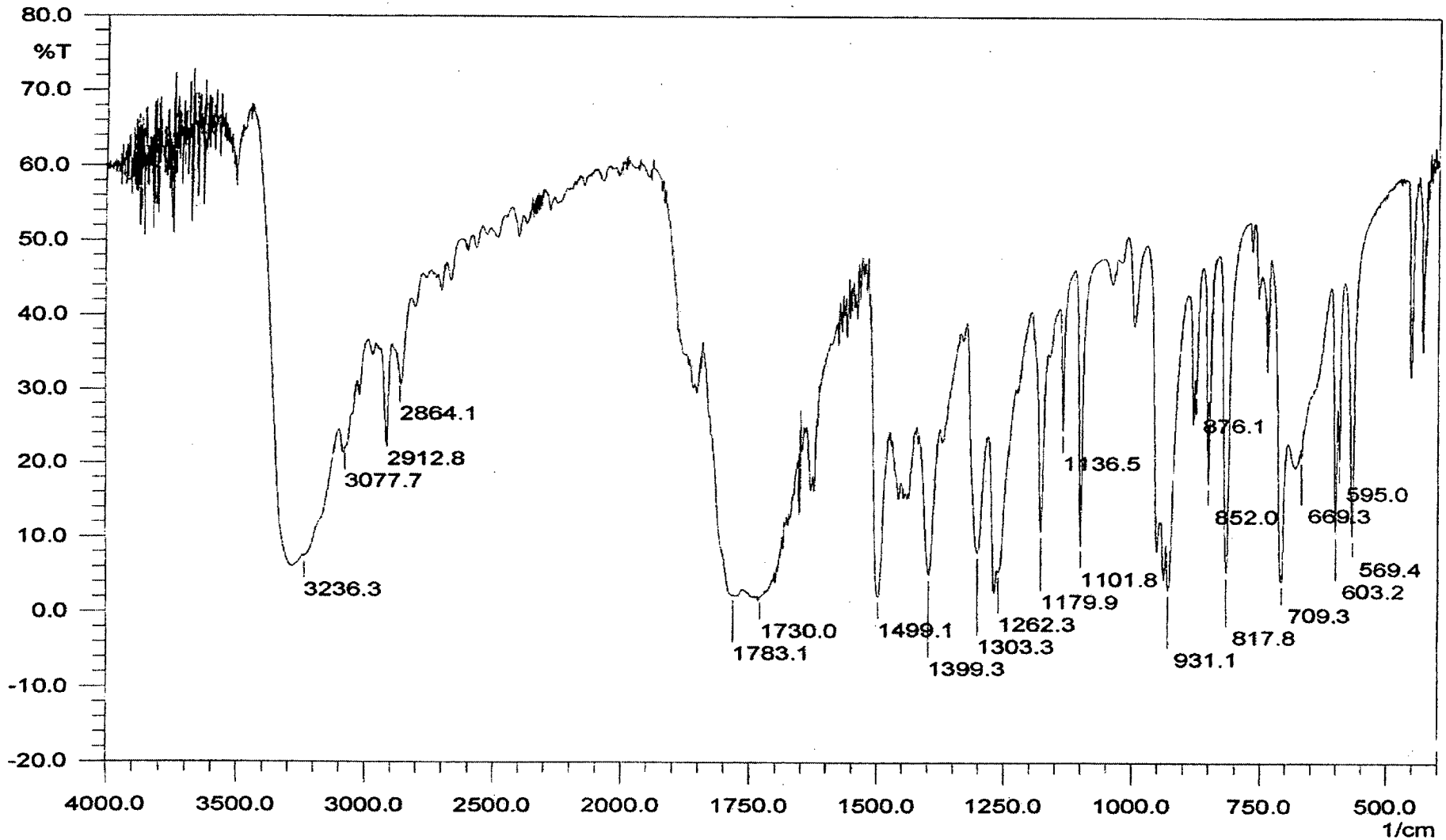


Fig.5a: IR spectrum of 6-methylbenzoxazoline-2-one

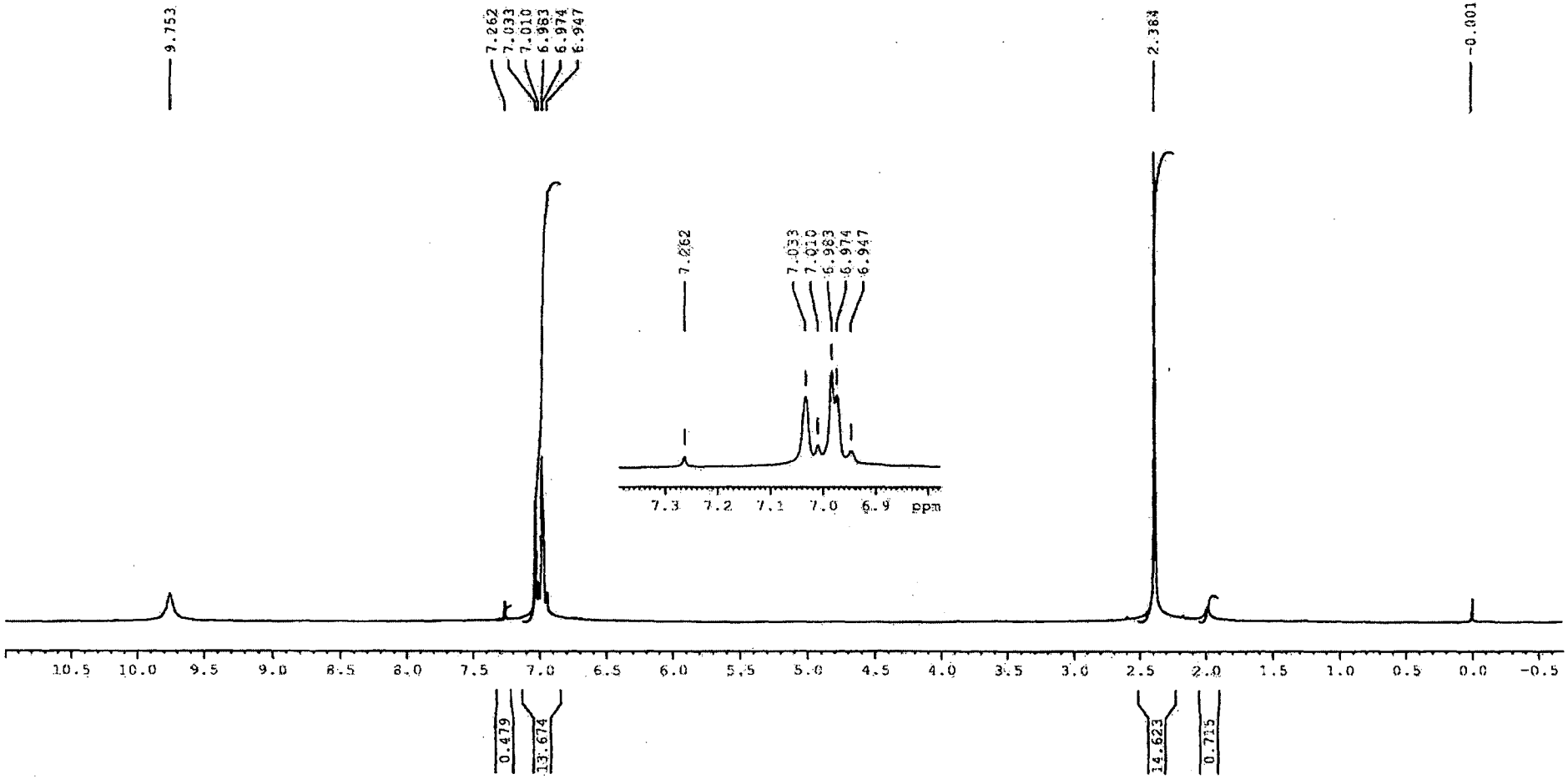


Fig.5b: ^{1}H NMR spectrum of 6-methyl benzoxazoline-2-one

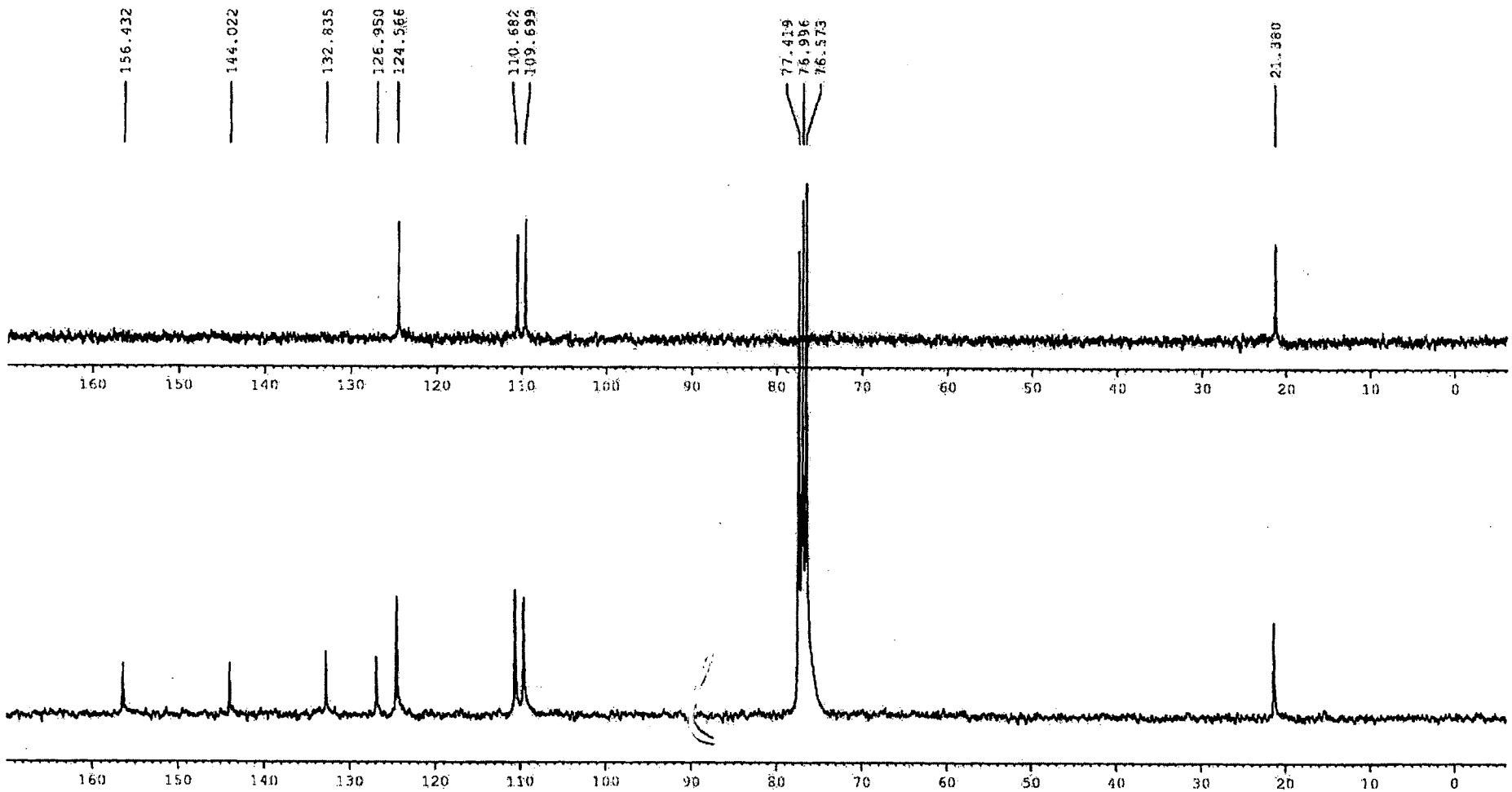


Fig.5c: ^{13}C NMR & DEPT spectra of 6-methyl benzoxazoline-2-one

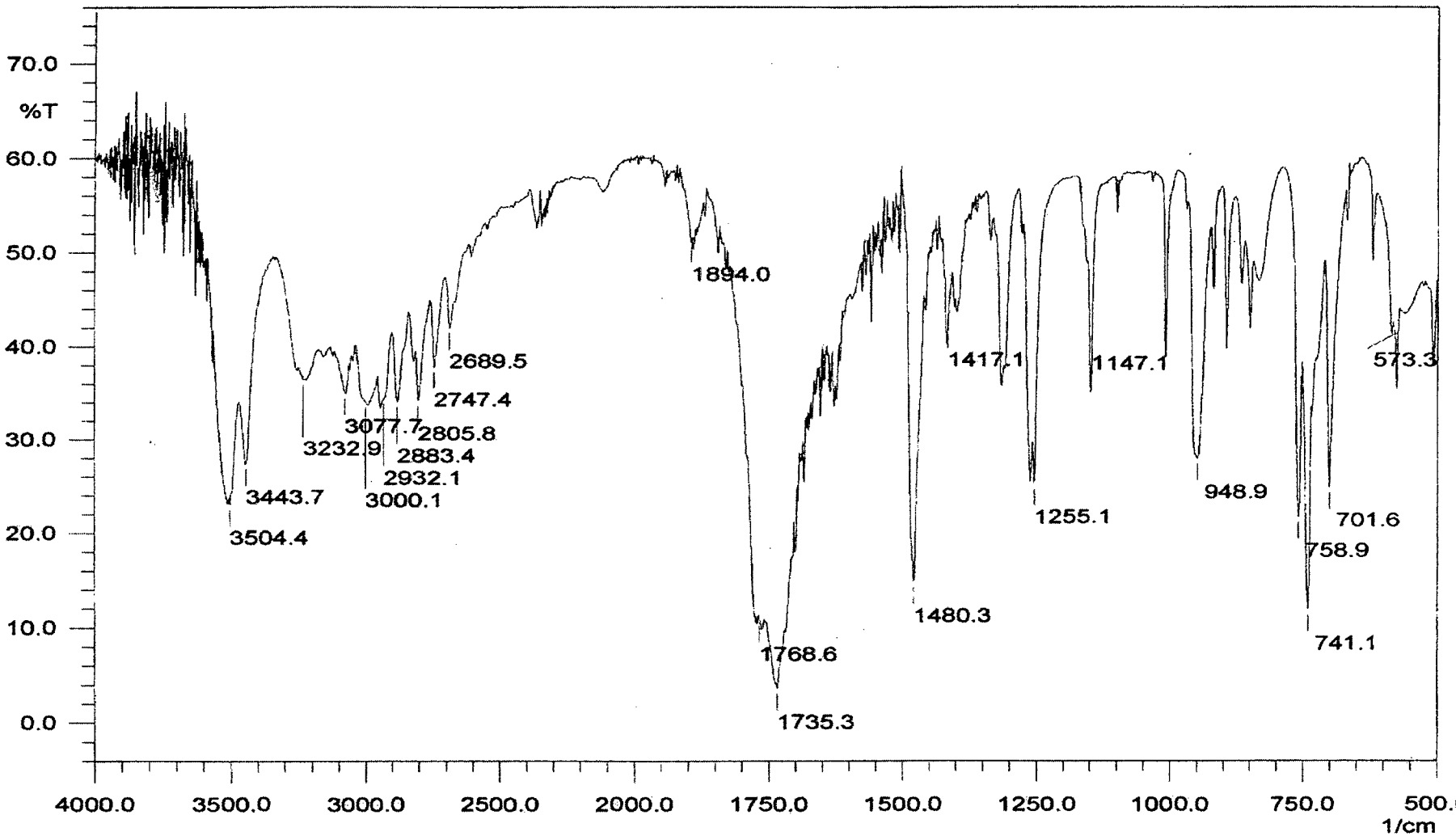


Fig.6a: IR spectrum of N-methylbenzoxazoline-2-one

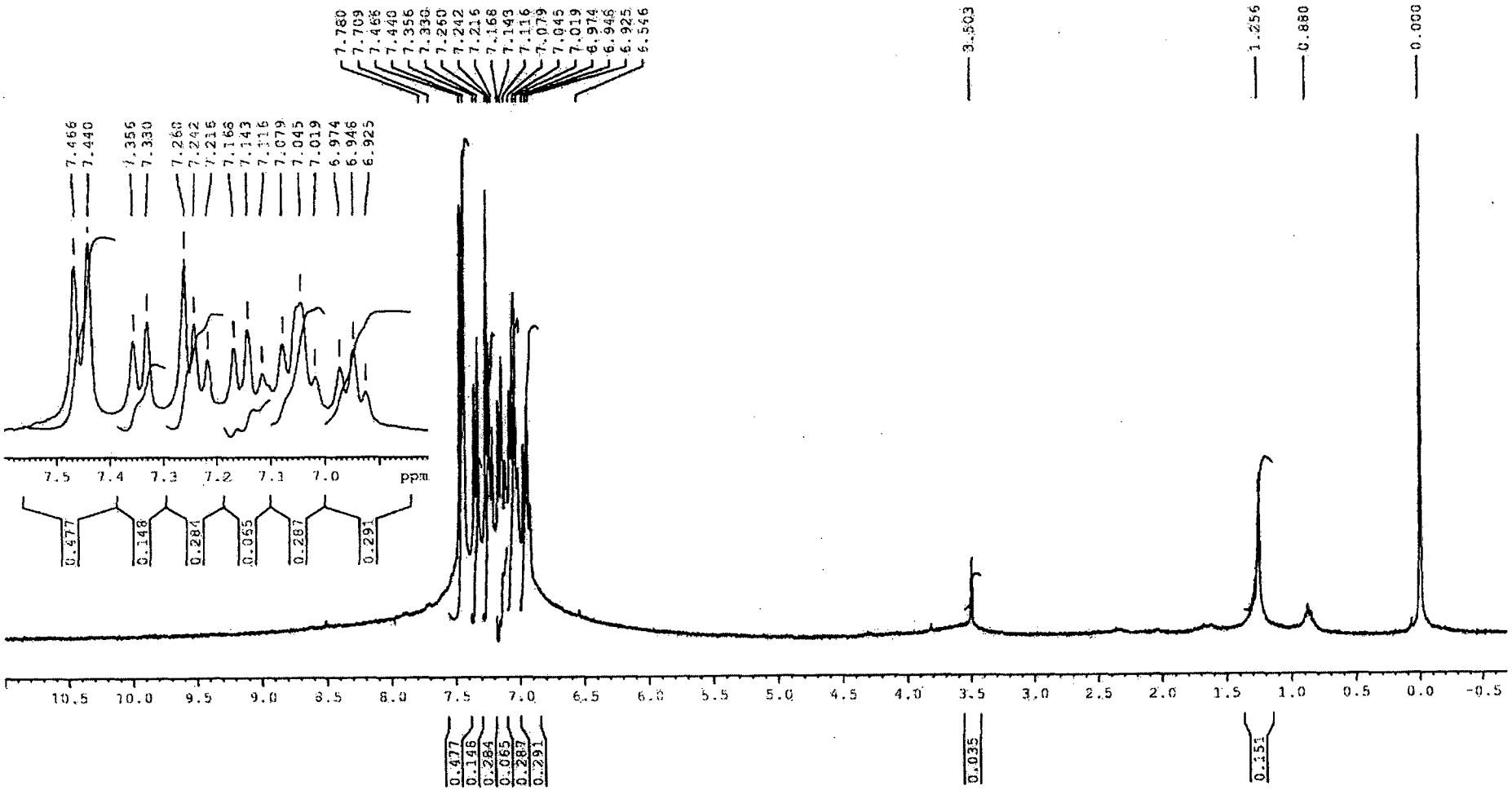


Fig.6b: ^{1}H NMR spectrum of N-methylbenzoxazoline-2-one

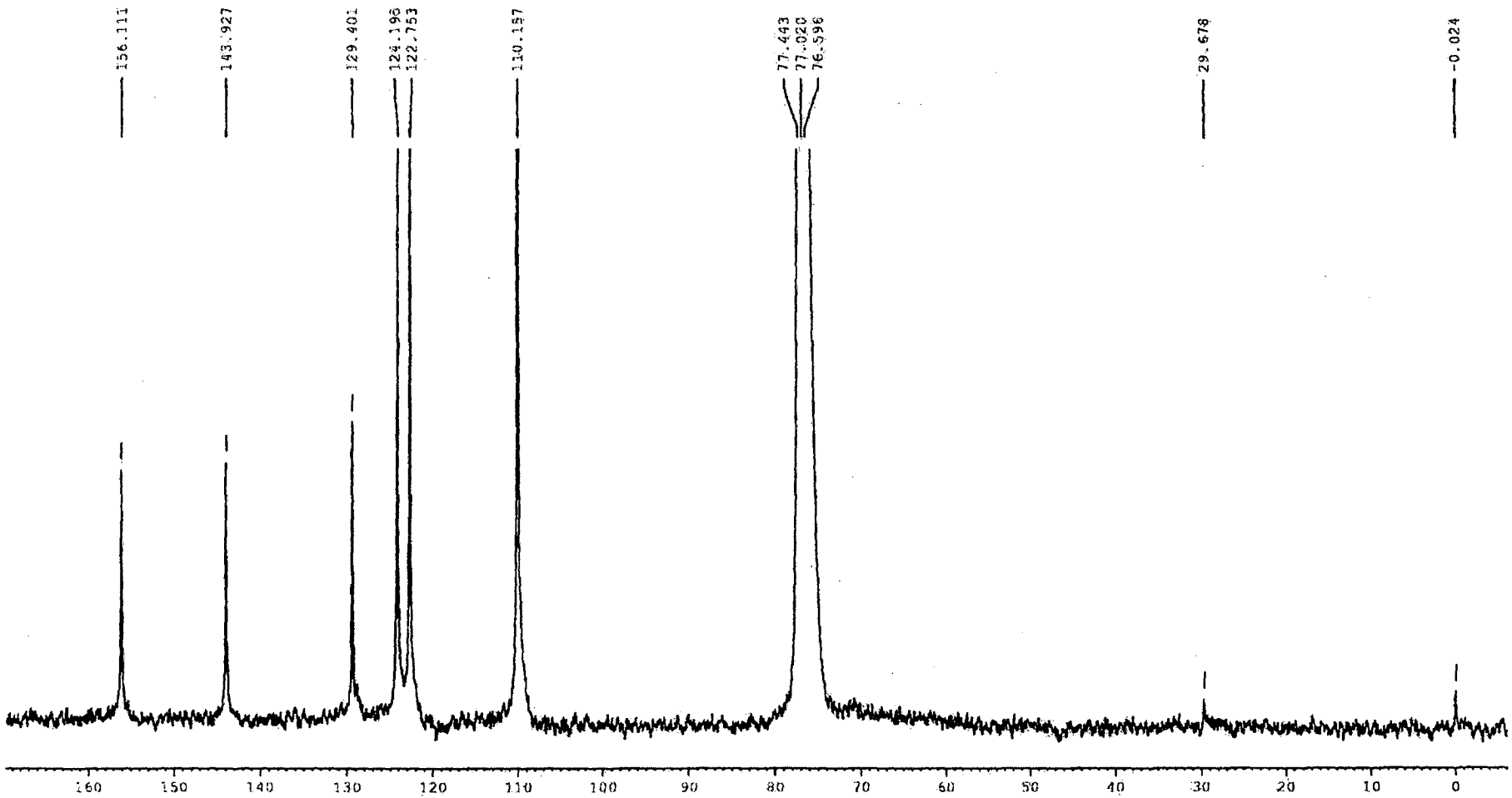


Fig.6c: ^{13}C NMR spectrum N-methyl benzoxazoline-2-one

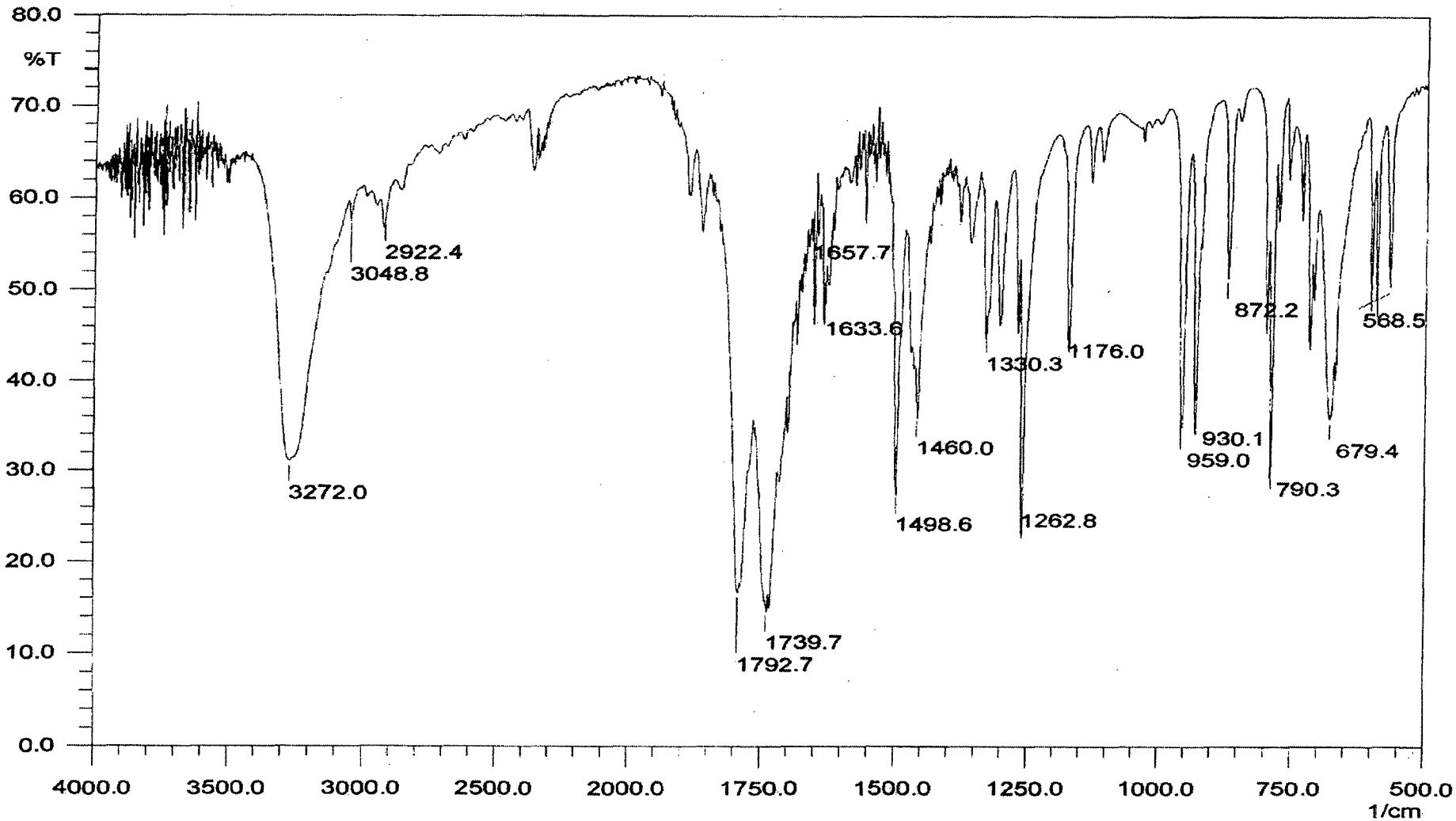


Fig.7a: IR spectrum of 5-methylbenzoxazoline-2-one

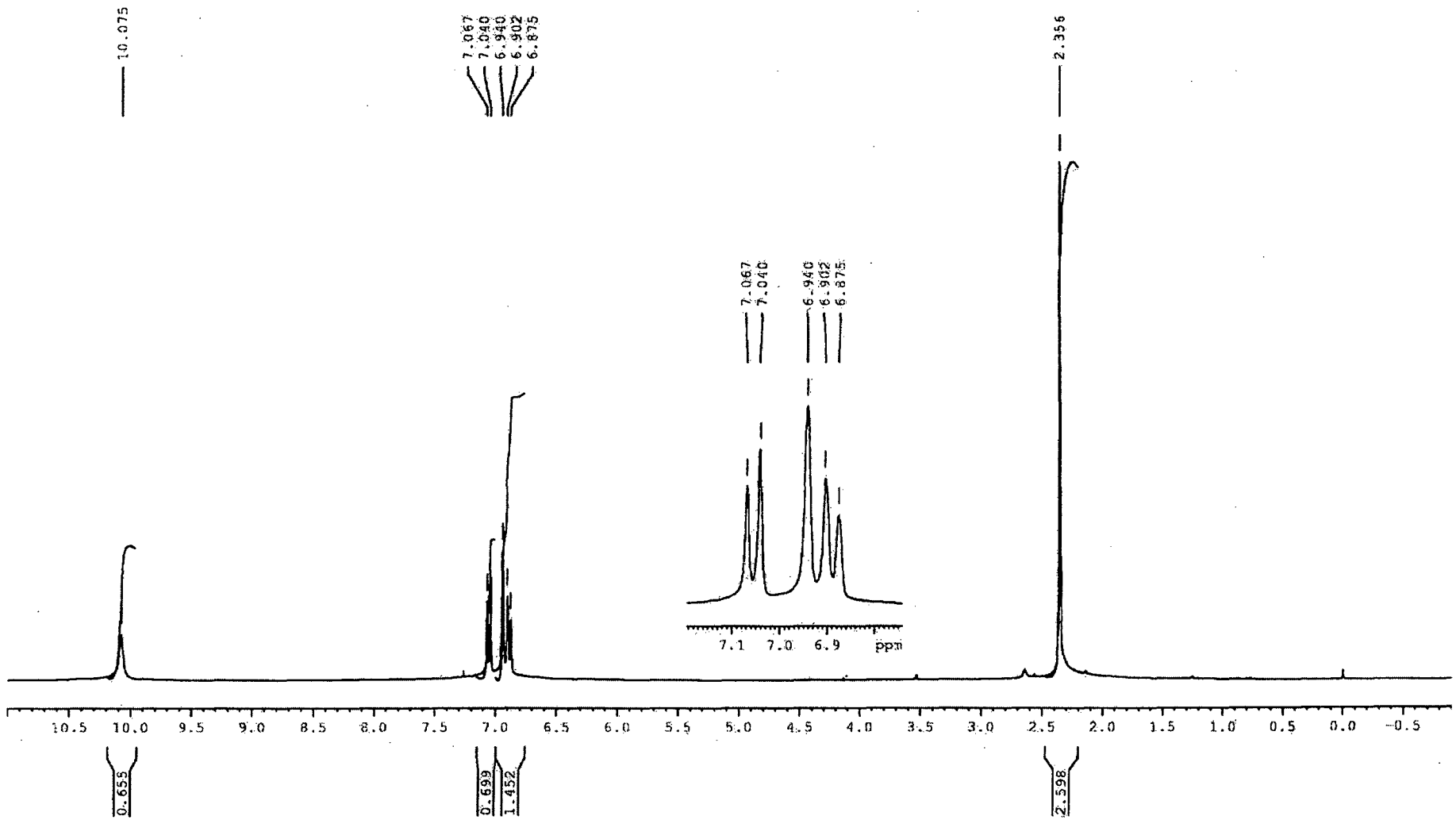


Fig.7b: ^{1}H NMR spectrum of 5-methyl Benzoxazoline-2-one

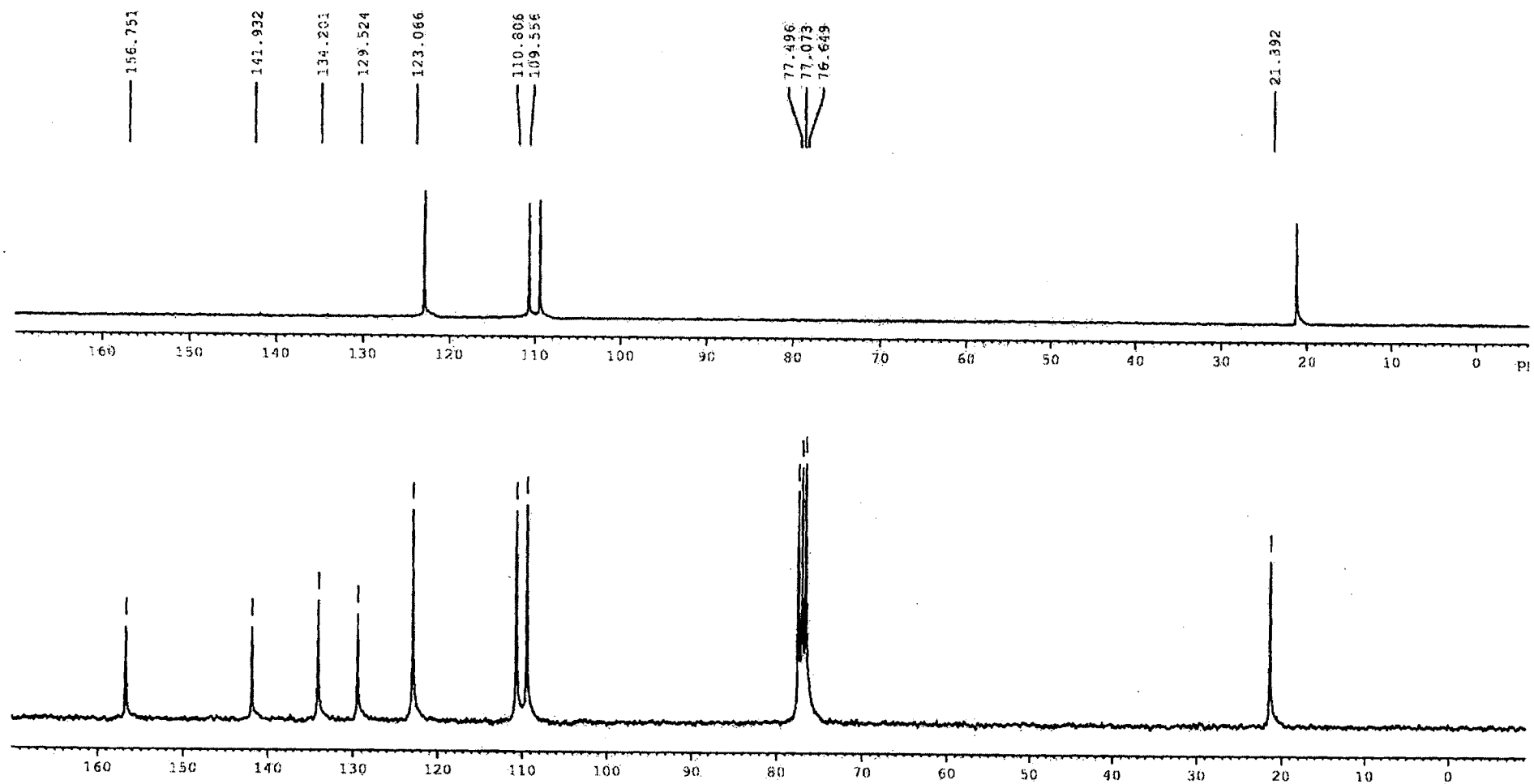


Fig.7c: ^{13}C NMR & DEPT spectra of 5-methyl Benzoxazoline-2-one

Table1: Melting point, Infra red and ^{1}H NMR spectral data of Benzoxazoline-2-one derivative

Compound	Melting point (°C)	IR (cm^{-1})		^{1}H NMR (δ)		
		Lactam (C=O)	NH	H4-H7	NH	Substituents
Benzoxazoline-2-one **(1)**	138-139	1765	3210	7.1-7.3	9.88	
6-chlorobenzoxazoline-2-one **(2)**	123-124	1778	3242	7.02-7.25	9.4	
5,7-dimethylbenzoxazoline-2-one **(3)**	177	1775	3038	6.75-6.726	9.6	2.337, 2.329
Benzoxazoline-2-thione **(4)**	156-58	1636	3229	7.09-7.26	9.6	
6-methylbenzoxazoline-2-one **(5)**	146-147	1783	3236	6.94-7.0	9.75	2.38
N-methylbenzoxazoline-2-one **(6)**	137-138	1768	-			3.5
5-methylbenzoxazoline-2-one **(7)**	127-128	1792	3272	7.1-6.92	9.95	2.35

Table 2: ^{13}C NMR spectral data of Benzoxazoline-2-one derivative

Compounds	^{13}C NMR (δ)							
	Lactam (C=O)	C-4	C-5	C-6	C-7	C-8	C-9	Substituents
Benzoxazoline-2-one **(1)**	156.2	122.7	124	110.1	110.1	143.9	129.4	
6-chlorobenzoxazoline-2-one **(2)**	156.2	122.7	124	128.1	110.1	143.9	129.3	
5,7-dimethylbenzoxazoline-2-one **(3)**	156.5	124.7	120.1	108	128.9	140.6	133.8	21.3, 14.4
Benzoxazoline-2-thione **(4)**	180.2	122.7	124	110.1	110.1	143.9	129.4	
6-methylbenzoxazoline-2-one **(5)**	156.4	124.5	109.7	126.9	110.6	144	132.8	21.3
N-methylbenzoxazoline-2-one **(6)**	156.1	124.1	122.7	110.1	110.1	143.9	129.3	29.6
5-methylbenzoxazoline-2-one **(7)**	156.7	123.0	129.5	109.5	110.8	141.9	134	21.3

Table 3: Leishmanicidal activity of Benzoxazoline-2-one

Parasites/compounds	Activity (%)
Promastigotes +Benzoxazoline-2-one	100
Promastigotes +6-chlorobenzoxazoline-2-one	35.4
Promastigotes +5,7-dimethylbenzoxazoline-2-one	47.5
Promastigotes +6-methylbenzoxazoline-2-one	Toxic to cell layers
Promastigotes +5-methylbenzoxazoline-2-one	56.4
Promastigotes +N-methylbenzoxazoline-2-one	21.27
Promastigotes +Benzoxazoline-2-thione	28.32
Promastigotes +Pentamidines (Standard)	99.5

Table 4: Anti bacterial activity of BOA and its analogs

Compounds	*E. coli*	*Sh. flexineri*	*Ps. aeruginosa*	*Klebsiella*	*S. aureus*	*S. typhi*	*V. cholerae*
Benzoxazoline-2-one	-	+ (<1mm)	-	+ (1-2 mm)	-	-	-
6-chloroBenzoxazoline-2-one	-	+ (2 mm)	-	+ (1-2 mm)	-	-	-
5,7-dimethyl Benzoxazoline-2-one	-	+ (3 mm)	-	+ (1-2 mm)	-	-	+ (2 mm)
Benzoxazoline-2-thione	-	+ (1mm)	-	+ (1-2 mm)	-	-	-
6-methyl Benzoxazoline-2-one	-	+ (1-2 mm)	-	-	-	-	-
N-methyl Benzoxazoline-2-one	-	+ (2 mm)	-	-	-	-	-
5-methyl Benzoxazoline-2-one	+ (<1mm)	+ (2 mm)	-	+ (2 mm)	-	-	+ (<1mm)
Standard (Streptomycin)	7-8 mm	7mm	3mm	6mm	2mm	1-2mm	1-2mm

Table 5: Anti bacterial activity of BOA and its analogs against Multi Drug Resistant Strains.

Compounds	*S. pyogenes*	*Acinetobacter*	*Sal. typhi*
Benzoxazoline-2-one	+ (1mm)	-	+ (<1mm)
6-chlorobenzoxazoline-2-one	-	-	+ (<1mm)
5,7-dimethylbenzoxazoline-2-one	-	-	+ (<1mm)
Benzoxazoline-2-thione	+ (<1mm)	-	+ (<1mm)
6-methylbenzoxazoline-2-one	+ (<1mm)	-	-
N-methylbenzoxazoline-2-one	-	-	-
5-methylbenzoxazoline-2-one	-	-	-
Standard (streptomycin)	8mm	4-5mm	4-5mm

Table 6: Anti fungal activity of BOA and its analogs

Compounds	*A. fumigatus*	*Fusarium sp*	*Cry. neoformans*	*A. niger*	*Rhodotorula*	*Nocardia*	*C. albicans*
Benzoxazoline-2-one	-	-	-	-	-	-	-
6-chloroBenzoxazoline-2-one	-	-	-	-	-	-	-
5,7-dimethyl Benzoxazoline-2-one	-	-	-	-	-	-	-
Benzoxazoline-2-thione	-	-	-	-	-	-	-
6-methyl Benzoxazoline-2-one	-	-	-	-	-	+ (2mm)	+ (1mm)
N-methyl Benzoxazoline-2-one	-	-	-	-	-	-	+ (1-2mm)
5-methyl Benzoxazoline-2-one	-	-	-	+ (1-2mm)	-	-	+ (<1mm)
Standard (Nystatin)	-	-	-	-	-	-	-

EXPERIMENTAL:

Melting points were determined on a digital melting point apparatus (Electothermal 9100) and were uncorrected.

Instrumental analysis (IR and NMR) was performed as explained in **Chapter 1, Section II.**

Purity of the compounds was assessed by TLC on Merck silica coated aluminium sheets Si gel 60 F_{254}. Silica gel 60-120 mesh was used for column chromatography.

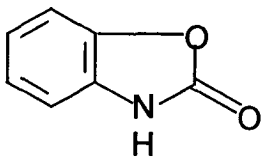
All the chemicals required for synthesis were obtained from Sigma chemicals, USA.

Synthesis of BOA analogs:

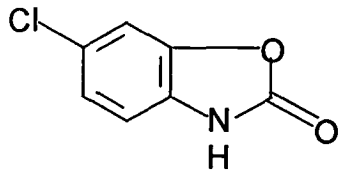
For example, 5-methylbenzoxazoline-2-one **(7)** was prepared by the reaction of 2-amino-4-methyl phenol with urea. 2-amino-4-methyl phenol (2g) and urea (1.2g) were refluxed for 14 hours in dry pyridine. Excess pyridine was removed by distillation under reduced pressure and the residue obtained was column chromatographed over silica gel. Elution of column with ethyl acetate: petroleum ether (20:80) yielded (compound **5**) showing a melting point of 126°C. The compound was purified by repeated chromatography over silica gel till it gave a single spot on TLC (solvent system: i) ethyl acetate: petroleum ether (20:80);

ii) MeOH:$CHCl_3$ (2:98) and spots visualized with iodine. Other analogs were prepared in a similar manner by condensation of respective o-amino phenols with urea or thiourea.

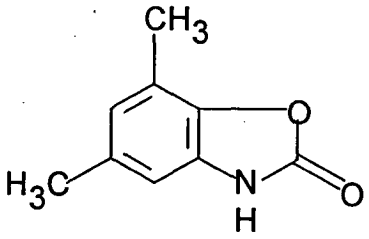
Structures of BOA and its analogs:



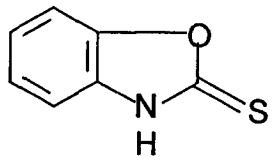
Benzoxazoline-2-one **(1)**



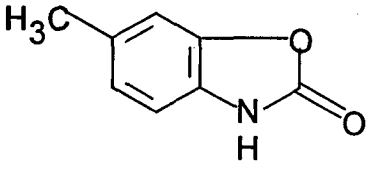
6-chlorobenzoxazoline-2-one **(2)**



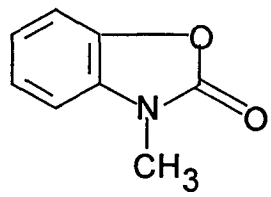
5, 7-dimethylbenzoxazoline-2-one **(3)**



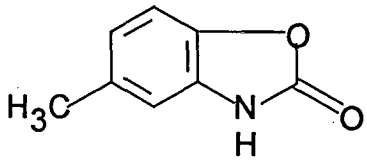
Benzoxazoline-2-thione **(4)**



6-methylbenzoxazoline-2-one **(5)**



N-methylbenzoxazoline-2-one **(6)**



5-methylbenzoxazoline-2-one **(7)**

Bioassay:

The bioassay was performed as explained in **Chapter 1, Section IV**.

Antiprotozoal activity:

The antiprotozoal activity was performed at CDRI*, Lucknow.

Procedure:

Organisms and media:

Human isolates of *Leishmania donovani* non-pathogenic strain UR6 were obtained from the Indian Institute of Chemical Biology, Kolkata, India. Promastigotes in the stationary phase of growth derived from in vitro cultures were used for the assay (Wright & Amin, 1989). The culture was cultivated at 22°C in culture tubes containing as the solid part brain heart infusion- agar (BHI) (supplemented with 10%rabbit blood drawn aseptically) and Hank's balance salt solution (HBSS) (Hi Media Laboratories, Mumbai) as the liquid part. Parasites were passaged every 5 days. Pentamidine was obtained from the Sigma-Aldrich chemicals, USA.

Promastigote parasites in the stationary phase of growth ($1x10^5$ parasite/ml) were inoculated into culture tubes containing BHI-agar as solid part and HBSS as liquid part. To these tubes different concentrations (10 to 100 μg/ml, DMSO) of compounds and

* We thank Dr.Shalini Sharma for tesing the samples for leishmanicidal activity.

pentamidine were added respectively. The inhibitory effect of compounds was compared with pentamidine, which is known to be active against leishmaniasis, and with untreated control cultures. The whole operation was carried out aseptically in an ultra violet chamber and the tubes were incubated for 5 days at 22 °C. The toxicity of DMSO was determined by adding different amounts of solvent (0.1 to 1.5%) to suspensions of promastigotes under the conditions described above. After five days the value of the non-toxic dilution was found to be 0.6% and the growth inhibition (upto 15%) was observed between 0.6% to 1%; therefore, higher concentrations of DMSO were never used. Leishmanicidal activity of each of the compounds was determined by counting the number of live parasites per field microscopically. The LC_{50} values were calculated by *probit analysis* and no mortality was observed in mice when the parent (LC_{50} = 40 μg/ml) molecule was administered (i.p) in the doses ranging from 0.25-1 g/kg. The mice were kept under observation for one week. Since the analogs had less potency its LC_{50} values were not determined.

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Summary

The search for bioactive molecules from marine organisms has taken a new turn with the discovery that the metabolites with the biological activity produced by some of the marine macroorganisms are not their true chemical constituents but derived from the associated microorganisms.

In Chapter I efforts have been made to study the marine fungus, *Aspergillus sulphureus*, isolated from the sediments of sea grass environment for the production of different metabolites, under different culture conditions. The metabolites have been identified on the basis of spectral data. With mycological broth as the culture media it was found to be a new source of kojic acid, an industrially important chemical used in cosmetics and in flavour industry. Triglycerides with saturated and unsaturated fatty acids as their substituents were produced by this filamentous fungus. The importance of polyunsaturated fatty acids is well established with their clinical applications in various diseases including eczema, osteoporosis, some immunological abnormalities, cancer, cardiovascular disease etc. The fungus also yielded ergosterol peroxide and its dihydro derivative. The steroid is well known for its immunosuppressive, antitumor and antiviral activities. There are recent reports of it exhibiting better antioxidant activity as compared to the well known antioxidants α-tocopherol and thiourea.

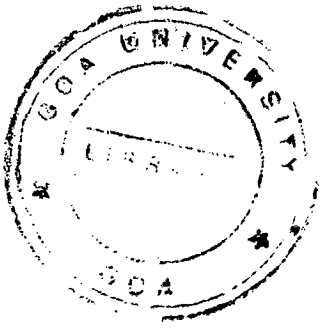
The kojic acid being an industrially important chemical, enhancement of its production, under different culture conditions using different nitrogen source and elicitors have been investigated. Fermentation medium with peptone as the nitrogen source and alginate elicitor was found to be the best producer of the chemical.

During the course of investigations on enhancement of kojic acid peptone bile sorbitol broth was one of the commercial medium tested. Although, the fungus under the conditions of the medium did not yield, kojic acid, but a new antifungal metabolite related to cyclopiazonic acid was obtained. Cyclopiazonic acid, a specific inhibitor of Ca^{2+}-ATPase in the intracellular Ca^{2+} storage sites, is a metabolite reported from *Aspergillus tamarii* and *Penicillium cyclopium.*

Organisms in the marine environment are constantly under stress due to pollution caused by the dumping of industrial waste, oil spills etc. Polycyclic aromatic hydrocarbons (PAHs) are one of the most toxic organic pollutants, which enter the marine environment via various anthropogenic sources. The second chapter of the thesis deals mainly with the studies made on the synergistic effects of PAH and other environmental stresses like elevation of nitrites on the estuarine fish, *Oreochromis mossambicus.* Damage caused to DNA of the fish has been evaluated by Comet assay. This damage is associated with the formation of DNA adducts which are biomarkers of pollution. These

adducts have been identified using the latest ESI-MS technique. An attempt to combat the above effects of PAH by the use of phytogenic surfactants has been dealt in this chapter.

Resistance of microbes to the existing drugs has led to the study of benzoxazoline-2-one and its analogs for their antiprotozoal and antimicrobial activities. These studies are reported in the third chapter. None of the analogs were as effective as the parent molecule benzoxazoline-2-one, when tested for leishmanicidal activity. Benzoxazoline-2-one being as effective as pentamidine the only drug, though toxic, being used clinically due to non availability of better drugs. None of the compounds exhibited promising antimicrobial activity.



PUBLICATIONS AND PRESENTATIONS

Publications:

1. Solimabi Wahidulla, **Rani Rajamanickam** and Lisette D'Souza (2002) Metabolites from marine fungus *Aspergillus sp.* Sree, A. Rao, Y. R., Nanda, B & Mishra, B. N (Eds) In: *Proceedings of the Symposium: Utilization of bioresources.* Pp.464-469.

2. **Rani Rajamanickam**, M.S. Shailaja, and Solimabi Wahidulla (2004) Biliary PAH metabolites and DNA damage in estuarine fish *Oreochromis mossambicus* exposed simultaneously to chrysene and nitrite. *Extended Abstracts:* International Workshop on Marine Pollution and Ecotoxicology. Pp. 218-220

3. **Rani Rajamanickam**, M.S. Shailaja, and Solimabi Wahidulla
   Biliary PAH metabolites and DNA damage in estuarine fish *Oreochromis mossambicus* exposed simultaneously to chrysene and nitrite.

   (**Ecotoxicology** (I.F=1.468) *in press*)

Presented paper at the

1. **National Conference on Utilization Of Bioresources (NATCUB 2002),** October 24-25, 2002, Organised by RRL (CSIR) & I IChE Bhubaneshwar, India.

   Title of the paper: Metabolites from marine fungus *Aspergillus sp.*

2. **International Workshop on Marine Pollution and Ecotoxicology.** 25-26 February, 2004, organized by National Institute of Oceanography, Dona Paula, Goa, India in association with International Water Association, UK.

   Title of the paper: Biliary PAH metabolites and DNA damage in estuarine fish *Oreochromis mossambicus* exposed simultaneously to chrysene and Nitrite.

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