

ISOLATION AND STRUCTURAL ELUCIDATION OF THE SECONDARY
METABOLITES OF *TRICHODERMA* SPECIES; POSSIBLE BIOLOGICAL
ANTAGONISTS AGAINST THE *ARMILLARIA MELLEA*

BY

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A thesis submitted in partial fulfilment for the Degree of
Master of Science of Kenyatta University.

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Isolation and
structural



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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with my approval as University Supervisor.

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DEDICATION

To my mother and Sheikh Abdillahi Nassir for their ever encouraging support.

His private attention to the matters relating to this work is highly appreciated.

Special thanks should however go to Richard Munn formerly of the University of Manchester, Institute of Science and Technology, Bolton for his contribution to the acquisition of equipment.

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ABSTRACT

The thesis describes the extraction, isolation and structural elucidation of the secondary metabolites of *Trichoderma* isolates (*T. longibrachiatum* Rifai and *T. harzianum* Rifai).

The *Trichoderma* culture broths were extracted with n-hexane, diethyl ether and ethyl acetate successively. The mycellium of *T. harzianum* was extracted by the above mentioned solvents and also with methanol. The crude hexane and diethyl ether extracts of *T. longibrachiatum* showed small but significant effects against the rhizomorphal vegetative radial growth of *A. mellea*, a causal agent of the disease *Armillaria* root rot in tea plants. The crude ether, hexane mycellium and methanol extracts of *T. harzianum* also showed small but significant bioactive effects against the mycelial radial growth of *A. mellea*.

Six purified substances were isolated from the various solvent extracts of *T. longibrachiatum* and *T. harzianum*. The first substance was positively identified as 1,2-dinonyl phthalate whilst the second substance was partially identified as a long chain saturated hydrocarbon which may contain cyclic structures. The third substance was positively identified as butanedioic (succinic) acid. The fourth and fifth substances were partially identified as unsaturated monocarboxylic acids. The sixth was an

interesting crystalline substance whose structure was not elucidated due to the puzzling spectral data.

Structural elucidation of the compounds were determined by the spectral data analysis. ^1H -nmr and ^{13}C -nmr chemical shifts led to the formulation of the molecular structures which were confirmed by the characteristic fragmentation pattern shown in the mass spectral data. Functional groups were inferred from the ir data.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

The tea plant, *Camellia sinensis* (L) O. Kuntze is a very important cash crop in the agriculturally based economy of Kenya, as it is not only an earner of foreign exchange but also provides livelihood to more than a million people¹.

There are a number of diseases affecting the tea plant but the most prevalent in Kenya (as well as in other tea growing countries) is the *Armillaria* root rot caused by the fungus *Armillaria mellea* Vahl ex Fr.².

1.1 *Armillaria mellea* Vahl ex Fr.

A. mellea is a fungus which constrains tea production as it is the cause of *Armillaria* root rot, one of the major diseases affecting many tea species and fruit trees through out the world³. *A. mellea*, though a destructive pathogen is known to have a low rate of spread⁴ and spreads mainly by means of rhizomorphs, mycellia and root contact with indigenous host roots.

Rhizomorphs, which are also referred to as boot laces and are either black or brown, are important in the epidemiology of *Armillaria* root rot^(5,6) as they are responsible for the underground spread of the fungus^(7,8). As for the mycellia, its transfer by root contacts

constitute a second means of spread of *A. mellea* particularly in areas where rhizomorphs are rare or absent⁹.

1.2 Current methods used in the control of Armillaria root rot

In plantations, the distribution of *Armillaria* root rot is concentrated in distinct patches and the pattern of spread is predominantly radial, emanating from the source of infection¹⁰. A tea bush infected with the fungus is usually yellow and shorter than the adjacent healthy bushes. *A. mellea* causes the greatest problem when new areas of land are prepared for tea planting. A lot of effort has been made to minimise the spread of *Armillaria* root rot such as avoidance of sites with high incidence¹¹, cultural practices, biological control, removal of all substrates from the soil by uprooting and destroying all root remnants,¹² ring barking of forest trees (by which ~~new~~ experienced personnel) for a period of 1 to 2 years prior to tea planting and chemical application of soil fumigants such as Armillatox, a phenolic emulsion containing 48% of active ingredient¹³ and other fumigants such as sodium pentachlorophenate, methyl bromide, Votex chloropicrin carbon disulphide and Vapam¹⁴ have been extensively used against *Armillaria* root rot. Although the above have been extensively tried, very few have proved both highly successful and convenient as a result of economic constraints and the complexity of the soil environment¹⁵

especially in the application of the volatile fumigants such as liquid methyl bromide.

In the sixties, the presence of a soil toxin from the savanna regions of Zimbabwe was reported. The toxin, which is a partially water soluble, thermo-labile substance, inhibited the growth of *Armillaria* rhizormorphs but the nature and origin of the inhibitor was not determined due to the difficulties of characterisation at the time¹⁶.

Soil fungi, *Trichoderma* have been reported to act as biological control agents against *A. mellea* as well as other soil borne plant pathogens. The proliferation of *Trichoderma* species can be enhanced by organic amendments such as coffee pulp and husks in the ratio of 10:1¹⁷. The biocontrol activity could be due to the production of secondary metabolites by the *Trichoderma* species which have antagonistic effects against pathogenic fungi and microbial organisms. The control could also be due to the increase in competition for soil nutrients as a result of the increase in production of *Trichoderma* species which predominate over *A. mellea*. *Trichoderma* species as biological control agents may in future prove to be both environmentally sound and economically sustainable in minimising the spread of the tea disease, *Armillaria* root rot.

Some secondary metabolites derived from *Trichoderma* species have previously been reported to significantly inhibit both mycelial and rhizomorphal growth of *A. mellea* as well as limit significantly the biomass accumulation of *A. mellea* and can thus be potentially useful for both control and management of *Armillaria* root rot¹⁷. Also many metabolites derived from *Trichoderma* species have been shown to be active against a wide range of fungi and microbial organisms and these observations have in part prompted the present study.

1.3 TRICHODERMA SPECIES

The genus *Trichoderma* which is present in the majority of soils is one of the commonest and widespread genera of fungi but their taxonomical classification is difficult resulting in the development of a species aggregate system of classification which groups together several similar species.

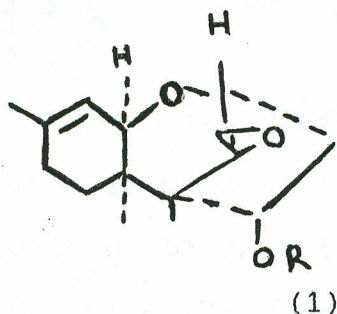
Isolates belonging to a distinct species aggregate may be morphologically similar but behave quite differently under different growth conditions. It is thus not surprising that there is no clear pattern among the wide variety of secondary metabolites produced by *Trichoderma* strains¹⁸.

Examples of classes of metabolites are sesquiterpenes, isonitrile diepoxides, isonitrins, cyano, alkyl pyrones, unsaturated monobasic acids and polypeptides.

A number of the *Trichoderma* species (*T. koningii* Oudem, *T. viride* Pers ex Fries, *T. longibrachiatum* Rifai, *T. harzianum* Rifai, *T. polysporum* Link ex Pers, and *T. hamatum* (Ben) Bain, have been associated with mycoparasitism of a wide range of soil borne plant pathogens (*Bacillus subtilis*, *Fomes annosus*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Mycena citricolor*) as *Trichoderma* are able to multiply on and colonize the plant surface and produce both non-volatile as well as volatile metabolites some of which can inhibit the growth of pathogenic fungi¹⁸. The use of the soil fungus *Trichoderma* for biological control of micro organism on pruning wounds has been reported to be successful because the growth of *Trichoderma* is favoured by the presence of readily available nutrients¹⁹ and the resultant increase in *Trichoderma* population competes effectively with the small population of pathogenic organisms. This is illustrated by the use of *T. harzianum* and its mutants in the control of damping off induced by *Pythium aphanidermatum* in peas, cucumber, tomatoes and pepper²⁰.

Considerable work has been done on some of the isolates of *Trichoderma* and have shown that *Trichoderma* species produce both non-volatile and volatile antibiotics which inhibit the growth of pathogenic fungi thus playing a crucial role in microbial antagonism²¹.

Trichodermin, (1) $C_{17}H_{24}O_4$, melting point (m.p) 45-46°C is a stable sesquiterpenoid metabolite of *T. viride* and *T. polysporum*.



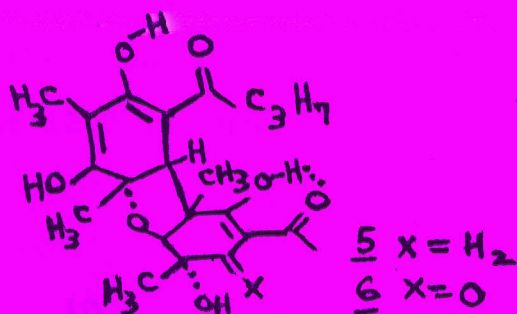
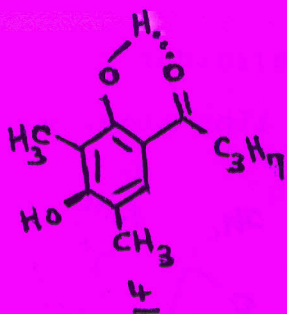
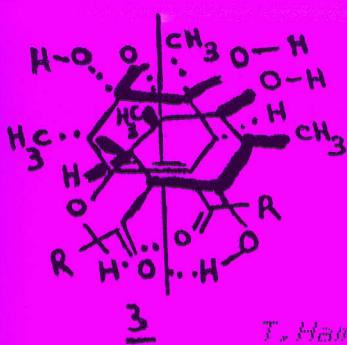
Trichodermin 1 R = acetyl

Trichodermol (2) R = H

Trichodermin, has been shown to have antibiotic activity against a variety of pathogenic fungi^(22, 23) including *Candida albicans* which it inhibits in low concentrations. The alcohol formed by the hydrolysis of (1) produces a sesquiterpene alcohol Trichodermol (2) $C_{15}H_{22}O_3$, m.p 118°C.

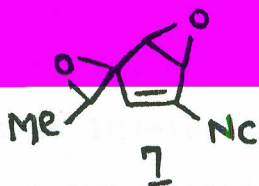
Trichodimerol (3), whose molecular formula is $C_{28}H_{32}O_8$ is an unusual natural product possessing a proper axis of symmetry and is produced by *T. longibrachiatum*. It is reported to be antagonistic to the fungus *Mycena citricolor*, a causal agent of the American leaf spot disease of coffee¹⁸.

Sorbicillin (4), bisvertinol (5), bisvertinolone (6) and the four polyketides trichodermolide, sorbiquonol, bislongiquinolide and 5-hydroxyvertinolide are also produced by the yellow *T. longibrachiatum* Rifai aggr. together with trichodimerol (3)¹⁸.

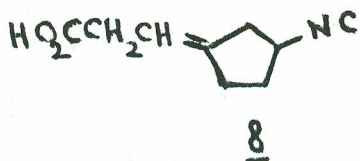


T. Hamatum produces metabolites that are

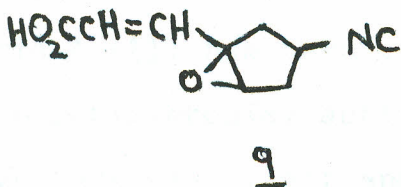
characterised by an iso cyano functionality. 3'-Methyl spiro (6-oxabicyclo [3,1,0] hexa-3-en-2, 2'-oxylan)- 4-ylisocyanide (7) which is also an isonitrile diepoxide.



and 3', (3-isocyanocyclopent-2-enyllic diene) propionic acid (8) which is also produced by all wild isolates and inhibits the growth of *Micrococcus luteus* and has antibiotic activity against rumen bacteria.

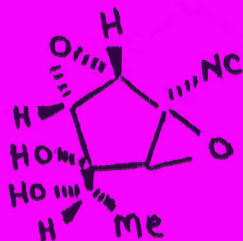


and lastly 3-(3-isocyano-6-oxabicyclo [3,1,0] hex-2-en-5-yl) acrylic acid (9)²³.



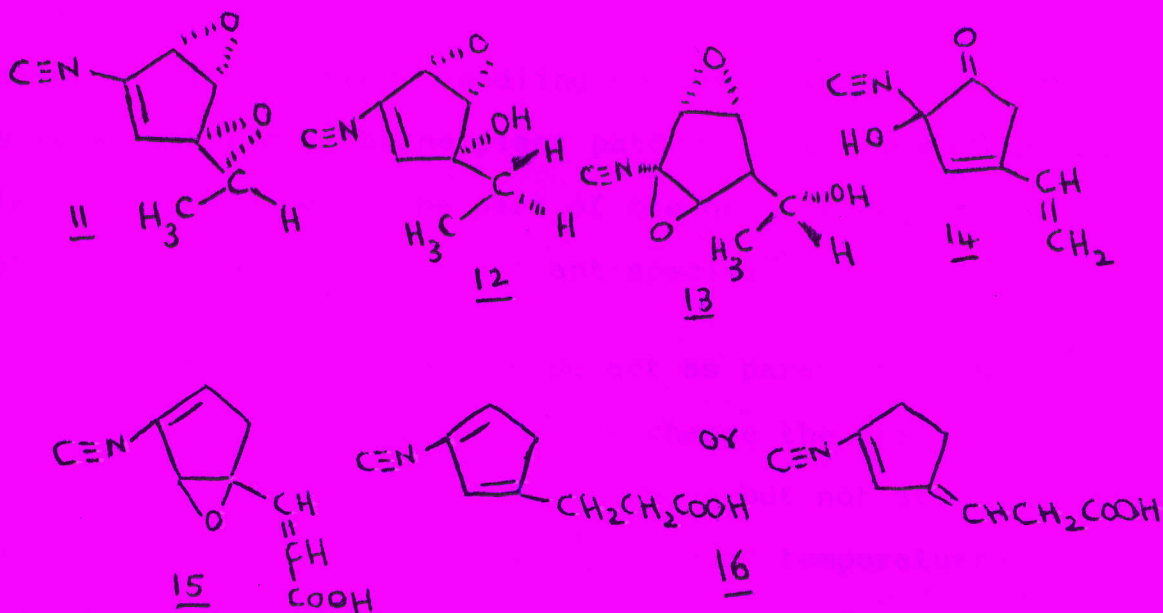
Trichoviridin (10), C₈H₉NO₄, m.p (95-96°C) is an unstable isonitrile diepoxide antibiotic isolated from

Trichoderma hamatum. It inhibits the growth of *Trichophyton asteroides* and *Escherichia coli* at 12.5–25 $\mu\text{g/ml}$ ²⁴.

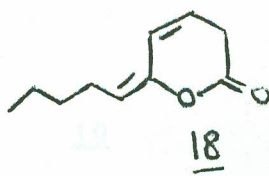
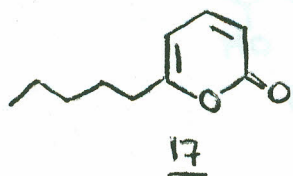
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Another fungal metabolite, possessing the same molecular formula and having an identical infrared to Trichoviridin (10) is also produced by a different unspecified strain of *Trichoderma*, this latter cyclopentane derivative also has two epoxides on the ring as does Trichoviridin. However, its melting point is 102–104°C²⁵. It is not clear whether the two metabolites are identical or not.

Trichoderma species have been reported to produce other antibiotics. These compounds also contain an isonitrile group and various oxygen functions and are referred to as isonitrin A (11), B (12), C (13) and D (14). The isonitrinic acids E (15) and F (16) are also described. Isonitrin A (11) which crystallises as colourless needles has its m.p at 91.0–91.5°C and its structure is similar to the previously reported structure (7) except that the stereochemistry has been defined. Isonitrin A (11) has been also reported to have the highest *in vitro* antimicrobial activities against gram positive and negative bacteria, yeast and also plant pathogenic fungi²⁶. Some of these compounds emit a characteristic coconut odour which is rarely found in species other than *T. viride*.



However Moss examined the volatile metabolites of several strains of *T. viride* and identified 6-pentenyl pyrone which he suggested was the compound responsible for the characteristic coconut aroma produced by some of the strains²⁶. 6-n-Pentenyl-2H-pyran-2-one (6-pentenyl-pyrone) (17) and the closely related analogue 6-n-pentenyl-2H-pyran-2-one (6-pentenyl-pyrone) (18) which are volatile oils, are also produced as secondary metabolites of some other *Trichoderma* species (27, 28, 29).



6-Pentenyl-pyrone, (18) principally a product of the spores was found together with some of the Trichorizianines.

Trichorizianine are biologically active peptides reputed to to be inhibitory to *Ceratocytic ulmi*, *Botrytis cinerea*,

Phytophthora cinnamomi,²⁸ *Gaeumannomyces graminis* (Sacc)

Arx and Olivier Var *tritici* Walker²⁹ and reduce the rate of

damping off in lettuce seedling by *Rhizoctonia solani* and several other soil borne plant pathogens. 6-Pentenyl pyrone is also considered to be part of the queen recognition pheromone complex of certain ant species³⁰.

The alkyl pyrones also act as paramorphogens in that they create conditions which change the spatial distribution of the organism's biomass but not it's rate of production. Their low volatility at soil temperatures compatible with plant growth ensures a high degree of soil permeation and persistence within the soil³¹.

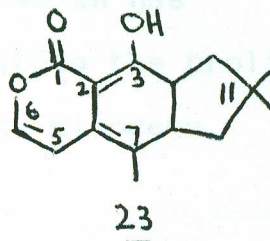
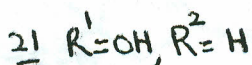
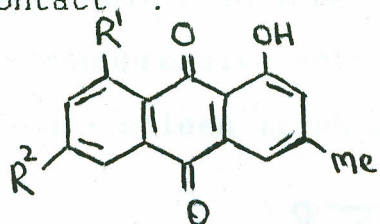
Trichorin A, $C_{20}H_{20}N_2O_8S_2$, colourless needles, (m.p 234-246°C) was obtained from acetone-n-hexane extract as the major product of the mycellium of *Trichoderma* species. It is an antibiotic active against gram negative bacteria. Its structure is not completely elucidated but is thought to be similar to dehydrogliotoxin³² (19).



Trichorin B, $C_{20}H_{20}N_2O_8S$ (yellow plates, m.p 230 - 238°C) was obtained as a minor product from the mycelial cake of *Trichoderma* (strain K-472) and was also obtained when trichorin A was dissolved in pyridine and recrystallised from ethanol³².

Gliotoxin, a non-volatile saturated form of (19) is reported to be one of the major metabolic products of *T. hamatum* Bainier and is known to be a highly antifungal and antibacterial metabolite of several *Fungi imperfecti*³³.

Anthraquinones *pachybasin* (20) (1-hydroxy-3-methyl anthraquinone, m.p 117°C), *Chrysophanol* (21) (1,8-dihydroxy-3-methyl anthraquinone, (m.p 195-196°C) and *Emodin* (22) (1,6,8-trihydroxy-3-methyl anthraquinone) are produced by *T. polysporus*, *T. viride* and *Phoma foresta* Forester. The crystalline compounds together with their O-acetyl and O-methyl derivatives have also been shown to suppress the production of the crystalline material, (3-hydroxy-7,11,11-trimethyl cyclopenta-(g)-benzopyran-1-one) (23), which is a sesquiterpene isocoumarin produced by the pathogenic basidiomycete fungus *Fomes annosus* at the line of mycelial contact³⁴.



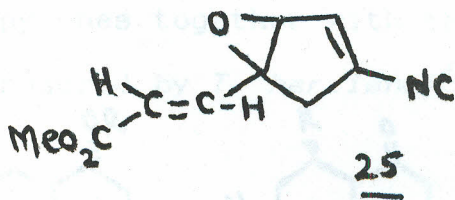
23

Heptelidic acid (24), C₁₅H₂₀O₅ also known as *Koningii acid*, is a sesquiterpene antibiotic with an epoxide structure is produced by several soil fungi such as *Anthostoma avocetta*³⁵, *Gliocladium virens*, *Chaetomium globosum* and *Trichoderma viride*^(36,37) as well as *Trichoderma koningii*³⁸.



Heptelidic acid which is an active metabolite of microbial origin is a potent inhibitor of ATP generation in the glycolytic pathway as it is a specific inhibitor of glyceraldehyde 3-phosphate dehydrogenase which catalyses the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate. It's mode of action is to bind to the sulfhydryl residue at the active site of the enzyme. It also inhibits lipid metabolism of animals as well as being an inhibitor of cholesterol biosynthesis in an *in vitro* rat liver enzyme system. Heptelidic acid also inhibits NAD⁺ oxidoreductase (phosphorylating) enzyme (37,38).

T. polysporus is also reported to produce isonitric acid E methyl ester (25) which has immunosuppressive activity as it inhibits the proliferation of mouse spleen lymphocytes induced by mitogens.

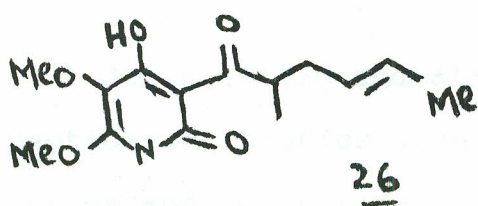


Carbon dioxide (CO₂) gas produced from one strain of *T. harzianus* Rifai was considered to be a biologically active volatile metabolite as it had inhibitory effects on *Pestalotia rhodendri* and *Aspergillus niger*.

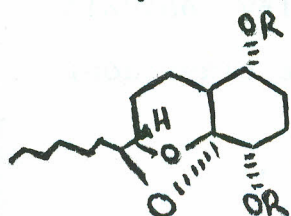
The production of ethanol, another volatile metabolite which was reported to inhibit growth and sporulation of *Aspergillus niger* and of *Pestalotia rhododendri* and the coloration and rate of elongation of seedlings of *Lactuca sativa* Var *sativa* has also been reported³⁹.

Dermadine, an unsaturated monobasic acid whose structure has not been published is an antibiotic from *T. viride*. It is known not only to inhibit the growth of *Bacillus subtilis* and aerobic aerogens but also to prevent odour in fish and fish crates and to treat bleeding places of silkworm⁴⁰.

Harzianopyridone (26) an antifungal metabolite of *T. harzianum* has been shown to be (E)-4-hydroxy-5,6-dimethoxy-3-(2-methyl-1-oxohex-4-enyl) pyridin-2-one (41,42).

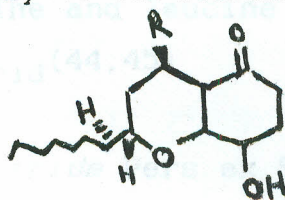


More recent investigations have shown that the alkyl pyrones together with the following compounds are also produced by *T. harzianum* (42,43)



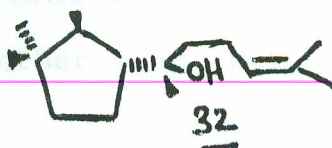
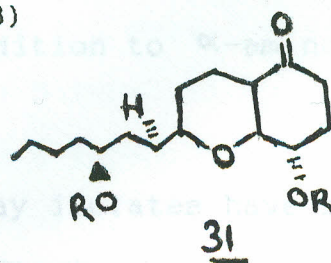
27 R=H

28 R=AC



29 R=OH

30 R=H





All the above have antibiotic activity against the growth of the take - all fungus *Gaeumannomyces graminis* Var *tritici*.

1.4 Peptide metabolites produced by *Trichoderma* species

A number of *Trichoderma* species have been reported to produce polypeptide metabolites which are non-volatile antibiotics against a range of fungi,⁴⁴ and most of the peptide antibiotics contain the unusual amino acid α -amino-isobutyric acid (Aib) ($H_2NC(CH_3)_2COOH$) (methyl alanine) as their major component.

Suzukacillin and alamethicine produced by *T. viride* are peptide antibiotics with antibacterial and antifungal properties and their mode of action is to modify the structure of the cell membranes. These peptide antibiotics contain glutamic acid (glutamine), proline, glycine, alanine, valine and leucine in addition to α -amino-isobutyric acid^(44,45).

T. viride Pers ex S.F Gray isolates have been reported to affect the growth of *Rhizoctonia solani* and *Armillaria mellea* together with other plant pathogenic fungi⁴⁴.

Trichorzianines A and B are separate groups of membrane active peptides as they form voltage-gated ion channels in black lipid membrane and also interact with phospholipid bilayers and induce membrane permeability modifications of liposomes^(45,46) in the absence of an applied voltage in a similar way to other peptaibols. They were isolated from the culture of the fungus *T. harzianum*. Trichorzianine A were neutral peptides while Trichozianine B were a group of acidic peptides.

The Trichorzianines antibiotics are 19- residue long hydrophobic peptides, structured in a right handed helix and contain a high proportion of α -amino isobutyric acid, an acetylated N-terminal extremity and a C-terminal amino alcohol either phenylalaninol or tryptophanol and thus belong to the peptaibol class^(47,48). Trichorzianines exhibit various biological activities for example antifungal and antibacterial properties and growth inhibition of the amoeba *Dictyostelium*⁴⁷.

Trichokindin i-x are other antibiotic peptides which belong to a class of peptaibols and were isolated from the spores of *T. harzianum*. The N-terminal is protected by an acetyl group and the C-terminal is linked with an isoleucinol. The peptides whose total structure have not yet been elucidated contain a high proportion of α -amino-isobutyric acid and isovaline.

The related peptides Trichokindin i-iii which are not protected by an acetyl group have their relative molecular mass of about 1200, while Trichokindin iv-ix have masses in the range of 1800 daltons and have a similar structure in which an amino alcohol is isoleucinol, in Trichokindin x the isoleucinol is replaced by leucinol⁵⁰.

Trichotoxin A, a new mycotoxin was found in a solvent extract of mycellium of *T. viride* NRRL 5242 isolated from Southern Leaf Blight, infected corn. Trichotoxin A is a cyclic peptide with the following amino acid composition (gluN)₂, (glu)₁ (pro)₂ (gly)₁ (ala)₃ (leu)₃ (Alb) it decomposes at 187°C and shows animal toxicity and some antimicrobial activity⁵¹.

Paracelsin is another peptide antibiotic containing α -amino isobutyric acid and is isolated from *T. reesei* *Simmions* QM 944. It forms voltage dependent ion conducting pores in lipid bilayer membrane in a similar way to alamethicine and exhibits antibiotic activity mainly against gram positive bacteria⁵².

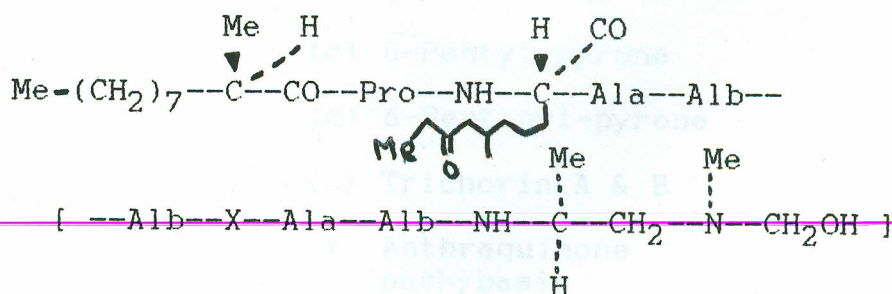
Trichosporins are a mixture of linear peptides isolated from the culture filtrate of *T. polysporum* and are antagonistic to *Leutinus edodes*. The peptides in which the N-terminal is protected by an acetyl group and the C-terminal by a phenylalaninol belongs to the class of peptaibols and contain a high proportion of α -amino

isobutyric acid^(53,54).

Cyclosporins A and C are antifungal antibiotics and are produced by submerged culture of *T. polysporum* (Link ex Pers) Rifai. The cyclosporin A is a non-polar cyclic peptide with a relative molecular mass of 1202.6 daltons. The cyclosporins inhibits cell wall synthesis as well as blocking chitin synthesis. They also exhibit a narrow spectrum of antifungal activity and in addition have immunosuppressive and antiphlogistic action⁵⁵.

Trichopolyns A and B are other polypeptide antibiotics isolated from the culture of *T. polysporum*. They are inhibitory against fungi, gram positive bacteria and acid-fast bacteria but ineffective against many other bacteria. They have a relative molecular mass of about 2000, similar infrared and amino acid components and can be readily interconverted. They differ in their counter anions, NO_3^- for Trichopolyn A and Cl^- for Trichopolyn B⁵⁶.

Trichopolyns i and ii are also produced by *T. polysporum*. The structures of Trichopolyns i and ii (i Alb = NHMe_2CO , X = ile and val respectively⁵⁷ are shown below.



It can thus be noted from the literature review that *Trichoderma* species are very important biocontrol agents not only for their antibiotic activity against a wide range of fungi but also for their antimicrobial activity against a wide range of bacteria.

The constitution of secondary metabolites of *Trichoderma* species reported to significantly inhibit mycelial and rhizomorphal growth and to hamper significantly the biomass accumulation of *A. solana* should be elucidated as accurate knowledge of the structures could in future lead to the possibility of producing, determining and modifying the structure activity relationships of the elucidated secondary metabolites for commercial uses as this venture may provide useful treatment of *Armillaria* root rot hence increase in tea production.

Table 1: A summary of the compounds produced by
Trichoderma species

<i>Trichoderma</i> species	Compound	Reference
<i>T. viride</i>	(a) Trichodermin	6
	(b) Trichodermol	6
	(c) 6-Pentyl-pyrone	9
	(d) 6-Pentenyl-pyrone	"
	(e) Trichorin A & B	10
	(f) Anthraquinone pachybasin	11

TABLE 1 CONTD...

	(g) Chrysophanol	11
	(h) Emodin	11
	(i) Heptelidic acid	"
	(j) Dermadine	13
	(k) Suzukacillin	14
	(l) Alamethicine	14
	(m) Trichotoxin A	16
	(n) Isonitrins A,B,C and D and isonitric acid E and F	8
<i>T. kanningii Gudem</i>	(a) 6-pentyl-pyrone	9
	(b) 6-pentenyl-pyrone	"
	(c) Heptelidic acid	11
	(d) Isonitrin C and D	8
<i>T. longibrachiatum</i>	(a) Trichodimerol	6
	(b) Sorbicillin	"
<i>T. longibrachiatum</i>	(c) Bisvertinol	"
	(d) Bisvertinolone	"
	(e) Trichodermolide "	"
	(f) Sorbiquinol	"
	(g) Bislongiquinolide	"
	(h) 5-hydroxyvertinolide	"
<i>T. polysporum</i>	(a) Trichodermin	6
	(b) Anthraquinone pachybasin	11
	(c) Chrysophanol	"
	(d) Emodin	"

TABLE 1 CONTD...

	(e) Isonitrinic acid E methyl ester	12
	(f) Trichosporins	16
	(g) Cyclosporins A & C	17
	(h) Trichopolyns A & B	"
	(i) Trichopolyns i & ii	"
	(j) Isonitrins D and isonitrinic acid E & F	8
<i>T. hamatum</i>	(a) Isocyano compounds	7
	(b) Trichoviridin	"
	(c) Gliotoxin	11
	(e) Isonitrin C and isonitrinic acid E & F	8
<i>T. reesei</i> Simons	(a) Paracelsin	16
<i>T. harzianum</i>	(a) Carbon dioxide	12
	(b) Ethanol	"
	(c) Harzianopyridone	13
	(d) Trichoizianine	15
	(e) Trichokindin 1-10	15 & 16
	(f) Isonitrin D and Isonitrinic acid E & F	8

1.5 Aim of the project

The literature reveals that *Trichoderma* species produce non volatile and volatile antibiotic substances against a wide range of pathogenic fungi and the secondary

metabolites of *T. longibrachiatum* Rifai, *T. harzianum* Rifai and *T. koningii* Oudem have been reported to significantly inhibit both the mycelial and rhizomorphal growth of *A. mellea* and also hamper significantly the biomass accumulation of *A. mellea* thus minimising *Armillaria* root rot and hence increasing tea production. The specific objectives of this study therefore were as follows;

1. To isolate secondary metabolites produced by Kenyan *Trichoderma* species.
2. To establish the structure of any pure compounds isolated from *Trichoderma* species by analysis of their spectral data i.e ir, ms, $^1\text{Hnmr}$, and $^{13}\text{C-nmr}$.
3. Should time have permitted, it was also hoped to investigate the biological antagonism of the purified metabolites against *A. mellea*. This work will be carried out in co-operation with the Tea Research Foundation of Kericho, Kenya with the help of their facilities.

in iodine tanks or spraying with the appropriate detecting

CHAPTER 2

EXPERIMENTAL

2.0 General experimental procedures

Glassware for collection and storage of the sample extracts were chemically cleaned by soaking in freshly prepared chromic acid overnight, rinsed with distilled water, dried and rinsed with appropriate solvents before using them.

All organic reagents were of the highest grade commercially available and were used as obtained. Analar grade or distilled reagent grade were used for all reactions and chromatographic separation.

Column chromatography was carried out on silica gel 60 (0.040-0.063 mm, 230-400 mesh ASTM). Separation and elution was achieved using different solvent systems. Analytical thin layer chromatography (tlc) was performed on either MN polygram sil G\UV₂₅₄ precoated plastic sheets of (40mm x 80mm, 0.25mm film thickness) or Allugram (R) sil G\UV₂₅₄ (20cm x 20cm, 0.25mm thickness silica gel 60 with fluorescent indicator) cut to appropriate sizes. Specially made glass teat pipettes were used to spot samples onto tlc plates. The loaded plates were developed at room temperature in glass jars by the ascending solvent technique. The developed spots were then visualized by exposing the plates to ultra-violet light followed by either developing in iodine tanks or spraying with the appropriate detecting

agents such as ceric sulphate in sulphuric acid, 0.5% solution of vanillin in sulphuric acid and ninhydrin/acetic acid and heating the sprayed plate at 110°C for seven minutes.

Melting points were determined on an Electrothermal digital melting point apparatus and a Griffin melting point apparatus and are reported uncorrected. Infrared (ir) spectra were run by the potassium bromide (KBr) pellet disc technique and as a liquid film between KBr plates using a Perkin Elmer 598 infrared spectrophotometer and expressed in wave numbers (cm^{-1}) and calibrated with a plastic film of polystyrene.

Low resolution mass spectra (eims) were recorded on a VG Masslab 12-250, VG-analytical mass spectrometer using a direct insertion probe (DIP) at ionization potential of 70 ev and are given as mass to charge ratio (m/e) with relative intensities in parenthesis.

Proton nuclear magnetic resonance (^1H -nmr) spectra were recorded in deuterated solvents with tetramethylsilane (tms) as the internal standard. Chemical shifts are reported in parts per millions (ppm) relative to tms. Multiplicities are indicated by the symbols s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (double of doublet). Carbon-13 nuclear magnetic resonance (^{13}C -nmr) spectra were recorded on AM 400 Bruker

instrument.

Solvent evaporation was done using a Rotatory film evaporator at 40°C under reduced pressure unless otherwise stated.

2.1 Assay for the effect of *Trichoderma* extracts on the growth of *Armillaria mellea*

The above assay was performed by the plant pathology section of the Tea Research Foundation of Kericho, Kenya.

2.1.1 Malt extract agar medium (mea)

(a) Composition : malt extract, 30.0g; glucose, 20g; peptone, 5.0g; agar no.2, 20.0g; distilled water, 1000ml.

(b) Preparation : All the ingredients, except the agar were first dissolved in distilled water with the help of a magnetic stirrer. One hundred and fifty millilitres of the solution was dispensed into several 250ml conical flask and 3.0g of agar no.2 added into each flask. The flasks were then plugged with cotton wool and sterilised at 121°C at 1.01325 NM^{-2} for 15 minutes.

2.1.2 Preparation of *Trichoderma* extracts for incorporation into the mea medium

Different *Trichoderma* extracts were dissolved in their respective solvents. The amount of solvent used were such that quantities of the *Trichoderma* extracts incorporated in 150ml of mea medium gave the concentration of the extract (w\v) of 100ppm.

2.1.3 Incorporation of the *Trichoderma* extracts into the mea medium

The *Trichoderma* extract was added to the autoclaved mea medium (see 2.1.1) at an approximate medium temperature of 55°C to give a concentration of 100ppm. The medium was then stirred and dispensed into sterile petri-dishes (150ml of medium into 6 petri-dishes) and left to set.

The dry solid medium in petri-dishes was inoculated with a 2mm diameter disc of *A. mellea* taken from the periphery of a young colony growing on mea medium.

The petri-dishes were then covered with aluminium foil and incubated at 22°C in an incubator. The growth of the *A. mellea* was then determined at regular intervals of time, measuring the colony diameters in millimeters less 2mm (the size of inoculum) to give the net growth.

The data on colony size was subjected to analysis of variance.

2.2 Trichoderma broth cultures

The *Trichoderma* isolates (*T. longibrachiatum*, 5 litres; *T. harzianum*, 10 litres; Control, 5 litres;) were cultured and supplied by the Tea Research Foundation of Kericho, Kenya.

The *Trichoderma* broth cultures were grown in Richard's nutrients solution (KNO₃, 10.0g; KH₂PO₄.3H₂O, 5.0g; MgSO₄.7H₂O 2.5g; FeCl₃, 0.02g; sucrose, 50g, distilled water, 1000ml).

Five litres of the solution was prepared and dispersed into one-litre conical flasks in 750ml aliquots. The flasks were then plugged with cotton wool then sealed upto the neck region with aluminium foil.

The medium was sterilized by autoclaving the flasks at 121°C at 1.01325 NM⁻² for 20 minutes.

After the autoclaved medium had cooled to room temperature, it was inoculated with *Trichoderma* species by aseptically transferring a 4-mm diameter disc of the *Trichoderma* culture grown on solid medium into the flasks. The broth cultures were then shaken at room temperature on an orbital shaker for 3 weeks when they were ready for extraction.

2.3 Extraction of secondary metabolites from *T. longibrachiatum*

Aliquots (140ml) of the culture broth were sequentially extracted with the use of a separating funnel with the following solvents (in increasing polarity) respectively.

1. 3x50ml of hexane.
2. 3x50ml diethyl ether.
3. 3x50ml of dichloromethane.
4. 3x50ml of ethyl acetate.

The extracts obtained were concentrated using a rotatory film evaporator at 40°C.

Table 2 : Shows the weights obtained of the different extracts.

Extract	n-hexane	diethyl ether	dichloromethane	ethyl acetate
Colour	light yellow oil	yellow oil	yellow oil	brown oil
Weights (mg)	23.9	66.3	19.8	51.4

2.3.1 Separation and purification of the n-hexane extract

Preparative tlc of the hexane extract using 25% CHCl₃ in hexane gave four distinct uv active bands. These bands were scraped off and eluted with CHCl₃. Bands 1,3 and

4 were rechromatographed using 25% CHCl_3 in hexane on a 0.25mm silica plates and revealed a complex mixture of components, which were not further separated. Band 2 was rechromatographed using 25% CHCl_3 in hexane on a 0.25mm silica plates to give a pale yellow oil (1.7mg) which appeared as a single spot with an RF of 0.4 on further examination by tlc (25% CHCl_3 in hexane).

Fraction 2

$^1\text{H-nmr}$ (CDCl_3 , δ ppm)

1.0 (s), 1.4(s), 1.65(s),

2.3.2 Diethyl ether extract

A preliminary examination of the diethyl ether extract using 25% chloroform in hexane showed six uv active spots of different Rf values. The extract was then separated on preparative scale using similar conditions (25% CHCl_3 in hexane). The 2nd fastest running fraction on further examination by tlc showed two UV active spots of Rf values 0.8 and 0.36 (CHCl_3 /hexane, 1:1 v/v) respectively. The high running fraction had a blue fluorescence when examined by uv. This fraction was further purified by tlc using CHCl_3 /hexane, 1:1 v/v, to give separated spots of Rf value 0.8 and 0.36 (<1mg). As the quantities of materials were exceedingly small, further examination of the spots was not made. The remaining fractions from the preparative tlc examination were complex mixtures and were set aside

for examination at a later date.

2.3.3 Ethyl acetate extract

Preparative tlc of the ethyl acetate extract using 25% chloroform in hexane gave four distinct uv active fractions. The fractions were scraped off and eluted with ethyl acetate and the solvent removed by evaporation using a rotatory film evaporator. Preliminary examination of the 1st, 2nd and 3rd fractions in 50% chloroform in hexane showed that each fraction contained a similar blue fluorescence spot (Rf value, 0.63) together with other spots of different Rf values. The fractions were thus pooled together and the fraction corresponding to the blue fluorescence (Rf value approximately 0.63) was separated by preparative tlc.

Since the diethyl ether extract, high running fraction had a similar blue fluorescence and similar Rf value under the same conditions, the partially purified material from the ethyl acetate extract (which gave the blue fluorescence) and that from diethyl ether were combined. This material was then further purified using preparative tlc (1:1, CHCl₃/hexane v/v) to give a colourless oil (4.7mg) which ran as a single spot on tlc (1:1, CHCl₃/hexane). The following are the δ values of ¹H-nmr in ppm.

^1H -nmr (CDCl_3 , δ ppm)

0.8 (t), 1.20(s), 1.4(s), 1.5(s), 4.75 (s), 6.6 (s).

The remaining fractions of ethyl acetate extract were set aside for further preliminary analysis.

2.3.4 Dichloromethane extract

Preparative tlc of the dichloromethane extract (19.8mg) using CHCl_3 : n-Hexane (1:3 v/v) showed three fractions which were not screened further as on preliminary analysis tlc they showed many closely overlapping spots with different tlc solvent systems.

2.4 Liquid - liquid Extraction

The *T. longibrachiatum* culture broth (200ml) was placed in a liquid - liquid extraction apparatus which was connected to a round bottom flask (250ml). The culture broth was sequentially extracted with 250ml of each of hexane, diethyl ether and ethyl acetate for a total of 27 hours in each case. The extracts were concentrated by removal of the solvent using a rotatory film evaporator under reduced pressure. The residual culture broth was stored at low temperature in a deep freezer for possible further examination.

This process was repeated until a total of 10 litres of the culture broth of *T. longibrachiatum* had been consumed. The description and masses of the extracts are

recorded in table 3.

Table 3. Shows the weights obtained by the above method.

Extract	:	n- hexane	:	diethyl ether	:	ethyl acetate
Colour	:	yellow oil	:	brown oil	:	red-brown oil
Weights (mg)	:	31.0	:	32.0	:	286

2.5 Separation and purification of the extract

2.5.1 Hexane extract

A sample of the hexane extract was examined by tlc using the solvent system 25% chloroform in hexane. This showed four spots which were all uv active and which developed well as distinct spots in iodine tanks. The extract was then separated on preparative TLC using the same solvent system.

Band 3 eluted from the silica by chloroform as a pale yellow oil and was further purified by preparative tlc using the same solvent system. Further examination of fraction 3 by analytical tlc showed a distinct reddish fluorescence spot with uv light which did not separate out when examined under different solvent systems. It was purified by repeated preparative tlc. The pale yellow oil obtained (19.5mg) had an Rf value of 0.57 (100% dichloromethane) and was assigned compound A.

Compound A

Pale yellow oil Rf :0.57 (CH₂Cl₂, 100%)

ir ν_{\max} (KBr liquid film, cm⁻¹) 2950, 2909, 1720, 1596, 1576, 1457, 1378, 1268, 1169, 1070, 1040, 961, 739 716.

Eims (m/e rel. int)

418 (<1) [C₂₆H₄₂O₄]⁺, 307(8) [C₁₈H₂₇O₄]⁺, 293(36) [C₁₇H₂₅O₄]⁺, 167(21) [C₈H₇O₄]⁺, 149(100) [C₈H₅O₃]⁺, 104(6) [C₇H₄O]⁺, 85(30) [C₆H₁₃]⁺, 71(63) [C₅H₁₁]⁺, 57(65) [C₄H₉]⁺ 43(57) [C₃H₇]⁺.

2.5.2 Diethyl ether and ethyl acetate extracts

Analytical tic of the above mentioned extracts showed loosely overlapping spots even with different tic solvent systems.

The extracts were combined with the remaining fractions previously obtained from the various extracts obtained by the use of a separating funnel and the solvent (methanol) was evaporated to give a dark red oily residue (TL₁) which was examined by column chromatography.

2.6 Chromatographic separation

2.6.1 Column chromatography of the TL₁

Silica gel (10g) was packed onto a glass column (25cm x 1.2cm) using chloroform. The chloroform was later eluted by passing hexane down the column. TL₁ (0.33g) was dissolved in minimum amount of 30% chloroform in methanol

(v\v) and mixed with activated silica gel (1g) and the solvent removed under reduced pressure. The dried mixture of silica gel and the adsorbed sample was then introduced onto the packed column. The solvents of increasing polarity were used to elute the column. The eluates were collected in 15ml fractions. A total of 115 fractions were obtained. Fraction 1-6 collected from 100% n-hexane and fraction 7 collected from 5% chloroform in hexane showed a single spot by tlc. The spot was both uv active and developed as a distinct spot when placed in an iodine tank. The same spot was visualized by spraying with ceric sulphate reagent and heating the plate at 110°C for seven minutes. The fractions 1-7 were then pooled together, the solvent removed by evaporation and the fraction 1-7 was purified by preparative tlc using 15% chloroform in hexane as the solvent system, to give a clear, colourless, viscous oil labelled compound B.

Compound B (15mg), clear, colourless viscous oil, R_f : 0.68
(CHCl₃\hexane 3:17 v\v)

ir ν_{max} (KBr liquid film. cm^{-1}) 2940, 2876, 1457, 1375, 787.

¹H-nmr (CDCl₃, δ ppm)

0.9(m), 1.3(s).

¹³C-nmr (CDCl₃, δ ppm)

14(s), 19(s), 23(d), 29(m), 32(q), 37(d).

Eims (rel. int %)

774(<1), 685(<1), 211(5), 191(11), 165(20), 149(21), 97(58),
95(85), 81(90), 57(100). Cims 795(<1), 579(<1), 419(14),
330(55), 191(23), 163(24), 151(26), 96(25), 95(69), 81(72),
58(100).

The remaining fractions were examined by tlc and those having similar Rf values combined. The combined fractions are recorded in table 4.

Table 4. shows the combined fractions and their respective weights.

Fraction No.	Weight (mg)	Fraction No.	Weights (mg)
1-7	16	100	2.6
8-11	3.78	101	3.5
12-15	1.0	102	5.1
16-21	0.7	103	12.6
22-24	9.8	104	58.5
27-31	3.2	105	55.8
32-38	6.1	106	9.6
39-44	4.5	107	1.9
45-56	12.1	108	5.2
57-64	2.3	109	32.5
65-78	15.25	110	51.0
79-82	1.5	111	14.5
83-88	2.3	112	10.3

TABLE 4 CONTD...

89-94	4.0	113	22.1
95-96	0.5	114	13.3
97-98	0.1	115	10.3

The combined fractions when examined by analytical tlc showed poor separation and were not examined further.

2.7 *Trichoderma harzianum*

2.7.1 Extraction of secondary metabolites from *T. harzianum*

The *Trichoderma harzianum* culture broth was filtered under vacuum and the mycellium obtained placed in a dessicator. The culture broth was subjected to liquid-liquid extraction sequentially with hexane, diethyl ether and ethyl acetate. The results are recorded in table 5.

Table 5: Shows the weights of the extracts obtained

Extract	n-hexane	diethyl ether	ethyl acetate
Colour	yellow oil	brown oil/ crystals	red oil
Weight (mg)	31.9	160	128

2.8 Separation and purification of the extracts

2.8.1 Hexane extract

Analytical tlc of the hexane extract using solvent system 5% chloroform in hexane showed seven UV active spots and one non uv active spot (iodine active) and these spots showed different colours when sprayed with vanillin/sulphuric acid and heated at 110°C for seven minutes. The eight spots had different Rf values and were separated by preparative tlc. The results are recorded in table 6.

Table 6: Fractions obtained from preparative tlc

Fraction	Rf	Amount (mg)	UV	Colour (Vanillin\H ₂ SO ₄)
2	0.15	2.5	✓	Blue
3	0.35	2.1	✓	Brown
4	0.48	4.9	✓	Black
5	0.57	2.0	✓	Blue
6	0.70	1.1	x	Purple
7	0.86	2.5	✓	Light blue
8	0.97	2.1	✓	Purple

On further tlc examination, the fractions showed two or more closely overlapping spots with different tlc solvent systems except fraction 5 which had a single spot.

Fraction 5

Rf : 0.568 (CHCl₃\n-Hexane. 1:19 v\v)

Eims probe (70 ev, m\e rel. int)

326(<1), 288(3), 259(7), 248(14), 185(4), 166(24), 158(20),
148(24), 113(21), 112(31), 97(20), 85(28), 83(50), 73(12),
71(51), 70(34), 69(34), 57(66), 55(60), 43(100), 41(55).

11.4mg of the remaining hexane extract was sent to the Tea Research Foundation of Kericho, Kenya for bioassay.

2.8.2 Diethyl ether extract

The dark brown extract showed evidence of crystals. Acetone was added to the extract to aid crystallisation and the mixture left in the deep freezer overnight. This gave a brown solution and colourless clear crystals settled out. The supernatant solution was removed and the crystals recrystallised from acetone. The final crystals obtained were washed with cold acetone (1ml x 6) to give small colourless crystals (42mg). More crystals came down in the brown mother liquor solution. The crystals were examined on analytical tlc using 5% ethyl acetate in chloroform. A single spot of Rf value of 0.58 was obtained. This compound was labelled compound C.

Compound C

Colourless crystals, sublimes, Rf :0.58 (Ethyl acetate\CHCl₃
1:19 v\v)

ir ν_{\max} (KBr disc. cm⁻¹) 3356-2386, 1684, 1390, 1291, 1180

907.

 $^1\text{H-nmr}$ (CD_3COCD_3 , δ ppm)

2.6(s)

 $^{13}\text{C-nmr}$ (CD_3COCD_3 , δ ppm)

174(s), 29(s).

Eims probe (70 ev. m/e rel.int)

118(<1), 116(<1), 100(58), 74(42), 55(100), 56(23) 45(57),
43(6) 43(7).2.8.3 Ethyl acetate extract

The extract adhered strongly to the walls of the glass container and resisted attempts to remove it by adding various solvents. The extract was only partially soluble in methanol and was not further examined.

2.9 Extraction of mycellium from *T. harzianum*

The mycellium which had been stored in a dessicator was sequentially extracted by shaking with 500ml of each of hexane, diethyl ether, ethyl acetate and methanol in order of increasing polarity. The solvents each remained in contact with the mycellium for 4 days and the temperature was then raised to the boiling point of the solvent and the solvent removed by decantation. The solvent was then evaporated under reduced pressure. The masses of the extracts are recorded in table 7.

Table 7: Shows the weights of the mycellium extracts obtained

Extract	n-hexane	diethyl ether	ethyl acetate	methanol
Colour	colourless waxy crystals	colourless waxy solid	colourless flaky wax	pink needles
Weights (mg)	43.8	30.1	20.8	200

2.9.1 Hexane mycellium extract

After standing overnight in a deep freezer wax-like crystals (43.8mg) were deposited which were recrystallised from chloroform. These were separated and washed severally with cold chloroform giving compound D (colourless waxy crystals) of melting point range 46.0-46.3^oc. A small sample of compound D decolourised bromine water.

ir ν_{\max} (KBr liquid film. cm^{-1}) 3300-2500, 2910, 2850, 2668, 1702, 1632, 1551, 1460, 1410, 1290, 1087, 937, 756, 716.

¹H-nmr (CDCl₃. δ ppm)

5.3(s), 2.3(t, J=7.5 Hz), 1.6(t, J=7.0 Hz), 1.5(d, J=23 Hz), 1.2(d, J=14 Hz), 0.9(t, J=6.7 Hz).

¹³C-nmr (CDCl₃. δ ppm).

10(s), 14(s), 23(s), 27(s), 29(m), 31(s), 102(s), 130(s), 179(s).

Eims probe (70 ev. m/e rel. int)

285(2, [M+1]⁺), 256(100, [M-28]⁺), 239(10), 227(3), 213(8).

185(5), 129(5), 111(4), 57(23), 55(28).

2.9.2. Diethyl ether mycellium extract.

The oil from the diethyl ether extract was triturated with hexane and a colourless waxy substance (30.1mg) was obtained. This was crystallised from hexane, the crystals separated and washed with cold hexane. This gave compound E as a waxy solid. A small sample of compound E decolourised bromine water. The melting point range of compound E was 51.6-52.4°C.

Compound E

ir ν_{\max} (KBr, liquid film, cm^{-1}) 3500-2500, 2910, 2850, 1732, 1702, 1450, 1370, 1290, 1108, 927, 837, 716.

^1H -nmr (CDCl_3 , δ ppm) 5.4(dd, $J=2.7$ Hz), 4.3(q, $J=5.4$ Hz), 4.1(q, $J=6$ Hz), 3.5(d, $J=5.2$ Hz), 2.3(q, $J=8.6$ Hz), 2.0(q, $J=6$ Hz), 1.6 (t, $J=6.8$ Hz), 1.3(m), 0.9(d, $J=8$ Hz) 0.8(q, $J=4.4$ Hz)

^{13}C -nmr (CDCl_3 , δ ppm) 14(s), 23(s), 24(d), 25(s), 29(m), 31(d), 33(s), 76.6(s), 128(s), 130(d), 179(s).

Eims probe (70 ev m/e rel. int)

412(1), 397(1), 367(38), 340(12), 312(12), 256(5), 185(6), 171(6), 129(20), 111(8), 97(21), 81(30), 69(52), 55(82), 43(100).

A yellow oil of low yield (<1mg) which was insoluble in hexane was also obtained from the diethyl

ether extract.

2.9.3 Ethyl acetate mycellium extract

The extract on concentration gave a white flaky wax (20.8mg) which was dissolved in 20% ethyl acetate in hexane(v/v). This solution was placed in a deep freezer overnight. Colourless crystals settled down and the supernatant solution was removed. The crystals (<1mg) were washed with cold hexane and were recrystallised from 10% ethyl acetate in hexane.

Colourless crystals (<1mg)

ir ν_{\max} (KBr disc. cm^{-1}) 3537-3234, 2951, 2850, 1711, 1661, 1381, 1271, 1099.

2.9.4 Methanol mycellium extract

Removal of the solvent from the methanol extract produced long, pink, threadlike needles (200mg) with an irritating smell. The melting point of the partially purified material was determined and the following observation made.

148°C - turned red

220°C - started to charr

250°C - charred completely.

The methanol mycellium extract was slightly soluble in methanol but very soluble in hot water (60°C) and thus a mixture of ethanol-water or methanol-water was

used for crystallization and purification of the crystals. A solvent system of 40% methanol in water (v/v) was added to the crystals and instead of dissolving in the solution, the crystals cleared completely to a white crystals from their pale pinkish colouration and an amorphous powdered material tended to float in the solution thus this was removed and the crystals further washed with the 40:60 - methanol : water mixture. The total amount of the clear long threadlike crystals obtained was 190.6mg. A small amount of the crystals was dissolved in the 40:60 - methanol : water mixture using a hot water bath and a drop of the solution placed on a filter paper which was sprayed with ninhydrin/acetic acid. The dry sprayed filter paper was placed in the oven at a temperature of 110°C for 7 minutes, a purple colouration was attained.

The melting point of the purified material was determined and the following observation was made,

- 125.5°C - turned pale brown
- 138.8°C - brown
- 145.1°C - started to charr
- 200°C - charred completely

Compound F

White threadlike crystals. Rf : 0.52 (CHCl₃\MeOH 5:2 v/v)

ir ν_{\max} (KBr disc. cm⁻¹) 3650-2500, 2930, 2740, 2400, 1770.

¹H-nmr (D₂O. δ ppm)

3.8(m).

^{13}C -nmr (D_2O , δ ppm)

101, 182.153, 186.438.

Eims Probe (70 ev, m/e rel.int)

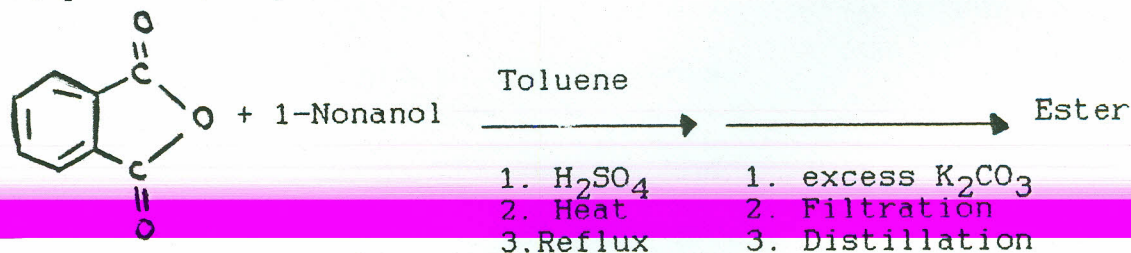
256(4), 149(38), 136(6), 123(6), 111(14), 97(34), 83(36),

69(53), 43(100), 55(74), 57(93), 71(40), 85(20).

2.9.5 Synthesis of 1,2-Dinonyl phthalate

Phthalic anhydride (14.8g) was placed in a round bottom flask. Toluene (100ml), 1-nonanol (40ml) and concentrated sulphuric acid (0.5ml) were then added. The phthalic anhydride dissolved completely in the mixture. The mixture was heated for eight hours and the water given off by the above reaction was collected by Dean-Stark apparatus. The reaction mixture was then refluxed for two days continuously. Anhydrous potassium carbonate was then added to the cooled reaction mixture to remove the excess water. The solution was filtered under vacuum.

The reaction product (10ml) was purified by fractional distillation under vacuum to yield a colourless waxy solid, m.p 174-176°C.



Ester (m.p 174-176°C), Colourless waxy solid, Rf 0.58 (100% CH_2Cl_2).

ir ν_{\max} (solid state, cm^{-1}) 3031, 2931, 2861, 1726, 1606, 1461, 1381, 1261, 1151, 1076, 1035, 971.

^1H -nmr (CDCl_3 , δ ppm)

1.3(m), 1.7(m), 2.1(q, $J=2.2\text{Hz}$), 3.4(t, $J=6.4\text{Hz}$), 4.3(m), 7.8(m).

^{13}C -nmr (CDCl_3 , δ ppm)

14(s), 23(s), 26(d), 29(m), 30(m), 32(s), 33(s), 65.6(s), 65.9(s), 136(m), 137(m), 168.3(s), 168.8(s).

ms (fab)

m/e 418(<1), 263(62), 167(9), 149(100), 85(7).

CHAPTER 3

RESULTS AND DISCUSSIONS

A number of *Trichoderma* isolates were investigated for their antibiotic activity against *A. mellea*. *T. longibrachiatum* Rifai, *T. harzianum* Rifai, and *T. koningii* Oudem were reported to be better inhibitors of *A. mellea* unlike other *Trichoderma* isolates as the secondary metabolites produced by the stated *Trichoderma* isolates significantly inhibited the mycelial and rhizomorphal growth and hampered significantly the biomass accumulation of *A. mellea*¹⁷. *T. longibrachiatum* and *T. harzianum* were chosen for the current study as these isolates were reported to have the highest significant effects against the *A. mellea*¹⁷.

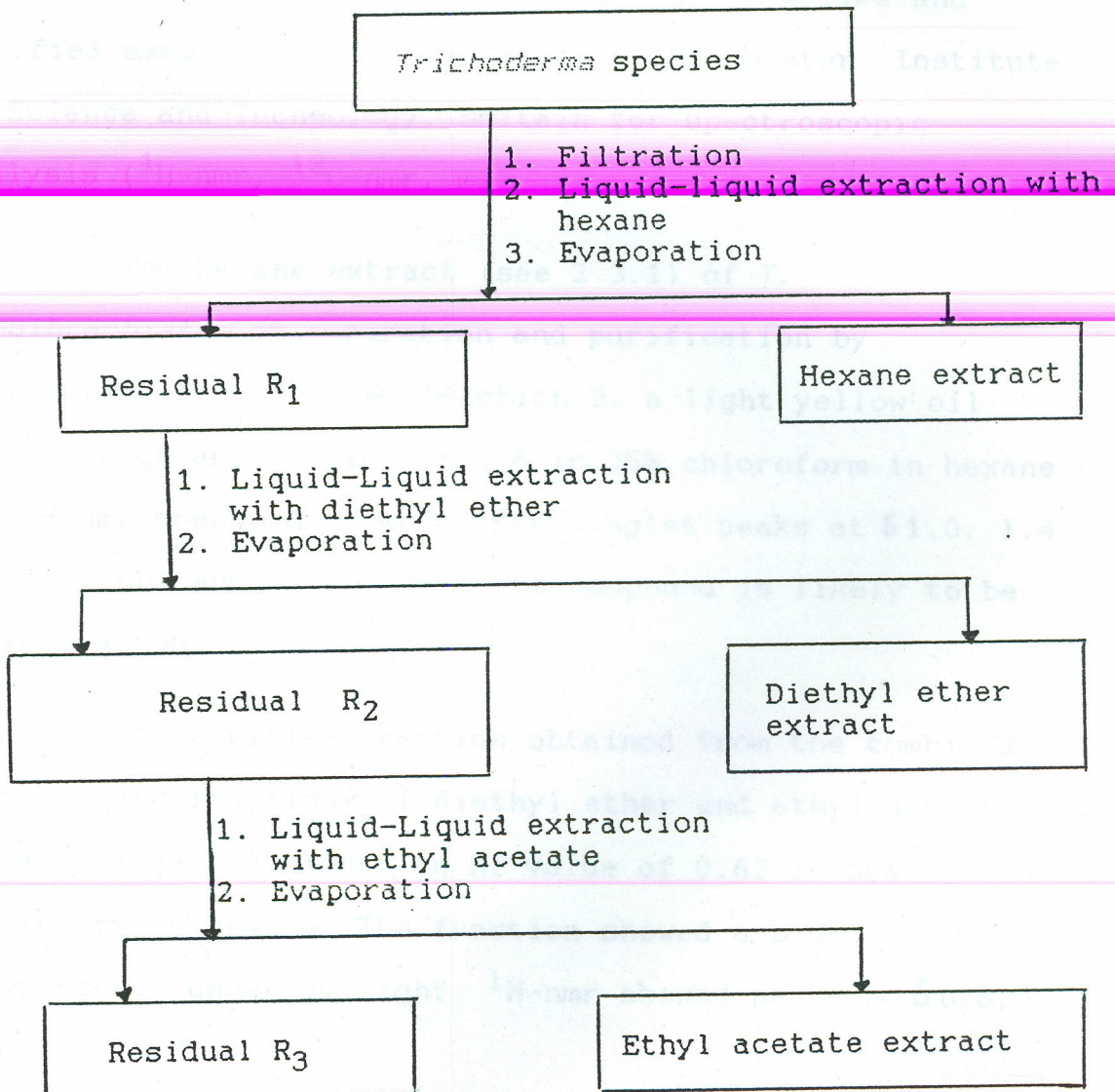
3.1 General extraction

The *Trichoderma* culture broths were filtered and successively extracted with hexane, diethyl ether and ethyl acetate solvents. Initially, a separating funnel was used but finally a purpose built extractor was used in the liquid - liquid extraction of the *Trichoderma* culture broths at the boiling points of the solvents.

The extracts obtained by the use of a separating funnel were in low yield and this coupled with the slow and tedious procedure involved with the technique, resulted in the discarding of the method and a better method of liquid-

liquid extraction which involved the use of a purpose built extractor was extensively used. This, was not only a superior technique but also increased the yield of the individual extracts. The resulting extracts were concentrated under reduced pressure using a rotatory evaporator. Figure 1 shows a flow diagram for the extraction of the *Trichoderma* species.

Fig. 1: Flow diagram for the extraction of the *Trichoderma* species.



A spot test survey using thin layer chromatography (tlc) revealed the presence of several uv active compounds in all the extracts of both *T. longibrachiatum* and *T. barzianum*.

3.2 Metabolites of *T. longibrachiatum* extracted by liquid - liquid extraction (use of a separating funnel)

Small fractions of the crude *Trichoderma longibrachiatum* extracts were sent to the Tea Research Foundation of Kenya for bioassay against *A. mellis* and purified samples to the University of Manchester, Institute of Science and Technology, Britain for spectroscopic analysis (^1H -nmr, ^{13}C -nmr, ms).

The hexane extract (see 2.3.1) of *T. longibrachiatum* on separation and purification by preparative tlc produced fraction 2, a light yellow oil (1.7mg) with an Rf value of 0.4 in 25% chloroform in hexane. The ^1H -nmr spectrum showed large singlet peaks at δ 1.0, 1.4 and 1.65 ppm suggesting that the compound is likely to be a hydrocarbon.

The purified fraction obtained from the combined high running fractions of diethyl ether and ethyl acetate extracts (see 2.3.3) had an Rf value of 0.63 in 50% chloroform in hexane. The fraction showed a blue fluorescence under uv light. ^1H -nmr showed peaks at δ 0.8,

1.20 1.4 and 1.5 ppm and extremely low intensity peaks at 4.75 and 6.6ppm which could be due to impurities. The ^1H -nmr spectra of the purified fractions of hexane and ethyl acetate extracts suggested the possibility of aliphatic hydrocarbon compounds.

Since the extracts were obtained by the use of a separating funnel and the yields obtained were extremely small no further structural determination could be achieved and also the purity of the samples may be less than 100% as a result of the low quantities of the isolated samples.

3.3 Bioassay results of *T. longibrachiatum*

Table 9: Shows the effects of each extract of *Trichoderma* at 100ppm after 37 days of inoculation of room temperature on radial growth (measured as colony diameter in mm) of *A. mellea*.

Treatment	Colony size (mm)
Control	39.60
Hexane extract	37.80
Diethyl ether extract	36.20
Ethyl acetate extract	39.00

Coefficient of variation (CV) % = 3

Least significant difference (LSD P=0.05) = 1.20

The hexane extract as well as the diethyl ether extract exhibited small but significant effects on the radial growth of *A. niger*.

3.4 Trichoderma extracts by the improved liquid - liquid extraction technique

Hexane extract obtained from liquid - liquid extraction using purpose built extractor was chromatographically separated by preparative tlc using 25% chloroform in hexane. Four distinct fractions were further purified by preparative tlc to give compound A.

3.5 Spectral Analysis of compound A

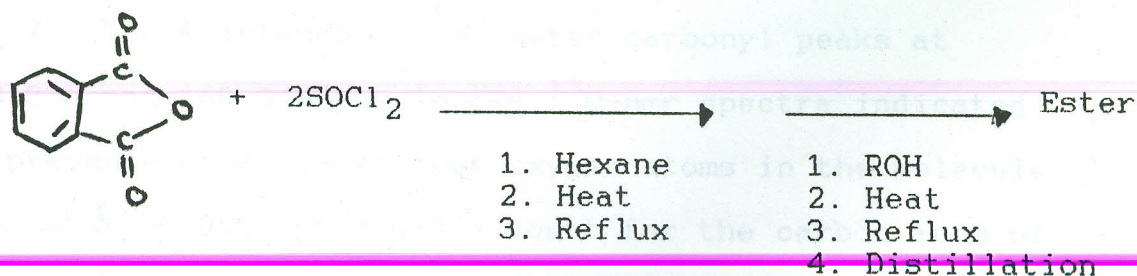
Compound A, a pale yellow oil (see 2.5.1) with an Rf value of 0.57 (100% dichloromethane) showed a large reddish fluorescence which did not separate out when examined under different solvent systems.

The compound showed a molecular ion at m/e 418 and a base peak at 149 in its mass spectrum (fig 2). The mass spectral computer library search suggested a number of possible structures for the compound and the closest fit suggested by the computer was for diisononyl phthalate ester (see fig 3). Although not suggested by the computer the ms of dinonyl phthalate ester was also examined⁵⁸. This spectrum proved to be an even better fit for the unknown. The literature Rf value of dinonyl phthalate of 0.60⁵⁹ and this compared well with that observed for

compound A (0.57) under similar conditions. The base peak of 149 is a characteristic peak for almost all 1,2 - benzene dicarboxylic acid esters (Phthalate esters).

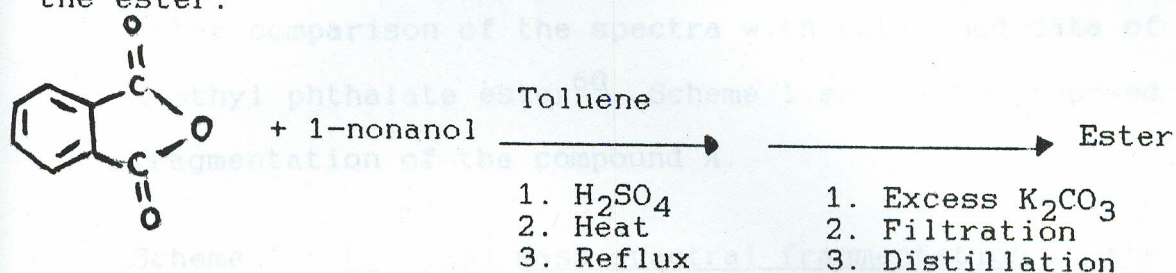
The ir spectrum (fig 4) gave a peak at 1721 cm^{-1} consistent with the presence of benzoate ester carbonyls ($\text{C}=\text{O}$ str, 1724 cm^{-1}) as conjugation of an aryl group with the carbonyl group causes the $\text{C}=\text{O}$ stretch to be at a lower than normal frequency (e.g aliphatic esters absorb about $1750\text{--}1735\text{ cm}^{-1}$)⁶⁰. Peaks at 1267 cm^{-1} and 1167 cm^{-1} are due to the $\text{C}-\text{O}$ stretch. The presence of a strong absorption at 739 cm^{-1} ($\text{C}-\text{H}$ def) further supported the presence of an ortho substituted benzene ring⁵⁹.

In order to confirm the identity of the compound A, dinonyl phthalate was synthesised. Initially thionyl chloride (2 moles) was reacted with phthalic anhydride (1 mole) in the presence of hexane (solvent), 1-nonanol was added to the unpurified diacid chloride in the hope of forming the diester.



but the method did not work as the end product obtained was a dark mass of intractable material.

A second method was devised for the synthesis of the ester.



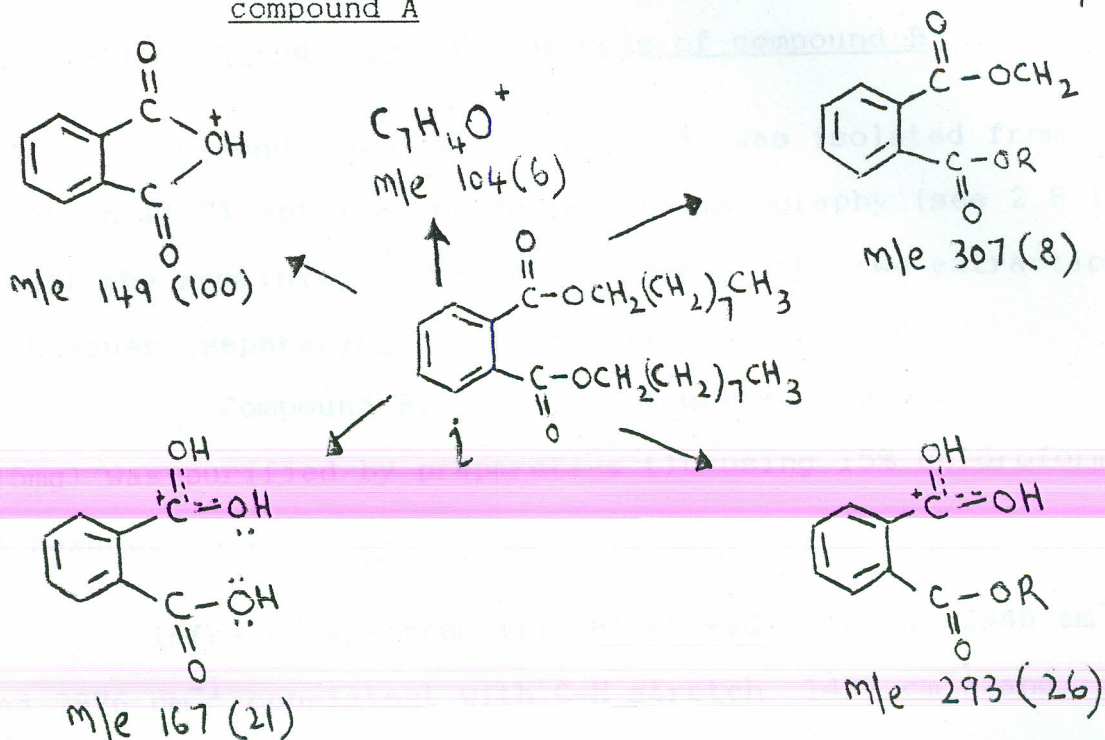
The ester obtained had a m.p range 174-176°C.

The ir spectrum (fig 5) as well as the mass spectrum of the synthesised dinonyl phthalate compared extremely well with those of compound A. Their Rf values were close within experimental error as the Rf value of the synthesised was 0.58. The slight difference in the Rf values could be due to a range of factors such as quality and activation grade of the stationary phase, humidity, quality of the solvent, impurities, tank saturation, technique used, development distance, temperature and amounts of samples.

The ^{13}C -nmr spectra (fig 6) of the synthesised compound was consistent with that given by ^1H -nmr spectra (fig 7). The existance of two ester carbonyl peaks at δ 168.835 and 168.348 ppm in the ^{13}C -nmr spectra indicated the presence of at least four oxygen atoms in the molecule. Peaks at δ 136.955 ppm could account for the carbon atom of the benzene ring. The two peaks at δ 65.066 and 65.452 ppm were assigned to the methylenes attached to the ether oxygen of the ester group.

The ^{13}C -nmr spectra assignments were arrived at after comparison of the spectra with published data of diethyl phthalate ester⁶⁰. Scheme 1 shows the proposed fragmentation of the compound A.

Scheme 1. Proposed mass spectral fragmentation of the compound A



Therefore based on the evidence presented structure (i) was proposed for the compound A.

Dinonyl phthalate is a very rare natural product but has been found in the aerosol samples of forest sites⁶¹. It is mainly used as a stationary phase in gas - liquid chromatography as well as a major oil in the procedure for the measurements of distribution spaces in the rat epididymal fat cells in which fat cells which had passed dinonyl phthalate exhibited normal lipogenesis from glucose

and normal sensitivity to insulin⁶². Dialkyls phthalates are mainly used in the high polymer technology as plasticisers.

Hence it is both unusual and interesting in obtaining dinonyl phthalate as one of the compounds exuded from *Trichoderma* species.

3.6 Isolation and spectral analysis of compound B

A second compound (compound B) was isolated from fraction (1-7) obtained by column chromatography (see 2.8.1) of all the remaining extracts obtained by the two extraction techniques (separating funnel and liquid - liquid extraction). Compound B, a clear colourless viscous oil (15mg) was purified by preparative tlc using 15% chloroform in hexane.

Infrared spectrum (fig 8) showed peaks at 2940 cm^{-1} and 2876 cm^{-1} consistent with C-H stretch, 1457 cm^{-1} and 1375 cm^{-1} due to C-H deformations. The lack of strong absorption bands in the $900\text{--}650\text{ cm}^{-1}$ region generally indicates a non-aromatic structure. The ir spectrum had no absorption bands in the assigned ranges expected for the functional groups such as carbonyl and hydroxyl indicating the absence of such groups in the molecule for example the absence of absorption in the $1850\text{--}1540\text{ cm}^{-1}$ region excludes a structure containing a carbonyl group⁶⁰.

Further evidence to identify the compound by the analysis of ^{13}C -nmr (fig 9) and the Dept spectra (fig 10) showed signals peaks at chemical shifts at δ 14, 19, 23, 26, 29, 32, and 37 ppm only consistent with the presence of aliphatic methyls and methylene carbon atoms of a long alkyl chain⁶². Lack of absorption peaks in the characteristic region confirms the absence of carbon containing functional groups.

^1H -nmr spectrum (fig 11) showed unresolved multiplets at δ 0.9 and a singlet at δ 1.3 ppm indicative of methyls and methylene protons. Absence of peaks in the downfield region (high δ values) suggests the absence of deshielded protons.

The mass spectra, both eims (fig 12) and cims (fig 13) showed a similar fragmentation pattern characterised by clusters of peaks and the corresponding peaks of each cluster separated by 14 (CH_2) mass units. The most abundant fragments are at C_3 and C_4 and the fragment abundance decreasing in a smooth curve making it difficult to get a clear value of the molecular ion M^\dagger or $\text{M} + 1$ or even $\text{M} - 1$. The presence of intense fragment peaks at $\text{M} - 1$ ($\text{C}_n\text{H}_{2n-1}$) such as 419, 279, 111 suggests the possibility of some cyclic hydrocarbons (C_nH_{2n})⁶³.

The presented data together with the nature of the compound (a clear viscous oil) indicates that the compound

could contain more than twenty carbon atoms in its structure⁶⁴.

Hence compound B could be a long chain saturated (aliphatic) hydrocarbon with the possibility of cyclic structures present.

3.7 Metabolites extracted from liquid - liquid extraction (use of a purpose built extractor) (see 2.4)

Table 10 shows the bioassay results of *T. harzianum*

Table 10: Shows the effect of *Trichoderma harzianum* on the radial growth (mycelial) of *A. mellea*.

: Treatment mean	: <u>Colony size (mm)</u>					
	: <u>Time of bioassay (days)</u>					
:	: 7 th	: 10 th	: 14 th	: 16 th	: 21 st	: 28 th
: Control	: 4.95	: 9.4	: 13.9	: 16.1	: 20.5	: 26.7
: Hexane (4)- extract	: 5.8	: 10.2	: 15.2	: 17.6	: 22.2	: 28.9
: Hexane (4) mycellium	: 3.4	: 7.4	: 13	: 15.2	: 20.6	: 27.2
: Diethyl ether (4) extract	: 4.4	: 8.2	: 12.6	: 15	: 18.6	: 23.2
: Methanol (4) extract	: 4.6	: 8.6	: 12.2	: 13.6	: 18.4	: 25.4

TABLE 10 CONTD...

Statistical treatment						
C.V %	22.6	12	8.1	7.1	6.8	7.6
LSD (P=0.05)	1.09	1.11	1.12	1.14	1.4	2.07

Note:

The assay was done on 6th June, 1993.

The diethyl ether extract of the culture broth together with the hexane, diethyl ether and the methanol mycellia inhibited the radial growth of *A. mellea* to different extents. The radial growth of *A. mellea* in the presence of the extracts showed a small but significant reduction in the growth (P=0.05) when compared with the control. The diethyl ether extract showed the highest significant (P=0.05) difference in the reduction of the mycelial growth.

The hexane extract had negative antibiotic activity as it induced further growth of the *A. mellea* as compared to the control.

The antibiotic activity decreased as the number of days (from the inoculation day) increased for hexane mycellium and methanol extracts. The ether extract continued showing some significant differences.

3.8 Isolation and spectral analysis of compound C

A third compound was isolated from the dark brown ether extract (see 2.8.2). Recrystallisation of the ether extract from acetone gave compound C which was a colourless crystalline solid. The compound had an R_f value of 0.58 (EtoAc\CHCl₃, 1:19 v\v) suggesting that the compound is fairly polar. The compound started to sublime above a temperature of 150°C indicating that the compound is a volatile compound.

The infrared spectrum (fig 14) suggested the presence of hydroxyl groups as it contained a broad band at 3356-2386 cm⁻¹ (O-H str of dimers), a strongly hydrogen bonded carboxylic acid or ester carbonyl (C=O str) was indicated by the absorption band at 1684 cm⁻¹ and strong absorption bands at 1390, 1290 and 1180 cm⁻¹ are consistent with C-O str or O-H def.

The ¹³C-nmr (fig 15) gave a singlet peak at δ 174 ppm corresponding to the carbon in carbonyl groups of carboxylic acids (δ 166-181 ppm)⁶². A singlet peak superimposed on the septet signal of the CD₃ group of the solvent acetone-d₆ at δ 29.1 ppm corresponding to the methylene carbon atom attached to a carboxylic group and a CH₂(-CH₂-CH₂-COOH) as the calculated chemical shift for a methylene carbon atom in a similar chemical environment is δ 28.7 ppm.

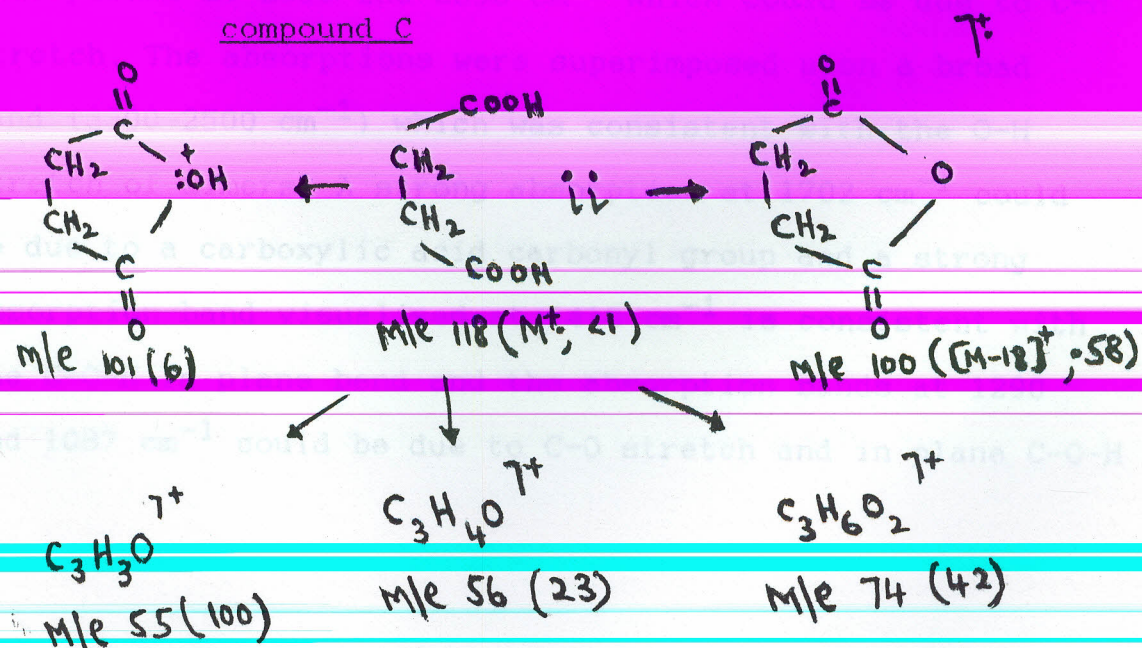
^1H -nmr spectrum (fig 16) showed a singlet peak at δ 2.6 ppm corresponding to the methylene protons influenced by a carboxylic acids attached to both the α -carbon atom and β -carbon atom in $\text{HO}_2\text{C}-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$ as the calculated chemical shift for a similar methylene proton is δ 2.5ppm. The chemical shift for a proton attached to a carboxylic acid group could not be visualised as the ^1H -nmr spectrum was not examined in the region of values higher than 9.0 ppm.

Both the ^1H -nmr and ^{13}C -nmr showed simple spectra of two singlet peaks for the entire compound suggesting the possibility of a high degree of symmetry present in the compound.

The mass spectrum, eims (fig 17) gave weak peaks at m/e 148 and 118. According to the ir spectrum, the functional groups present in the compound are O-H, C=O and C-O thus the possible structures for a mass ion of 148 are $\text{C}_6\text{H}_{12}\text{O}_4$ with a double bond equivalence (DBeq) of one, $\text{C}_7\text{H}_{16}\text{O}_3$ (DBeq = 0), $\text{C}_8\text{H}_4\text{O}_3$ (DBeq = 7) $\text{C}_9\text{H}_8\text{O}_3$ (DBeq = 6) and $\text{C}_{10}\text{H}_{12}\text{O}$ (DBeq = 5). $\text{C}_7\text{H}_{16}\text{O}_3$, $\text{C}_9\text{H}_8\text{O}_3$ and $\text{C}_8\text{H}_4\text{O}_3$ were discarded due to having no DBeq for $\text{C}_7\text{H}_{16}\text{O}_3$ and the other two for being highly conjugated and not supported by the ir spectrum due to the absence of alkenic absorption bands at around 1650 cm^{-1} (C=C str), this is also confirmed by the ^1H -nmr and ^{13}C -nmr spectra. As for $\text{C}_{10}\text{H}_{12}\text{O}$ not only does it have a high DBeq of 5 but also eliminates the presence of

an O-H and a C=O group being present in the same compound. The $C_6H_{12}O_4$ (DBEq = 1) is a possible structure but may have many C-O bonds. The m/e 148 peak is also not supported by the fragmentation pattern of the compound as m/e 100 (58%) a major peak could not be accounted for from the supposedly M^+ of 148 as a fragment ion of mass 48 [C_4 , CH_4O_2 , O_3] seems highly unlikely from the stated possible structures of M^+ (148) hence the m/e 148 peak was considered to be contributed by an impurity. The peak at m/e 118 was thus considered to be the molecular ion as it was completely supported by the fragmentation pattern of the compound as a peak at m/e 100 [$M-18$] $^+$ (58%) corresponding to the loss of water molecule. A peak at 72(4%) as a result of [$M-46$] $^+$ could be attributed to the loss of H_2O and CO groups. The peak at m/e 56(23%) could be due to the ketene fragment [C_3H_4O] $^+$ and the base peak at m/e 55(100%) consistent with [C_3H_3O] $^+$ fragment. Scheme 2 shows the proposed mass spectral fragmentation of compound C.

Scheme 2: Proposed mass spectral fragmentation of the compound C



Therefore based on the evidence presented, structure (ii), succinic acid (butanedioic acid) was proposed for compound C.

Confirmation of the above structure was made by comparing an ir spectrum of succinic acid (fig 18) obtained by the KBr disc technique. The ir spectra of the succinic acid and the extracted succinic acid were identical thus confirming the structure of the compound C as to that of succinic acid (butanedioic acid).

3.9 Isolation and spectral analysis of compound D

A fourth crystalline substance (compound D) was isolated from the hexane mycellium extract (see 2.9.1). This extract on dissolving in chloroform followed by standing in a deep freezer deposited colourless waxy crystals which on recrystallisation displayed a m.p of 46.0-46.3°C.

The infrared spectrum (solid state, fig 19) showed absorptions at 2910 and 2850 cm^{-1} which could be due to C-H stretch. The absorptions were superimposed upon a broad band (3300-2500 cm^{-1}) which was consistent with the O-H stretch of dimers. A strong absorption at 1702 cm^{-1} could be due to a carboxylic acid carbonyl group and a strong absorption band visualised at 1410 cm^{-1} is consistent with the C-O-H in plane bend and the absorption bands at 1290 and 1087 cm^{-1} could be due to C-O stretch and in plane C-O-H

bending of dimers and an O-H out of plane bend at 937 cm^{-1} . As the overall characteristic of the ir spectrum was similar to those of long chain carboxylic acids, the melting points of the saturated straight chain carboxylic acids were compared with compound 4 in the table 11 below.

Table 11: Comparison of the melting points of saturated straight chain carboxylic acids with compound 4

Aliphatic acid	Molecular Formular	M.P ($^{\circ}\text{C}$)
Decanoic acid	$\text{CH}_3(\text{CH}_2)_8\text{COOH}$	32°C
Undecilic acid	$\text{CH}_3(\text{CH}_2)_9\text{COOH}$	28.3°C
Dodecanoic acid	$\text{CH}_3(\text{CH}_2)_{10}\text{COOH}$	44°C
Tridecanoic acid	$\text{CH}_3(\text{CH}_2)_{11}\text{COOH}$	$45-46^{\circ}\text{C}$
Compound 4		$46-46.3^{\circ}\text{C}$
Tetradecanoic acid	$\text{CH}_3(\text{CH}_2)_{12}\text{COOH}$	54°C
Octadecanoic acid	$\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$	68.8°C

It can thus be observed that tridecanoic and dodecanoic acid have melting points similar to compound 4.

Comparison of the infrared spectra of compound 4 and some aliphatic acids revealed that compound 4 had significantly more peaks in the fingerprint region than standard ir spectra of tetradecanoic (myristic) and octadecanoic (stearic acids) indicating that other structural features may be present in compound D. An

extremely small absorption at 1634 cm^{-1} incorporated with the C=O str absorption at 1702 cm^{-1} could be due to C=C str and a strong band at 716 cm^{-1} (out of plane C-H def) which could be consistent with cis alkene absorptions ($1662\text{--}1626\text{ cm}^{-1}$ and $730\text{--}665\text{ cm}^{-1}$ respectively)⁶⁰. There was a slight evidence in support of the presence of an alkenic double bond by the medium absorption band at 1410 cm^{-1} which could correspond to C-H def of alkenes.

Comparison of the melting point of compound D with some unsaturated monocarboxylic acids revealed that the melting point of compound D ($46\text{--}46.3^\circ\text{C}$) was relatively close with those of 11-octadecenoic acid (44°C) and 9-octadecenoic acid (45°C). The unsaturation nature of compound D was further confirmed by the decolourisation of bromine water.

The mass spectrum of compound D was obtained under both chemical ionisation (cims, fig 20) and electron impact (eims, fig 21) conditions. Molecular ion peaks are usually difficult to observe for long chain carboxylic acids by the eims technique as carboxylic acids easily lose their carboxylic group upon electron impact hence the greatest peak in the eims spectrum was observed at $m/e\ 302$ Daltons with a base peak at $m/e\ 256$ Daltons. As for the chemical ionisation mass spectrum which usually produces simpler spectra as it gives either the molecular ion peaks $[M]^+$ or prominent quasi-molecular ion peaks $[M-1]^+$,⁶⁰ the CI gave a

wide range of peaks with the highest peak at m/e 419 which could be due to $[M-1]^+$ hence the $[M]^+$ would be at m/e 420. A very weak peak at m/e 420 is present in the cims spectrum. The base peak in the CI mass spectrum is at m/e 274 and other major peaks at 300, 274 and 206. Assuming that the molecular ion correspond to a M^+ of a long monocarboxylic acid of relative molecular mass of 420, the only possible molecular formula will be $C_{28}H_{52}O_2$ (DBeq = 3). Examination of the major fragmentation ions $M-120$ (300); $M-146$ (274); $M-214$ (206); give the following possible formula for the fragment ions.

For the fragment of mass 120, the possible structural formula are as follows, $C_4H_8O_4$, $C_5H_{12}O_3$, C_8H_8O , C_9H_{12} and $C_7H_4O_2$. None of the above mentioned could be the possible structures for the fragment of 120 mass units from the M^+ of 420 as $C_4H_8O_4$ and $C_5H_{12}O_3$ have more oxygen atoms as compared to the M^+ . As the C_8H_8O , C_9H_{12} and $C_7H_4O_2$, they all have higher DBeq of 5, 4 and 6 respectively as compared to the DBeq of 3 for the M^+ .

For the fragment of mass 146, the possible structures could be $C_6H_{10}O_4$, $C_7H_{14}O_3$, $C_8H_2O_3$, $C_8H_{18}O_2$, $C_9H_6O_2$, $C_{10}H_{10}O$, $C_{11}H_{14}$ and $C_{12}H_2$. Again none of the stated could be fragments coming from the M^+ giving rise to the 274 fragment ion (base peak) as $C_6H_{10}O_4$, $C_7H_{14}O_3$ and $C_8H_2O_3$ are eliminated on the basis of having more oxygen atoms than the initial M^+ . The remaining structures can not arise

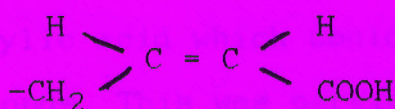
from the M^{\dagger} as they have high DBeq in comparison to the M^{\dagger} that is $C_9H_6O_2$ (DBeq = 7), $C_{10}H_{10}O$ (DBeq = 6), $C_{11}H_{14}$ (DBeq = 5) and lastly $C_{12}H_2$ (DBeq = 12).

As for the fragment of mass unit 214, the possible structures could be any of the following; $C_{11}H_{20}O_4$, $C_{12}H_{24}O_3$, $C_{12}H_8O_4$, $C_{13}H_{28}O_2$, $C_{13}H_{12}O_3$, $C_{16}H_8O$ and $C_{17}H_{12}$. Once again, none of the above could account for the fragment of mass units 214 as $C_{11}H_{20}O_4$, $C_{12}H_{24}O_3$, $C_{12}H_8O_4$ and $C_{13}H_{12}O_3$ have higher numbers of oxygen atoms than the M^{\dagger} . $C_{16}H_8O$ and $C_{17}H_{12}$ have greater DBeq of 4 and 12 respectively as compared to the M^{\dagger} as for $C_{13}H_{28}O_2$, this has zero DBeq suggesting that the presence of either etherial (-C-O-) or hydroxyl (C-O-H) bonds hence eliminating the existence of a carboxylic acid group.

On this evidence, the peak at m/e 420 is unlikely to be the M^{\dagger} ion of the compound (could be due to impurity) or that the assumption that the compound is a long chain carboxylic acid is incorrect.

The peak at m/e 302 is observed in both the eims as well as the cims mass spectra. Assuming that it represents the peak for the molecular ion M^{\dagger} . The peak at m/e 285 could be as a result of M-OH, peak at m/e 257 could be due to M-CO₂H and a further lose of a hydrogen atom giving rise to a major peak at m/e 256 (base peak) observed in the eims. There is also a characteristic peak at m/e 60

environment of δ 11 and 30 ppm respectively. Signal peaks at δ 14, 23, 27 and 29 could be due to carbon atoms in a long alkyl chain as the observed chemical shifts are equal to calculated chemical shifts. The singlet peak at δ 34 ppm could correspond to a CH attached to a branched ethyl group $-\text{CH}(\text{CH}_2\text{CH}_3)-$ as the calculated chemical shift for a carbon in a similar chemical environment is δ 35 ppm. A singlet peak at δ 32 ppm could be due to a methylene carbon atom attached to an alkenic carbon atom ($-\text{CH}_2-\text{CH}=\text{C}-$) as the chemical shift is close to the calculated shift of δ 32.4 ppm. The peaks at δ 130 and 102 ppm could correspond to the alkenic carbon atoms attached to carboxylic acid and CH_2 of a long alkyl chain



The ^1H -nmr spectrum (fig 23) showed a singlet peak at δ 5.3 ppm which could be due to alkenic proton as δ value for a proton in a similar chemical environment is around δ 4-10 ppm. Triplet peaks at δ 2.3 and 1.6 ppm which could correspond to methyl groups attached to alkenic carbon atoms in different chemical environments. The ppm (in the downfield region) could be due to a methyl group coupled with a neighbouring alkenic proton and the proton from the carboxylic acid group. The triplet at δ 1.6 ppm could be due to a methyl group coupled by the neighbouring ring alkenic

protons⁶⁰. A triplet peak at δ 0.9 ppm in the upfield region could actually be corresponding to normal terminal methyl protons. A doublet peak at δ 1.5 ppm could be due to a proton coupled by a single proton and a large signal peak at δ 1.2 ppm could correspond to methylene protons of a similar chemical environment. The chemical shift for a proton attached to a carboxylic acid group could not be visualised as the ¹H-nmr spectrum was not examined in the region of values higher than δ 9.0 ppm.

In the light of the facts presented, it was quite difficult to come up with a complete structure for compound D as the various spectra were not compatible with one another as the ir and ms spectra suggested the possibility of a long chain monocarboxylic acid which could be containing an alkenic double bonds. This was partially supported by the ¹³C-nmr as the spectrum suggested the possibility of a long chain α, β -unsaturated aliphatic carboxylic acids. As for ¹H-nmr, the spectrum not only suggested the existence of the alkenic double bonds but also suggested the possibility of a ring structure (possibly a steroid type) as well as long alkyl chain being present in the compound.

The uncertainty in the determination of the correct constitution of the mass ion and the fragment ions from the ms also made it difficult to interpret the ms spectral data. High resolution mass spectrum would have helped tremendously in confirming the partial

structures proposed but it was not available.

4.0 Isolation and spectral analysis of compound E

Compound E, a waxy solid was obtained by recrystallisation of the ether mycellium extract with hexane. The compound had a sharp melting point in the range of 51.6-52.4°C.

The infrared spectrum (fig 24) showed absorption bands at 2910 and 2850 cm^{-1} which could be due to C-H stretch. These absorptions were superimposed on a broad band (3500-2500 cm^{-1}) consistent with an O-H stretch of dimers. Absorption at 1732 cm^{-1} could correspond to an α,β -unsaturated ester or saturated 6-ring (δ -) lactone carbonyl group (C=O str). A strong absorption at 1702 cm^{-1} could be due to an α,β -unsaturated carboxylic acid carbonyl group (C=O str) as unsaturation in conjugation with carboxylic carbonyl group show a decrease in the frequency of normal aliphatic acids and thus shows absorptions in the dimers in the 1710-1680 cm^{-1} region. A medium absorption band at 1450 cm^{-1} could be due to CH def of alkenes. Absorption band at 1370 cm^{-1} could be consistent with the C-O-H in plane bend and other absorption bands at 1290 and 1109 cm^{-1} could be due to C-O stretch and in plane C-O-H bending of dimers and a medium absorption at 927 cm^{-1} could correspond to the O-H out of plane bend. A strong absorption at 716 cm^{-1} (out of plane C-H def) could be

reasonable for alkene absorptions. The unsaturation nature of compound E was confirmed by the decolourisation of bromine water.

The ^{13}C -nmr (fig 25) gave peaks which could correspond to carbon-carbon double bonds at δ 102, 128 and 130 ppm. A peak at δ 76.6 ppm could be due to a carbon atom attached to a hydroxyl group (C-OH) or an etherial group (-C-O-C-) respectively. The presence of a peak at δ 22.7 ppm could be due to a methyl ester. Peaks at δ 14, 23, 27 and 29 ppm could correspond to carbon atoms of a long alkyl chain successively. Two peaks at δ 24 and 25 ppm could be due to isobutane group. The peak at δ 33 could be due to a methylene carbon atom attached to the branched isobutane and the peak at δ 31 could be due to methylene carbon atoms attached to the alkenic carbon atoms.

The ^1H -nmr (fig 26) showed a double of doublet peaks at δ 5.4 ppm which could correspond to an alkenic proton which couples with a corresponding (neighbouring) alkenic proton and a carboxylic acid proton (-CH=CHCOOH). A multiplet peaks at δ 4.3 ppm may be due to an alkenic proton coupled with an alkenic proton, carboxylic acid proton (or a methyl carboxylate) and methylene protons in either $-\text{CH}_2-\text{CH}=\text{CHCOOH}$ or $-\text{CH}_2-\text{CH}=\text{CHCOOCH}_3$. A doublet peak at δ 3.5 ppm may be due to a methyl group in $\text{CH}_3\text{OOC}-\text{CH}=\text{C}-$. A quartet peak at δ 2.3 ppm and a triplet peak at δ 1.6 ppm may be due to a methyl group attached to alkenic carbon

atoms in different chemical environment. The δ 2.3 ppm signal could be due to $-\text{CH}_2-\text{CH}(\text{CH}_3)=\text{CH}-\text{CH}_2$ and δ 1.6 ppm could be due to a methyl group coupled by neighbouring ring alkenic protons. A large multiplet signal at δ 1.3 ppm could be corresponding to methylene protons of a similar chemical environment.

The mass spectrum, eims (fig.27) gave a weak peak at m/e 412 $[\text{M}]^+$ (2%) and a base peak at m/e 43 (100%). A peak at m/e 397 $[\text{M}-\text{OH}]^+$, 6% could be due to a loss of an OH group. A major peak at 367 (38%) could be due to $[\text{M}-\text{CO}_2\text{H}]^+$, another major peak at m/e 353 (18%) could be due to $[\text{M}-\text{CH}_2\text{CO}_2\text{H}]^+$, 339 $[\text{M}-(\text{CH}_2)_2\text{CO}_2\text{H}]^+$, 311 $[\text{M}-(\text{CH}_2)_4\text{CO}_2\text{H}]^+$, 283 $[\text{M}-(\text{CH}_2)_6\text{CO}_2\text{H}]^+$, 255 $[\text{M}-(\text{CH}_2)_8\text{CO}_2\text{H}]^+$ consistent with long chain aliphatic acids. There are also peaks corresponding to the various fragments. 43 $[\text{CH}_2\text{CO}_2\text{H}]^+$, 57 $[(\text{CH}_2)_2\text{CO}_2\text{H}]^+$, 101 $[(\text{CH}_2)_4\text{CO}_2\text{H}]^+$, 129 $[(\text{CH}_2)_6\text{CO}_2\text{H}]^+$ and 157 $[(\text{CH}_2)_8\text{CO}_2\text{H}]^+$ respectively. A characteristic McLafferty rearrangement peak at m/e 60 (40%) is also observed. The lower region of the ms spectrum is similar to a hydrocarbon spectrum due to the presence of a series of clusters at interval of 14 mass units (fragmentation of CH_2 units).

As can be deduced from the above presented facts, the ^1H -nmr, ir and ^{13}C -nmr are not consistent with the ms spectrum as ^1H -nmr, ir and ^{13}C -nmr suggests the possibility of the presence of both α,β -unsaturated conjugated carboxylic acid and methyl ester being present in the

compound E. In addition to partially supporting the ^{13}C -nmr and ir spectra, ^1H -nmr spectra also suggests the possibility of ring structures being present in the compound E. As for ms, the spectrum suggests a long alkyl chain aliphatic acid based on it's fragmentation pattern. Hence more spectral information is needed from other spectroscopic techniques such as Distortionless enhancement by polarisation transfer (Dept) and high resolution mass spectroscopy. The Dept spectrum could have eased the interpretation of the ^{13}C -nmr spectrum as it distinguishes between CH_3 and CH peaks (inverted down) or CH_2 (appear up). Quaternary carbon atoms give no signal in the Dept procedure thus Dept spectrum, also gives the number of ^1H -atoms attached to each carbon atom in a ^{13}C -nmr spectrum. As for high resolution ms, it would have confirmed the various partial structures proposed by the different spectral techniques (ir, ms, ^{13}C -nmr and ^1H -nmr). Unfortunately ^{13}C -dept and high resolution ms were not available.

4.1 Isolation and structural analysis of compound F

The fraction from methanol mycellium extract gave long pink threadlike needles which had an irritating smell similar to dried fish. The crystals were further recrystallised from 60% water in methanol giving compound F which was a colourless, crystalline solid. The compound F had an RF of 0.52 (29% methanol in chloroform, and developed in iodine tank) indicating that compound F was

reasonably polar. The compound was generally soluble in water but sparingly soluble in organic solvents.

On determining the melting point of the unpurified crystals, several changes were observed. The crystals first turned reddish at a temperature of 148°C and begun to charr at 220°C and by 250°C the crystals had charred completely (turned black) suggesting that it had a higher percentage of carbon atoms in it's composition.

The melting point of purified crystals was also determined and a similar observation was made. The crystals turned pale brown at 125.5°C and brown at 138.8°C . It started to charr at 145.1°C and by 200°C the crystals had charred completely suggesting the presence of a higher composition of carbon atoms.

The compound gave a positive test with ninhydrin as it gave a purple colouration. Normally it is only proteins, peptides and amino acids which give positive results (different colours) with ninhydrin⁶⁵.

The infrared spectrum (fig 28) was run as a mull of hexachloro-1,3-diene. The ir spectrum showed a broad band at $3650-2500\text{ cm}^{-1}$ which could be due to strong hydrogen bonding either due to O-H str or N-H str a sharp absorption at 2400 cm^{-1} , medium absorption at 1770 cm^{-1} and 1400 cm^{-1} may be due to C=O str of a possible amino acid carbonyl group.

The ^1H -nmr (fig 29) showed weak multiplet peaks at δ 3.8 ppm and a large signal for the presence of the impurity (HOD) in the solvent D_2O at δ 4.8 ppm.

The ^{13}C -nmr (fig 30) showed weak carbon signals at δ 101, 182 and 186 ppm only. This was quite surprising as a lot of the sample ($> 30\text{mg}$) had been utilised for this particular spectroscopic analysis and it would have been proper to have obtained better peaks for a highly concentrated sample but the weak carbon signals suggests the possibility of compound F being inorganic rather than organic.

The mass spectrum, eims (fig 31) showed a weak peak at m/e 256 (4%) which could be due to the molecular ion $[\text{M}]^+$, major peaks at 149 (38%), 136 (6%), 123 (6%), 111 (14%), 97 (34%), 83 (36%) and 69 (53%) and the lower region of the mass spectrum resembles the ms of a series of a hydrocarbon clusters as it shows peaks due to the fragmentation of CH_2 units of 14 mass units. Peaks at m/e 43 (100%, base peak), 55 (74%), 57 (93%), 71 (40%) and 85 (20%).

No other partial structures could be obtained from the available spectra. More of compound F is required for further chemical tests and structural activity studies in order to identify the nature of compound F as it seems to be a very interesting compound with major puzzling results

interesting in obtaining dionyl phthalate as one of the

since the ir suggested a possible amino acid compound which was supported by the positive ninhydrin test but the ^1H -nmr and ^{13}C -nmr spectra indicated that the compound might be inorganic in nature due to the absence of peaks in the upfield region of both ^1H -nmr and ^{13}C -nmr spectra which are normally observed for alkyl groups. The above even contradicts the compound's behaviour on ascertaining the melting point as it charred completely at around 250°C suggesting that compound F contained a high percentage of carbon atoms which is supported by the higher relative intensities of possible alkyl fragments as the lower region of the mass spectrum resembled that of a long chain hydrocarbon.

4.2 Conclusion and comments

The study established that *Trichoderma* species produces secondary metabolites that are bioactive against *A. niger* as the bioassay results showed that the hexane and diethyl ether extracts of *T. longibrachiatum* and diethyl ether, hexane mycellium and methanol extracts of *T. harzianum* had significant effects against the radial growth of both the mycellium and rhizormorphs of *A. niger*.

The compounds isolated from the hexane extracts of *T. longibrachiatum* were 1,2-dinonyl pthalate and an unknown long chain saturated (aliphatic) hydrocarbon attached to cyclic structures. It was quite unusual and at the same interesting in obtaining dinonyl pthalate as one of the

secondary metabolites of the *Trichoderma* isolate as it is a very rare natural product.

As for the various extracts of *T. harzianum*, diethyl ether extract produced Succinic acid (butanedioic acid) which is a very simple molecular compound. It is not surprising in isolating succinic acid in the *Trichoderma* isolates as it is a metabolic intermediate in the Kreb's cycle (tricarboxylic or citric cycle) as it participates in the biosynthesis of fumaric acid and succinyl co-enzyme A. Succinic acid is also a by product in the glyoxylate cycle which involves the anabolic conversion of acetate (oxaloacetate) to carbohydrates by bacteria, algae and higher plants.

The hexane diethyl ether mycellia and methanol extracts produced possible bioactive compounds whose structures could not be ascertained due to the conflicting spectral data obtained.

From this study, therefore more informative spectral data such as ^{13}C -Dept and high resolution mass spectroscopy are necessary for confirmation of partial structures (depicted by ir, ms, ^1H -nmr, ^{13}C -nmr) in order to establish a complete structure. Structural activity studies are also required in order to establish the class of the interesting compound isolated from the methanol extract.

Most reported studies of *Trichoderma* species resulted in high yields of extracts and ethyl acetate extracts were reported in most cases to be the most bioactive extracts as they showed significant inhibitory effects against a number of soil - borne pathogens unlike in this study where the yields of individual extracts obtained from large quantities of *Trichoderma* culture broths were extremely low and the ethyl acetate extract proved through bioassay to be the most inactive extract against the mycellial and rhizomorphal growth of *A. mellea*.

There were difficulties encountered with the slow and tedious liquid - liquid extraction technique which involved the use of a separating funnel a superior method of liquid - liquid extraction was sought which involved the use of a purpose built extractor. Even then, from large quantities of *Trichoderma* isolates, small quantities of pure samples were obtained which were just enough for the different spectroscopic analysis. Hence for further studies, a better liquid - liquid extraction technique has to be devised in order to ensure maximum extraction of samples so that further work can be done to establish the bioactivity of the compounds as all the compounds isolated from this study are only possible biological antagonists against the *Armillaria mellea* fungus.

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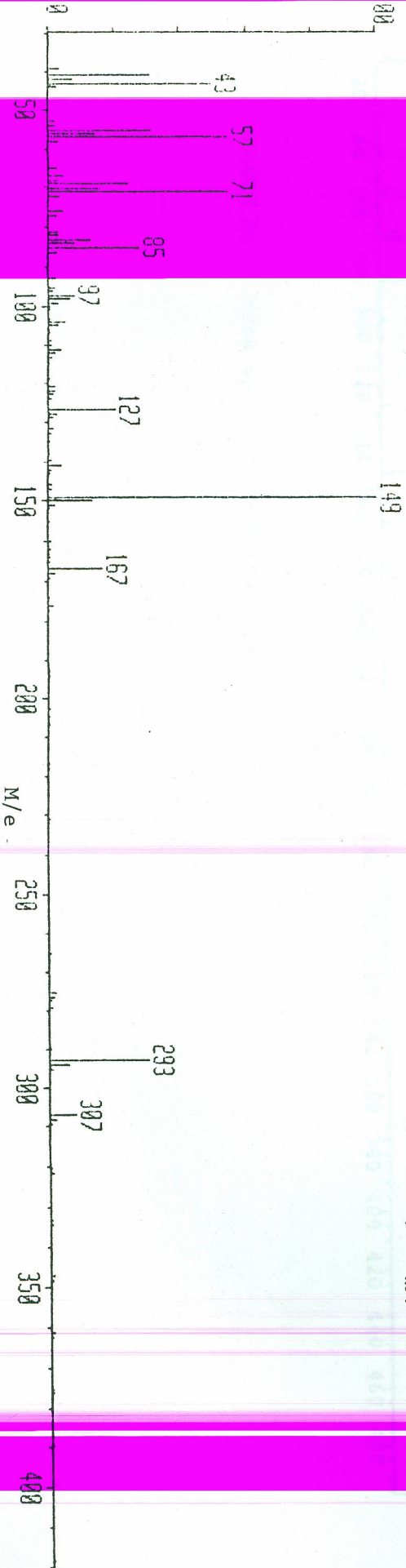


FIGURE 2: Mass spectrum of compound A

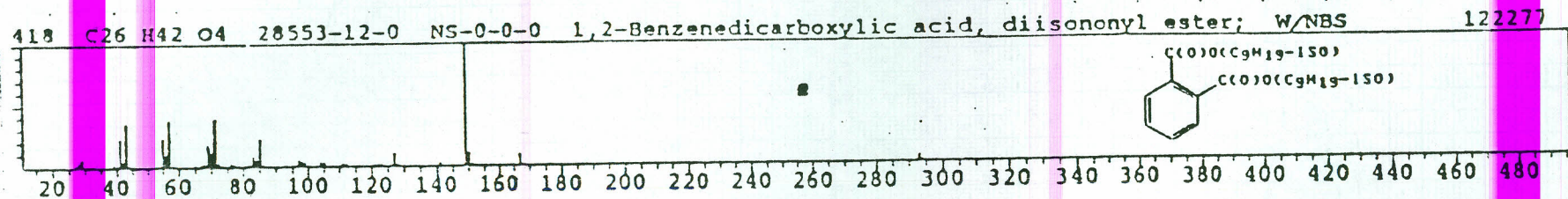


FIGURE 3: Mass spectrum of diisononyl phthalate

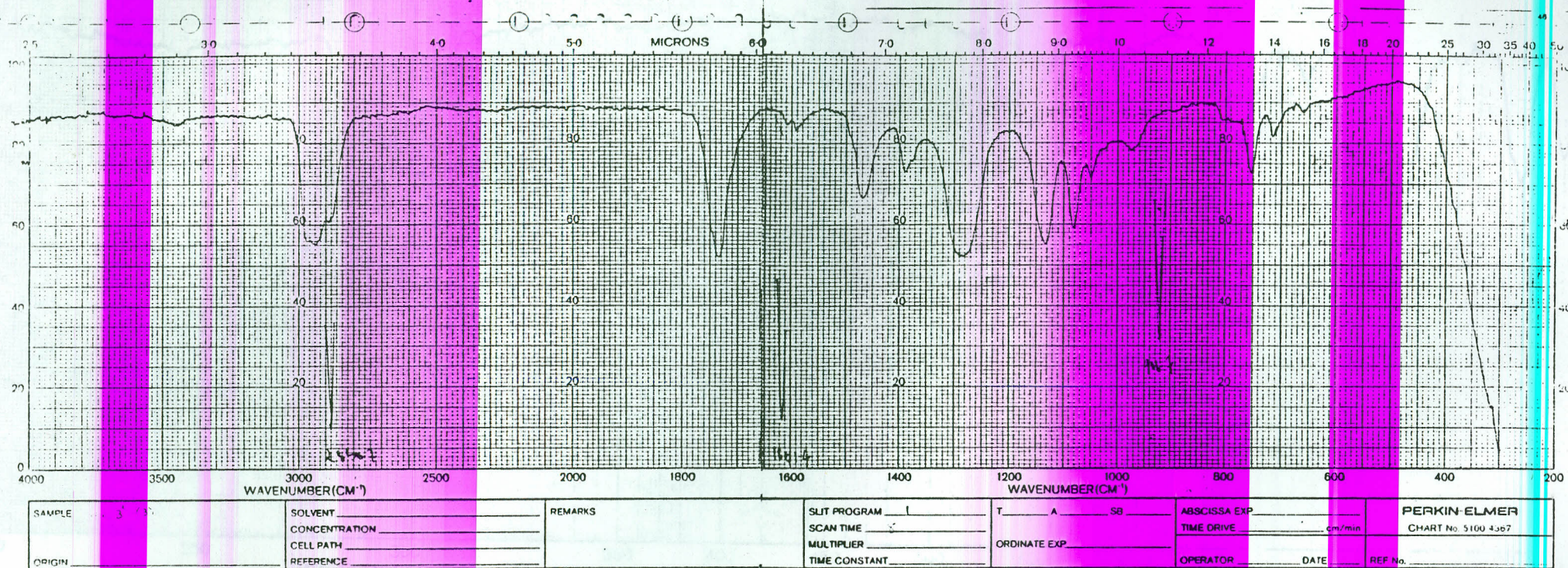
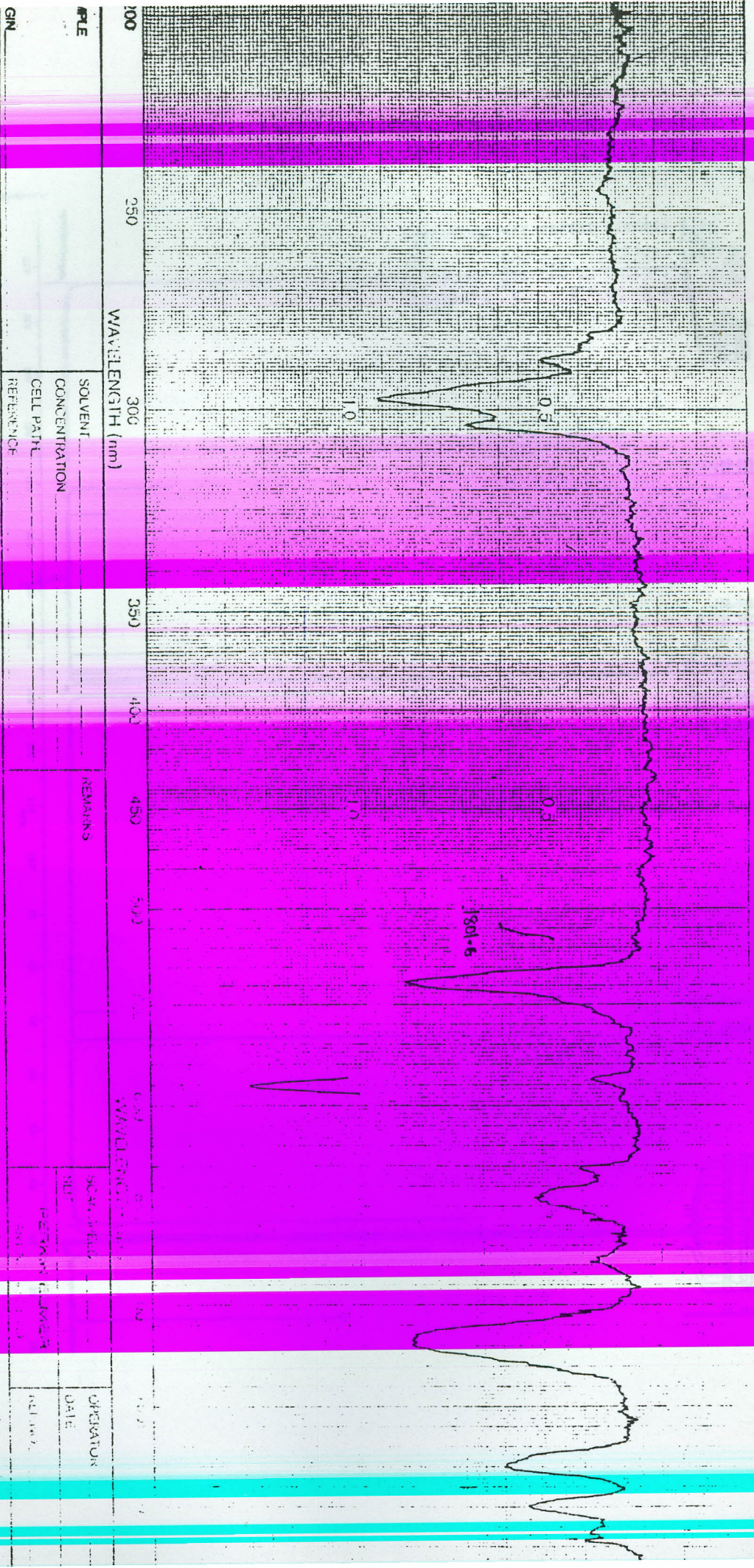


FIGURE 4: Ir spectrum of compound A



FILE _____
SOLVENT _____
CONCENTRATION _____
CELL PATH _____
REFERENCE _____

REMARKS _____

SEARCHED _____
SERIAL _____
INDEXED _____
FILED _____

OPERATOR _____
DATE _____
CELL NO. _____

FIGURE 5: Ir spectrum of the synthesised ester

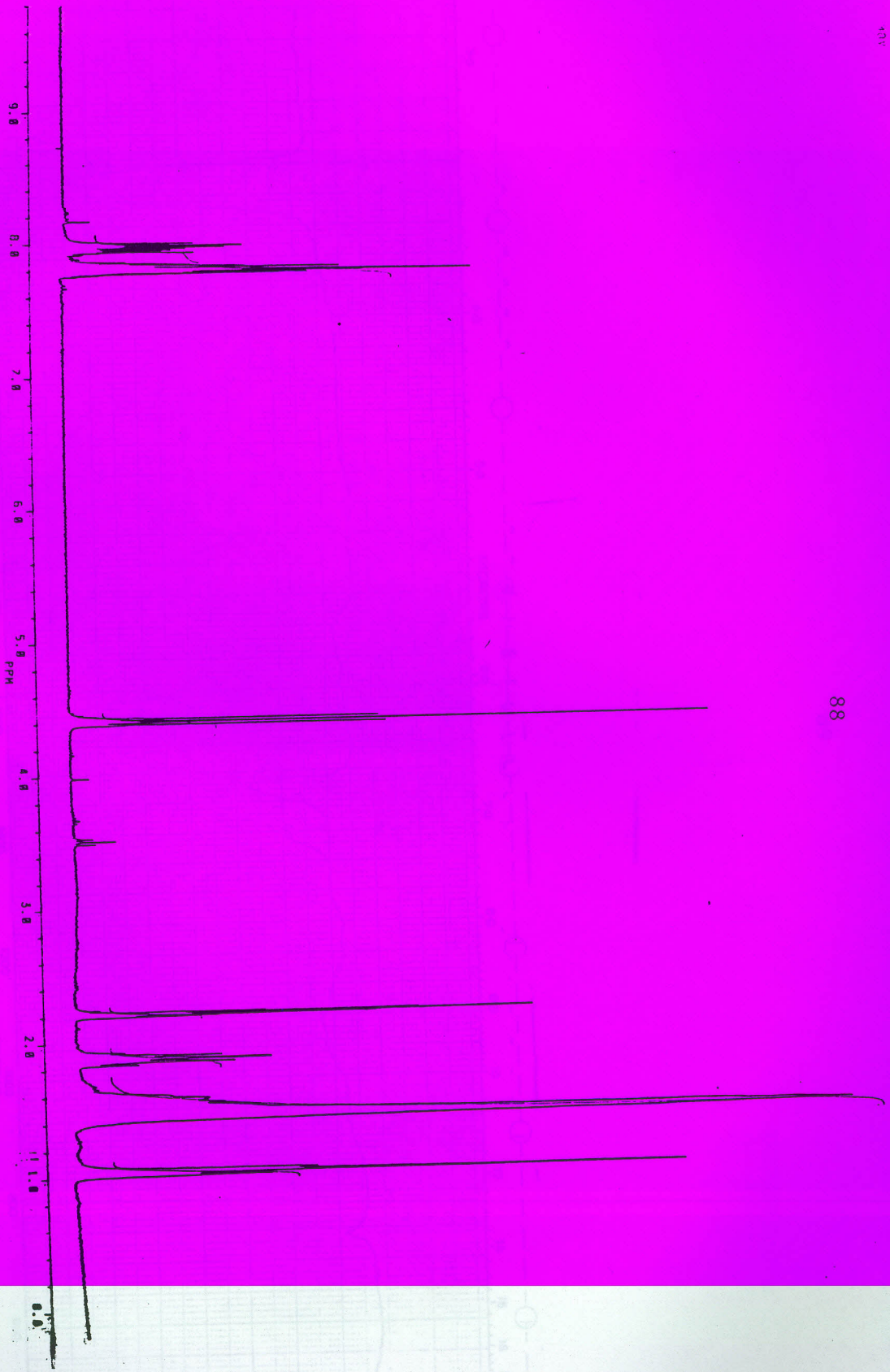
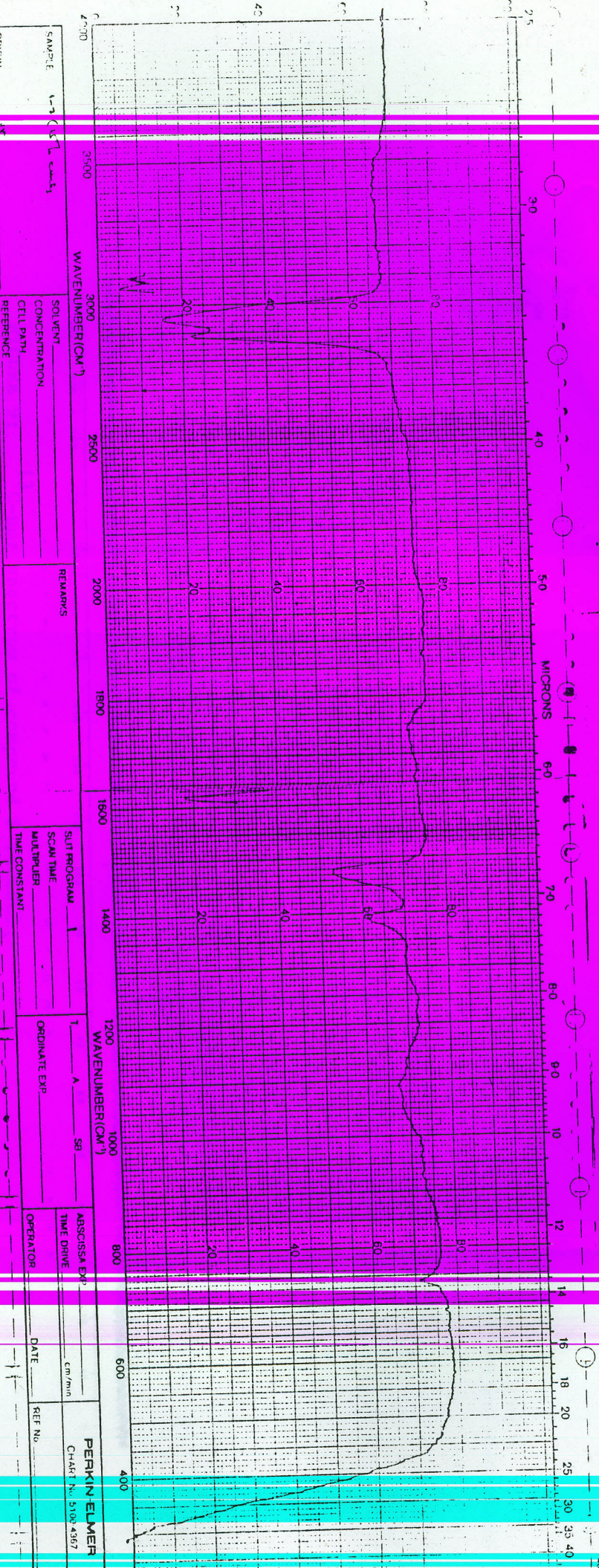


FIGURE 7: $^1\text{H-NMR}$ of the synthesised ester in CDCl_3



SAMPLE: <u>1-3 (ST) C-5</u> SOLVENT: _____ CONCENTRATION: _____ CELL PATH: _____ REFERENCE: _____	REMARKS: _____ SUI PROGRAM: <u>1</u> SCAN TIME: _____ MULTIPLIER: _____ TIME CONSTANT: _____	WAVELENGTH (CM⁻¹): 3500, 3000, 2500, 2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400	ABSORSSA EXP: _____ TIME DRIVE: _____ OPERATOR: _____ DATE: _____ REF No: _____
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FIGURE 8: Ir spectrum of compound B

27.112

90

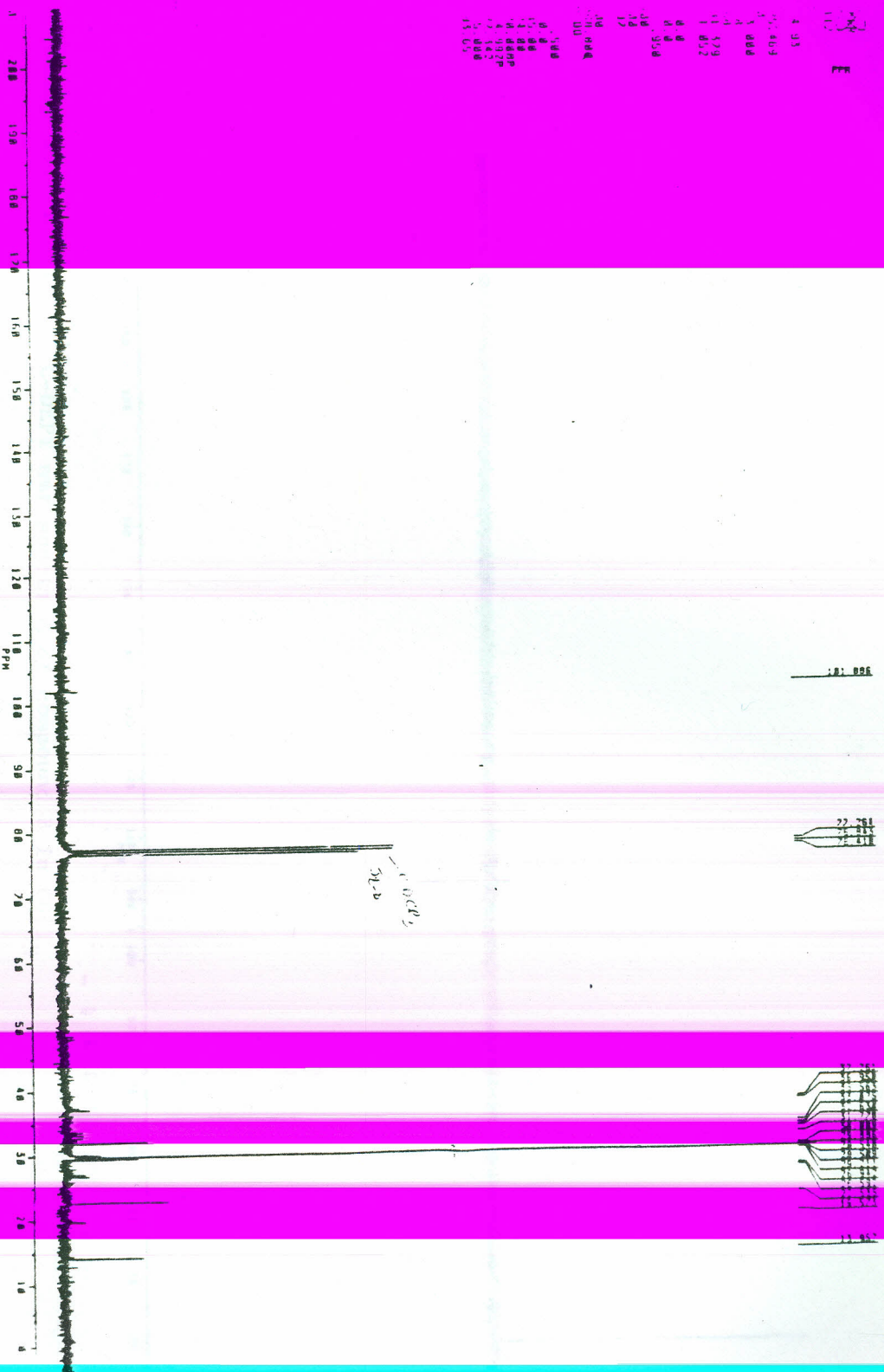


FIGURE 9: ¹³C-nmr spectrum of compound B in CDCl₃

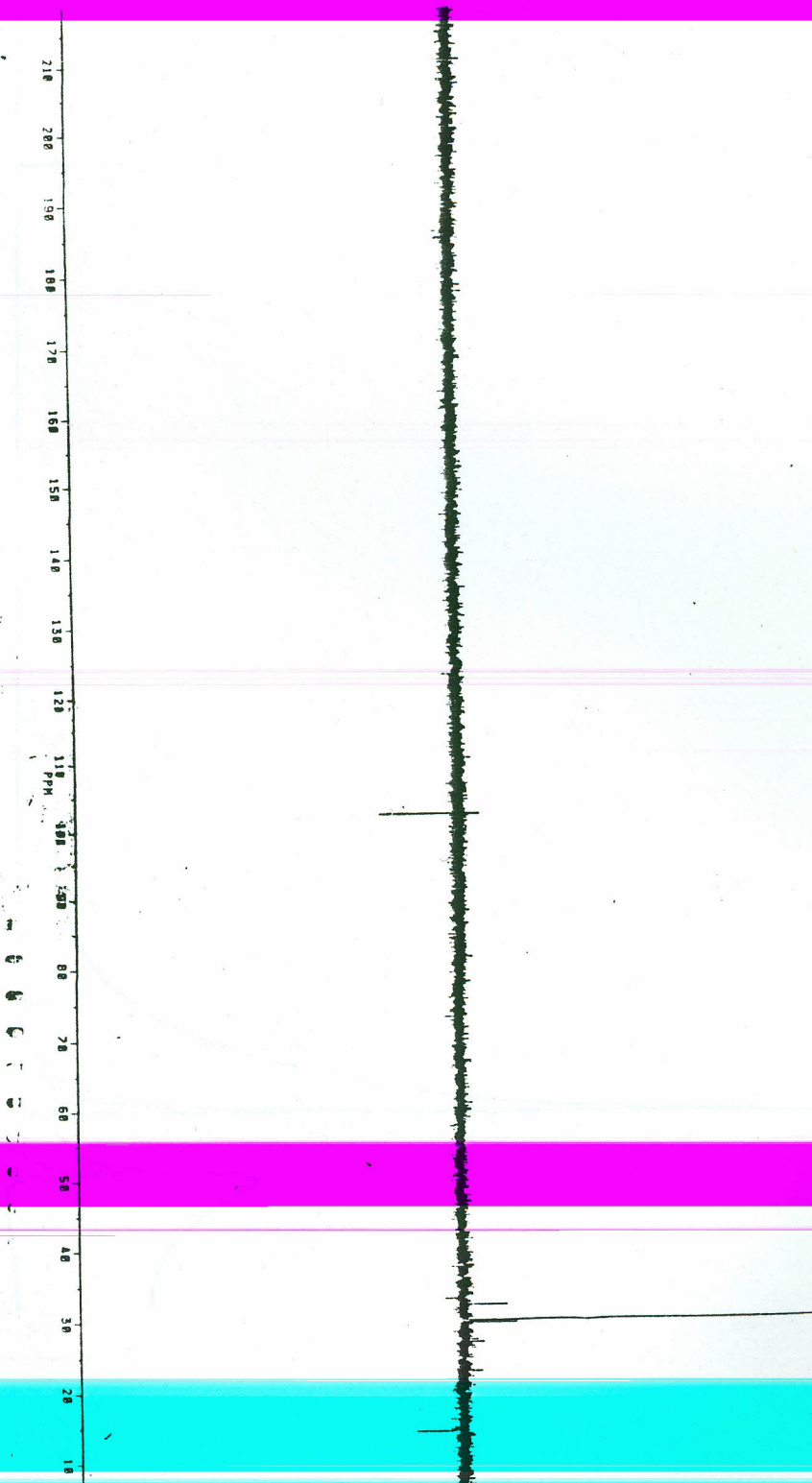


FIGURE 10: ^{13}C -DEPT nmr spectrum of compound B in CDCl_3

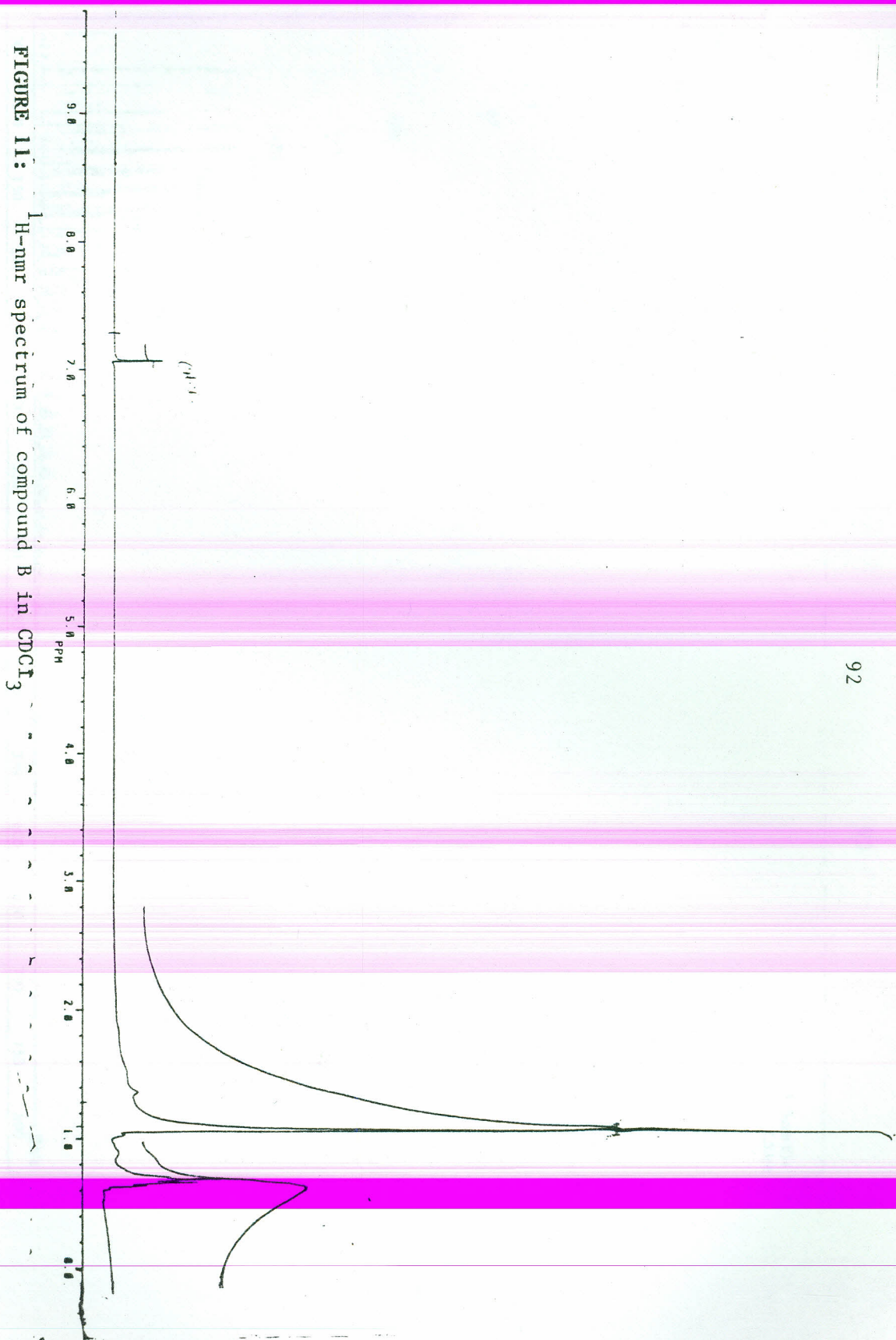




FIGURE 12: Mass spectrum (EI-MS) of compound B

1: Scan EI+
1.53e6

2 Scan Cl+
1.7465

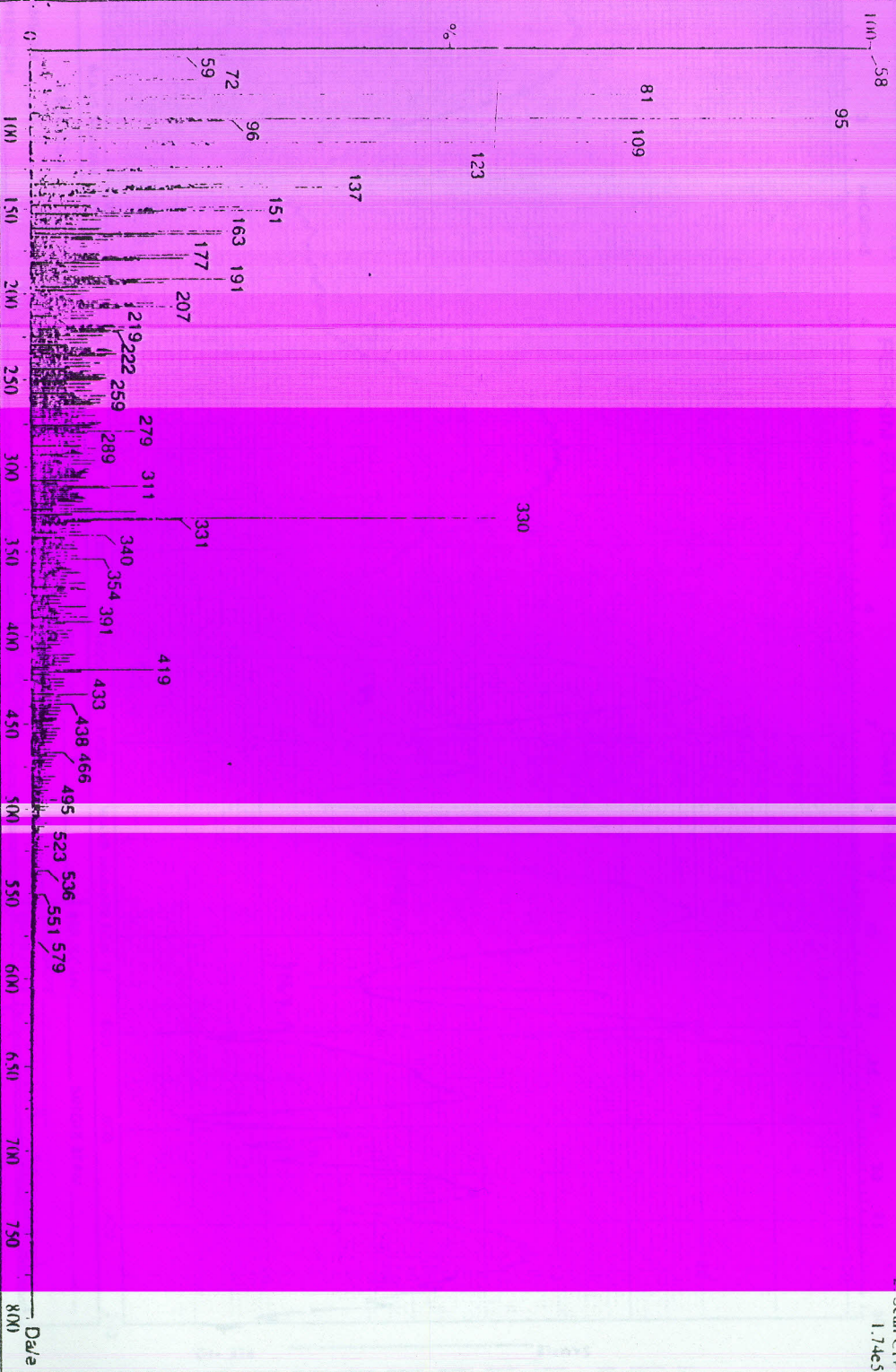


FIGURE 13: Mass spectrum (cims) of compound B

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WAVE NUMBER (CM ⁻¹) 4000 2500 2000 1800 1600 1400 1200 1000 800 600 400 200		WAVE NUMBER (CM ⁻¹) 2500 2000 1800 1600 1400 1200 1000 800 600 400 200	
ABSCISSA EXPANSION 0.5 SUPPRESSION		ORDINATE EXPANSION % T 85	
SAMPLE <u>EtOH</u> ORIGIN <u>Germany</u>		REMARKS SOLVENT CONCENTRATION	
SCAN TIME 12 MULTIPLIER SUT PROGRAM		REP. SCAN TIME DRIVE OPERATOR <u>Fuller</u>	
CELL PATH REFERENCE <u>EtOH</u>		SINGLE BEAM DATE <u>11/1/53</u>	

FIGURE 14: Ir spectrum of compound C



FIGURE 15: ^{13}C -nmr spectrum of compound C in CD_3COCD_3

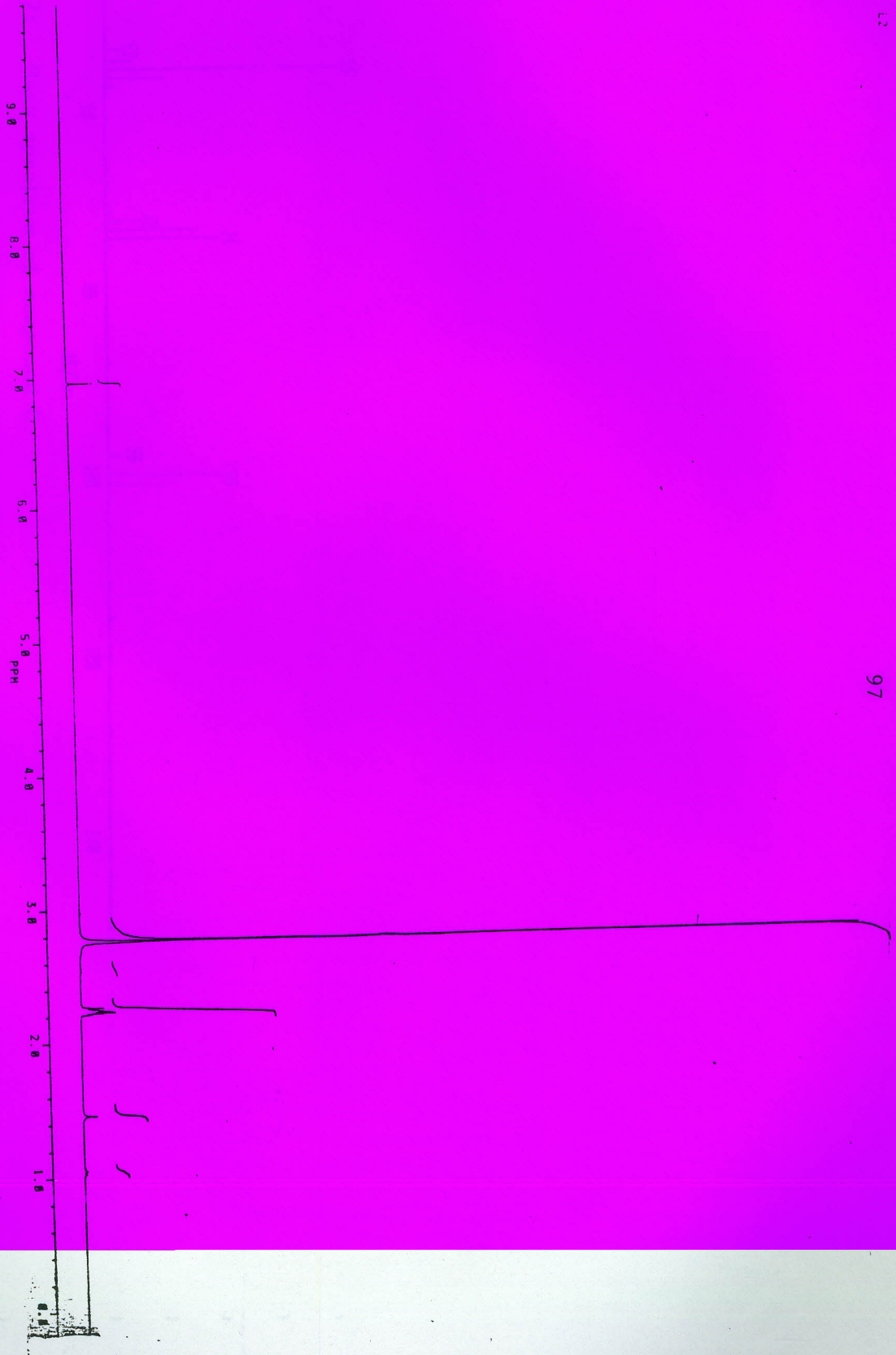


FIGURE 16: 1H-nmr spectrum of compound C in CD₃COCD₃

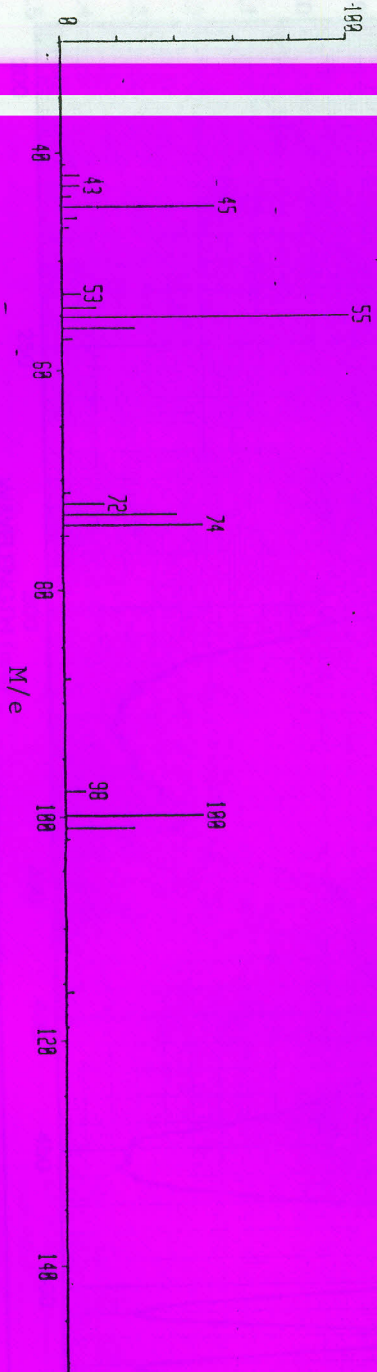
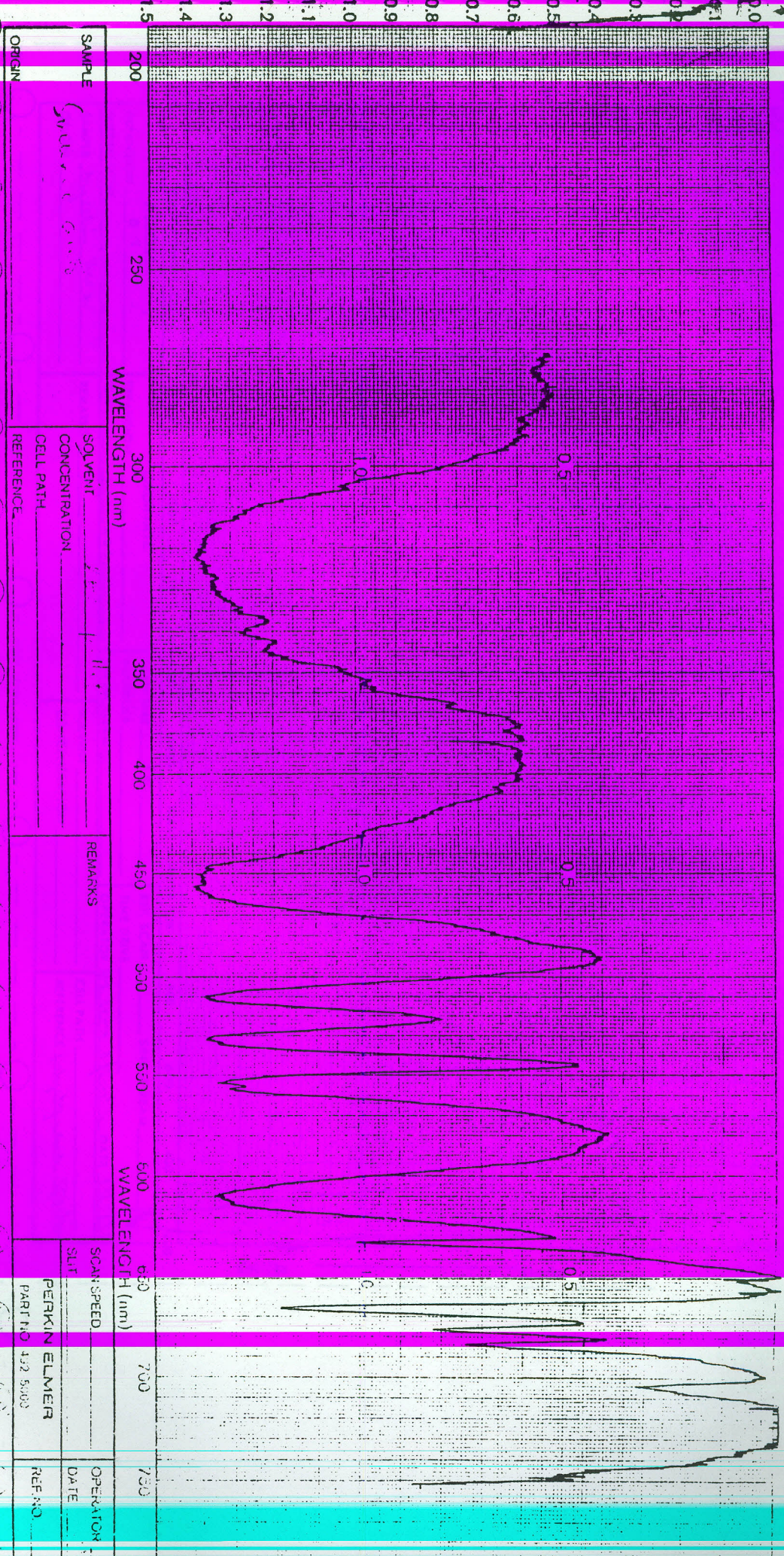


FIGURE 17: Mass spectrum of compound C



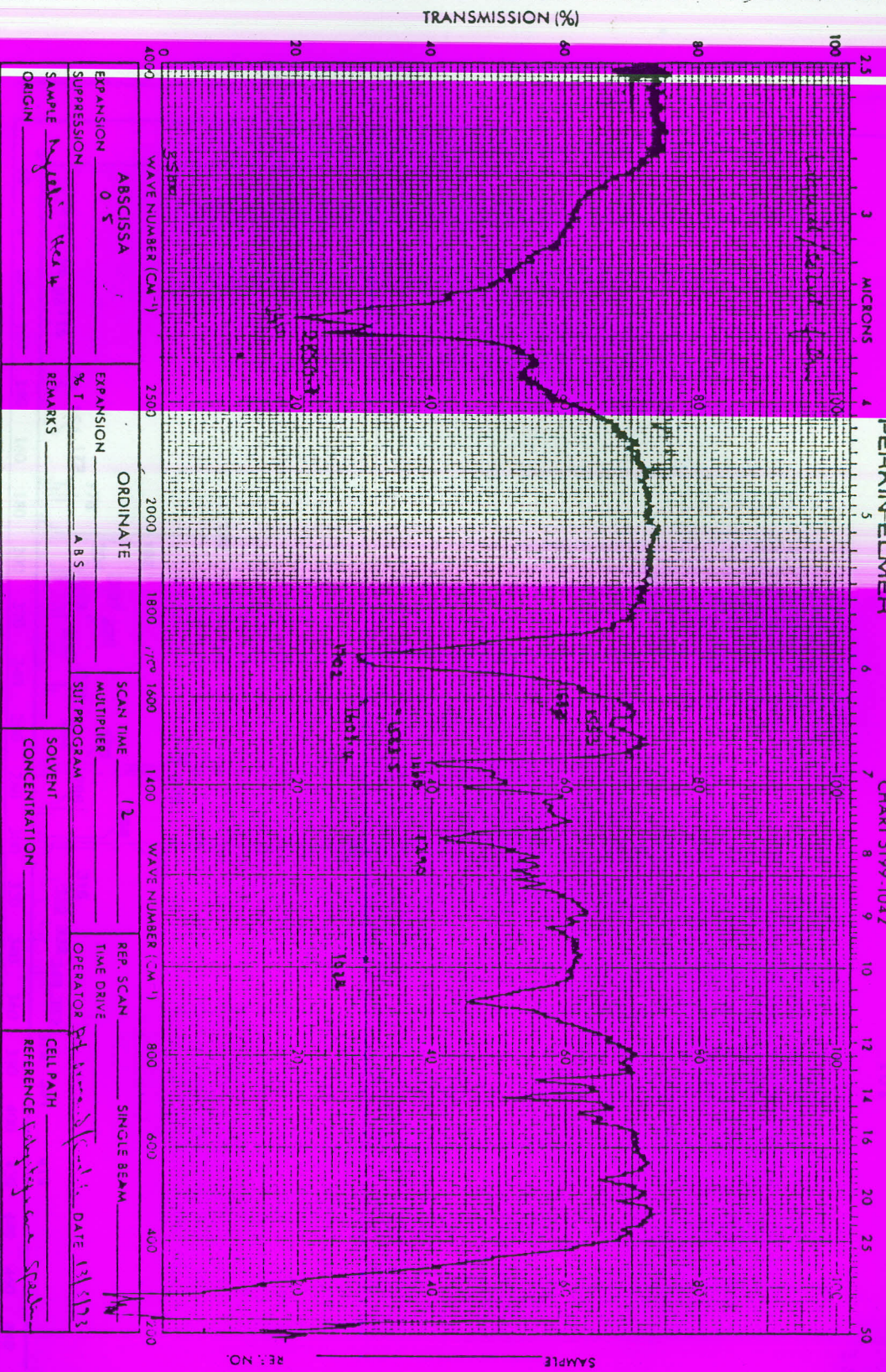
SAMPLE <i>Succinic acid</i>	SOLVENT	<i>CH₂Cl₂</i>	REMARKS
	CONCENTRATION	<i>1.1%</i>	
	CELL PATH		
	REFERENCE		
ORIGIN			
	SCANS/SPEED		OPERATOR
	SLOT		DATE
	PERKIN ELMER		REF. NO.
	PART NO. 102 5000		

FIGURE 18: Ir spectrum of succinic acid

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EXPANSION 0.5
 SUPPRESSION
 SAMPLE N-methylacetamide
 ORIGIN

ORDINATE
 % T
 A.B.S.

SCAN TIME 12
 MULTIPLIER
 SUT PROGRAM

REP. SCAN
 TIME DRIVE
 OPERATOR P. J. ... DATE 12/1/73

CELL PATH
 REFERENCE Acetylene Spade

FIGURE 19: Ir spectrum of compound D

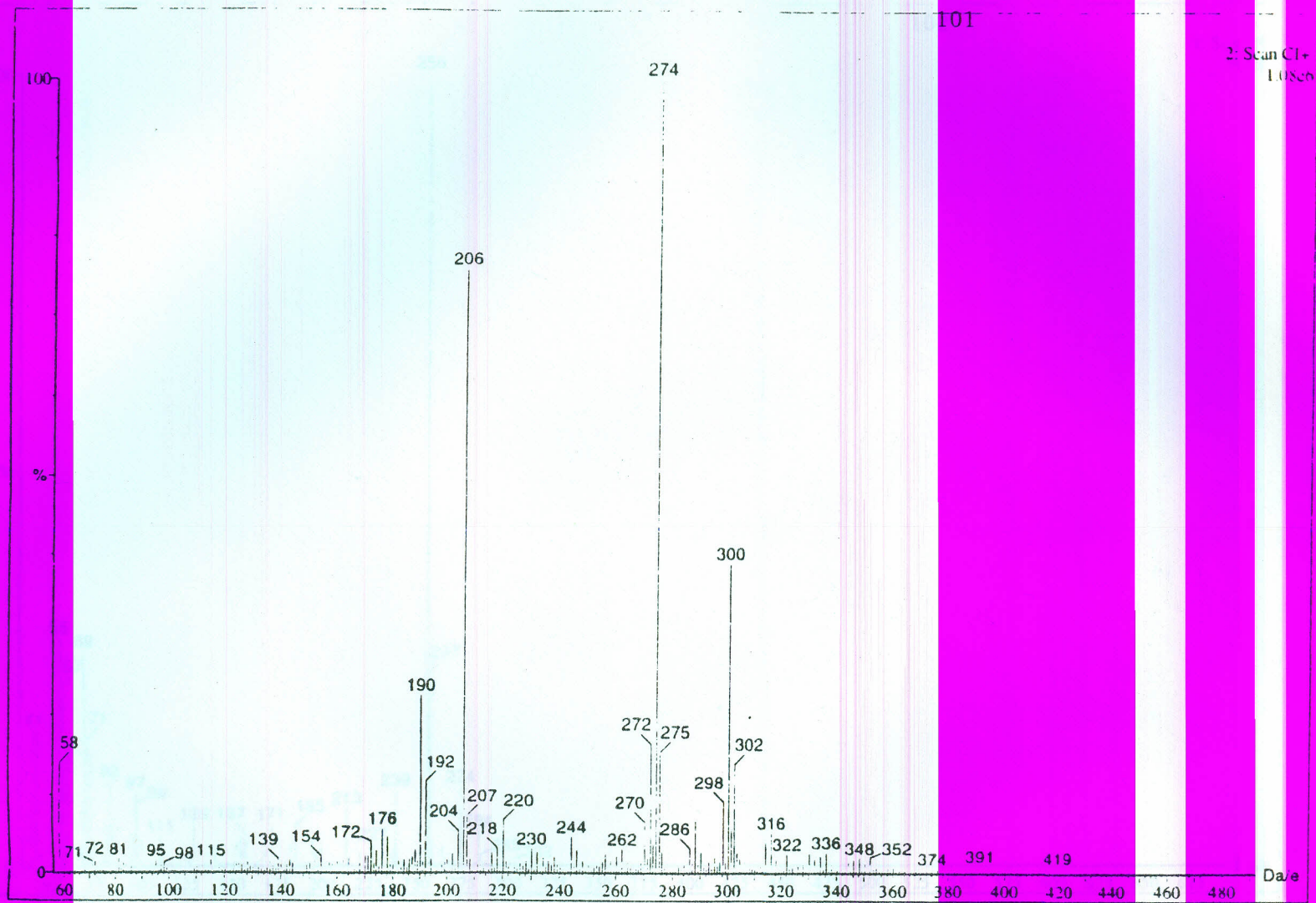


FIGURE 20: Mass spectrum (cims) of compound D

102

1: Scan EI+
9.31e5

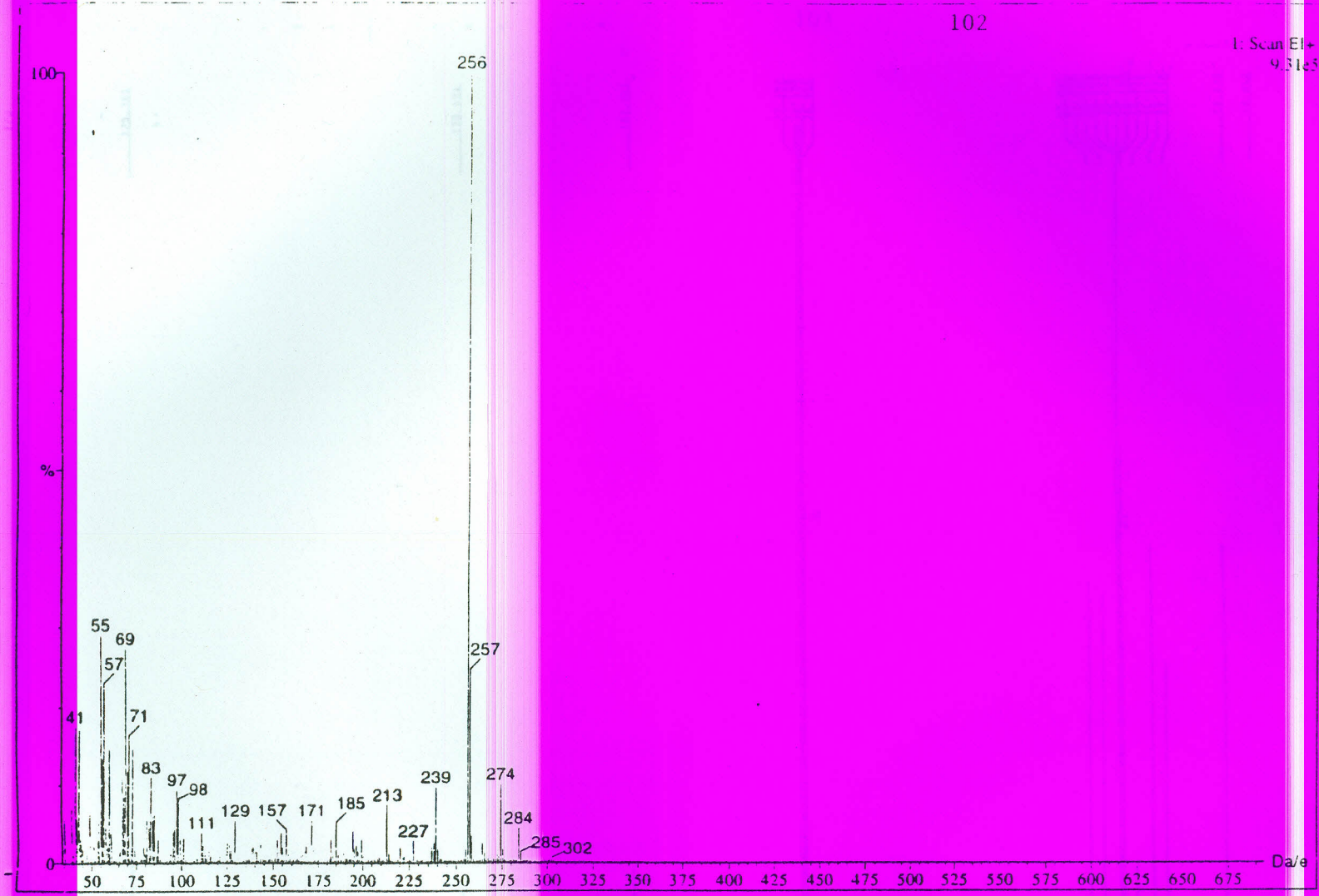


FIGURE 21: Mass spectrum (eims) of compound D

22



AP2709.259
 RU PR30.
 Y02.4U
 DATE 28-4-35
 SF 75.469
 SV 75.0
 OI 6625.000
 SI 32750
 TO 32750
 SW 17241.379
 HZ/PT 1.852
 PW 0.0
 RD 0.0
 AG 0.950
 RG 400
 NS 120
 TE 297
 FW 21500
 OZ 4060.000
 OP 16H 00
 LB .500
 GB 0.0
 CX 45.00
 CY 24.00
 F1 10.000P
 F2 4.997P
 HZ/CM 377.142
 PPM/CM 5.000
 SR -1405.12

PPM

179.281

179.681

102.211

22.377
22.377
22.377

15.428

18.423

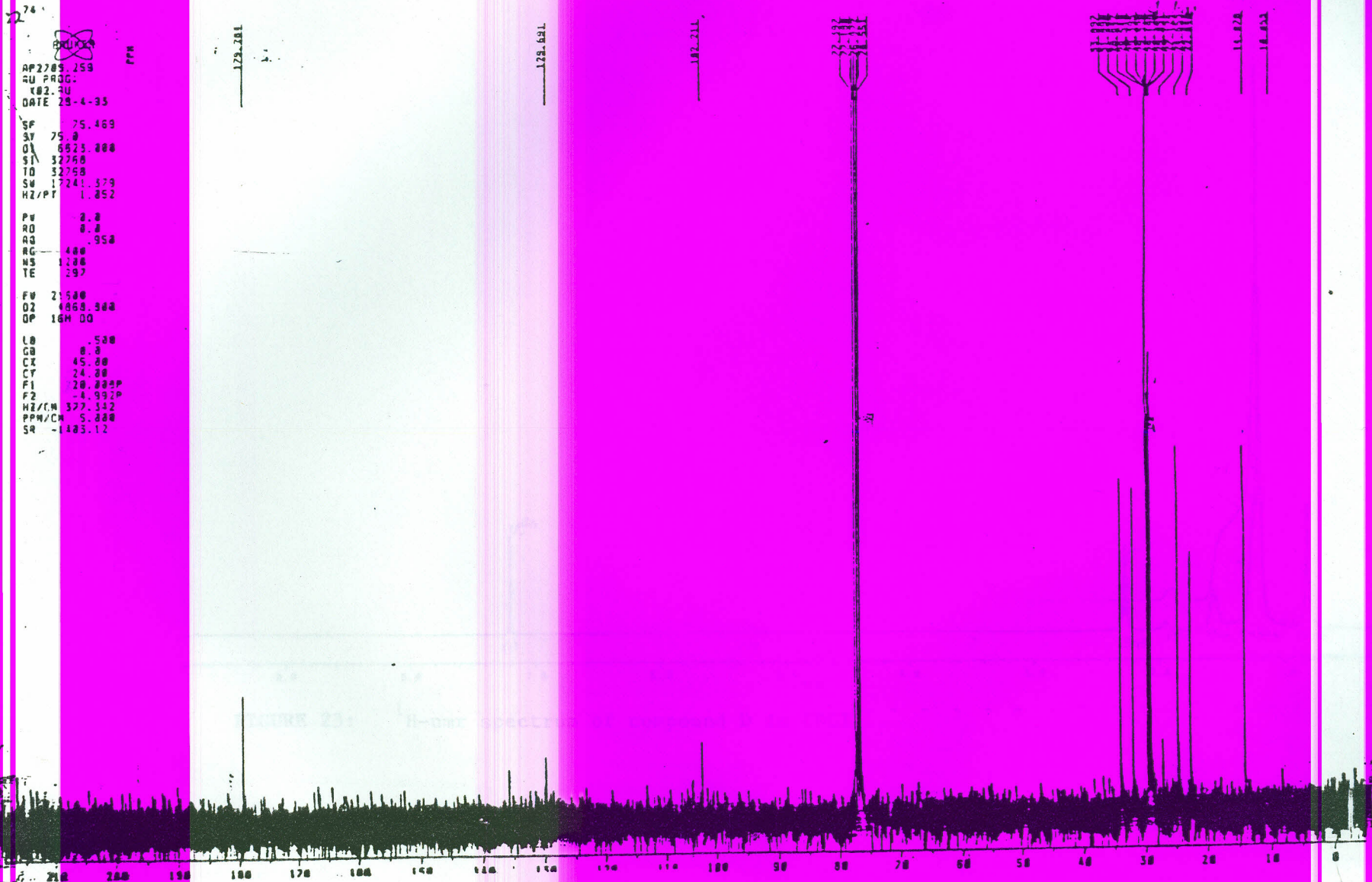


FIGURE 22: ^{13}C -NMR spectrum of compound D in CDCl_3

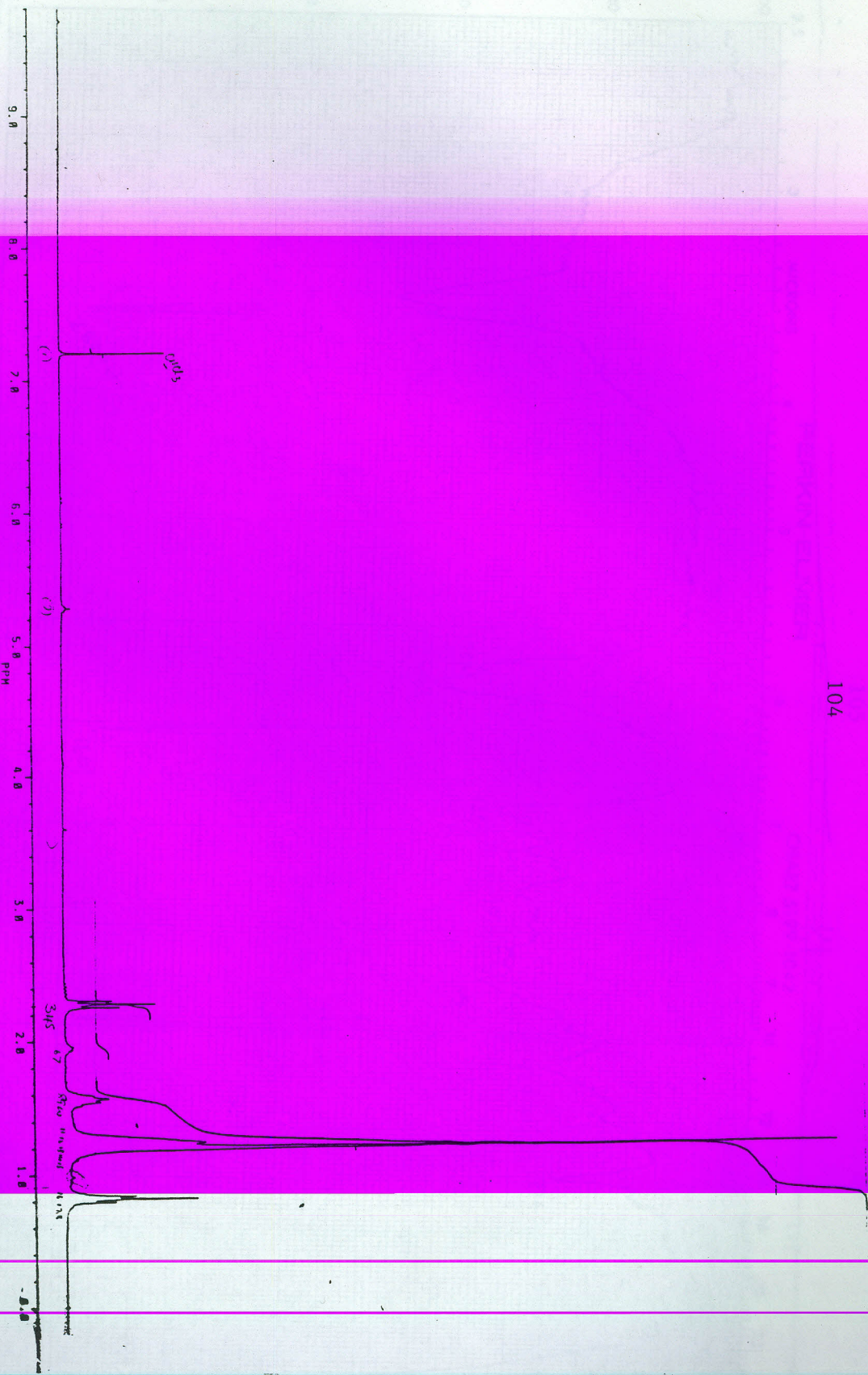
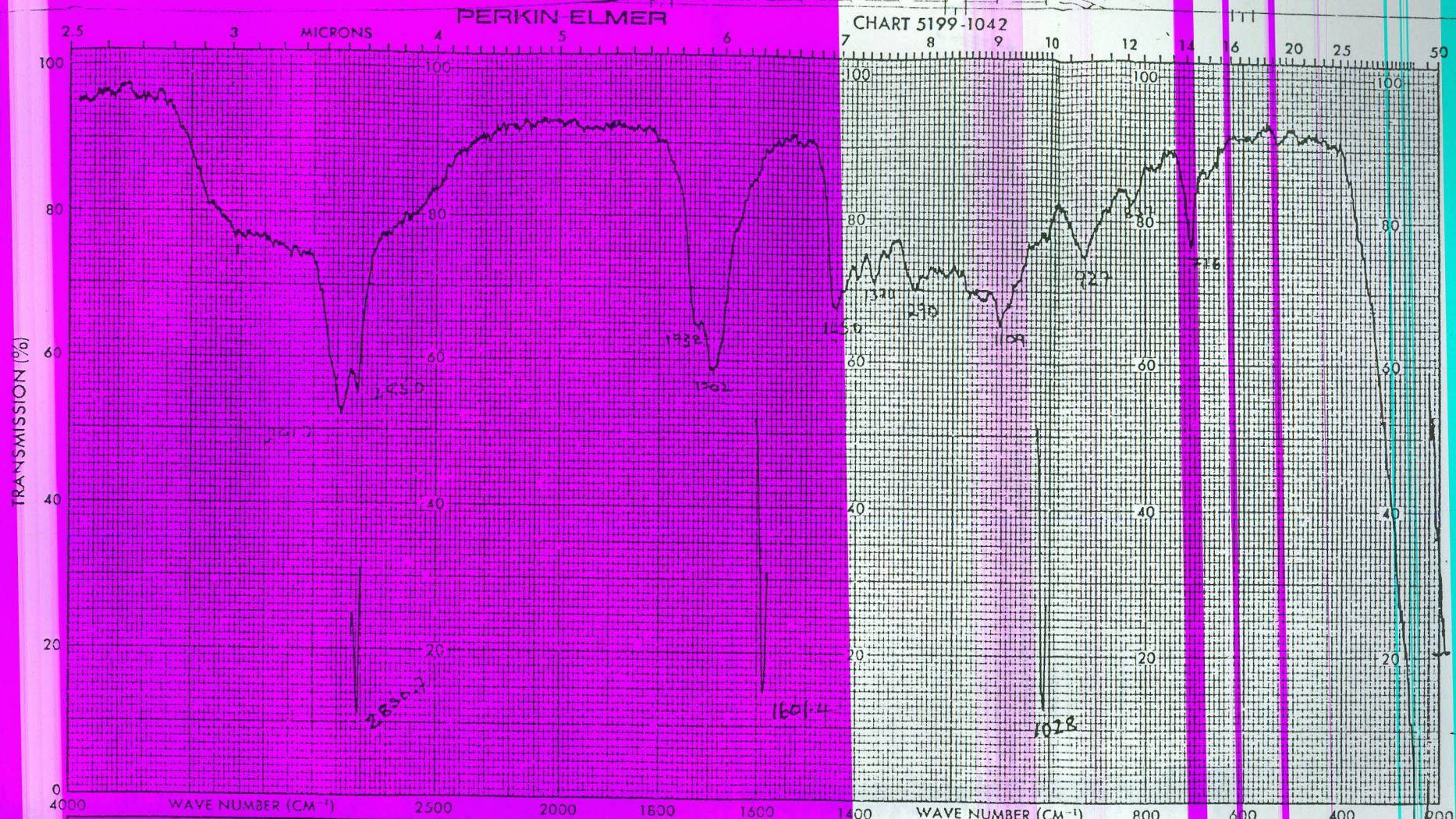


FIGURE 23: 1H-nmr spectrum of compound D in CDCl₃

SAMPLE
 ORIGIN
 ANALYST
 SUPERVISOR

DATE

TIME



SAMPLE _____
REF. NO. _____

ABSCISSA		ORDINATE		SCAN TIME	REP. SCAN
EXPANSION <u>0.5</u>		EXPANSION _____		<u>8</u>	<u>SINGLE BEAM</u>
SUPPRESSION _____		% T _____		MULTIPLIER _____	TIME DRIVE _____
		ABS _____		SLIT PROGRAM _____	OPERATOR _____
SAMPLE		REMARKS _____		SOLVENT	CELL PATH
ORIGIN <u>Ether 4 Mycelium</u>				<u>4</u>	REFERENCE _____
				CONCENTRATION _____	DATE _____

FIGURE 24: Ir spectrum of compound E

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2
1
0

PPA



FIGURE 25: ^{13}C -nmr spectrum of compound E in CDCl_3

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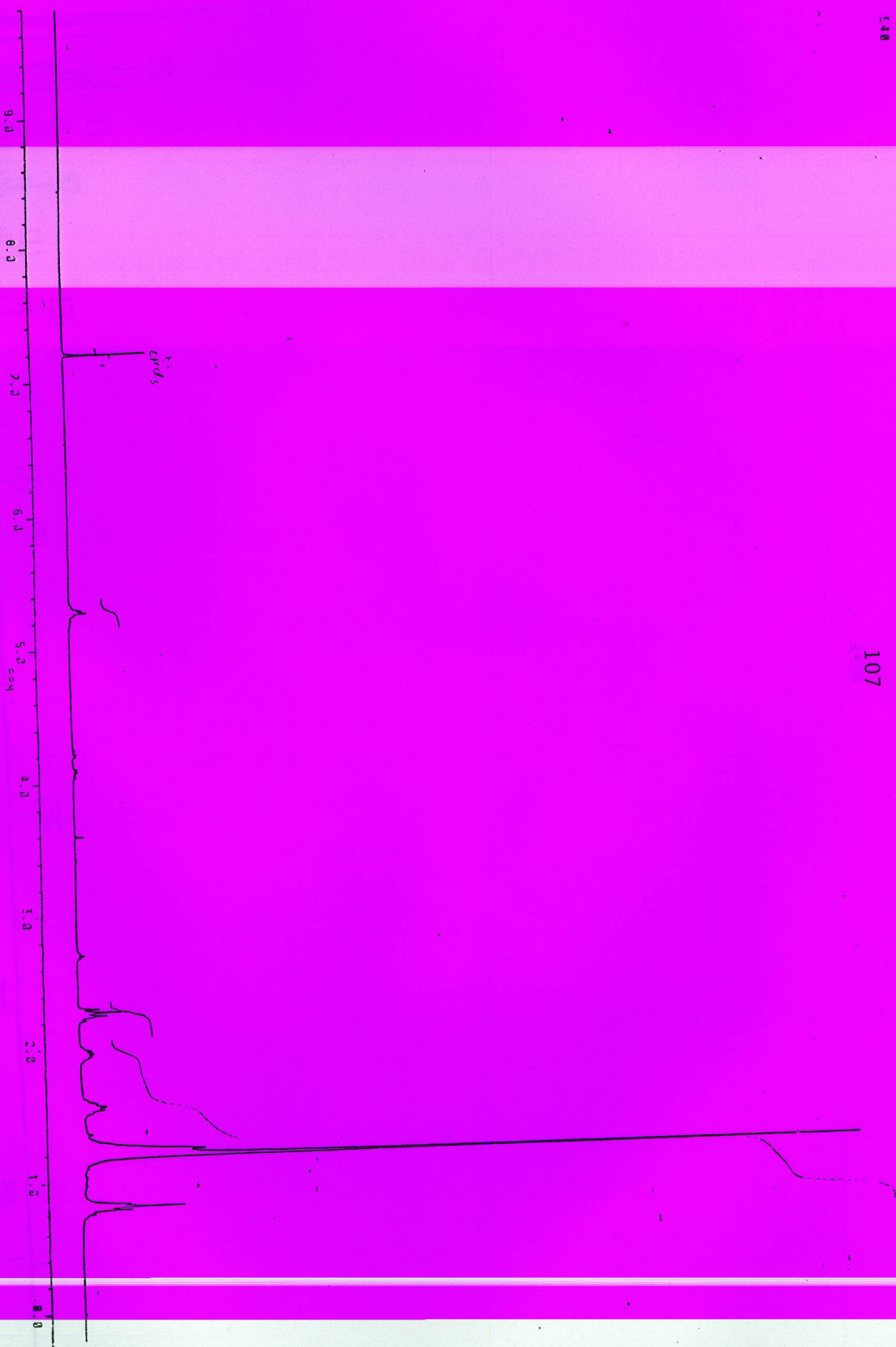


FIGURE 26: $^1\text{H-NMR}$ spectrum of compound E in CDCl_3

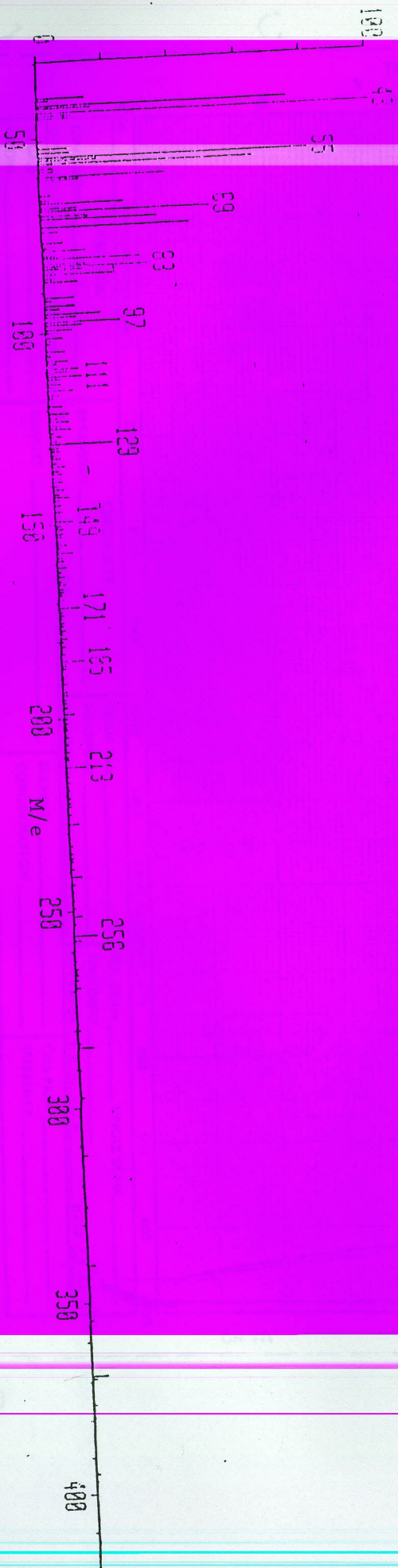
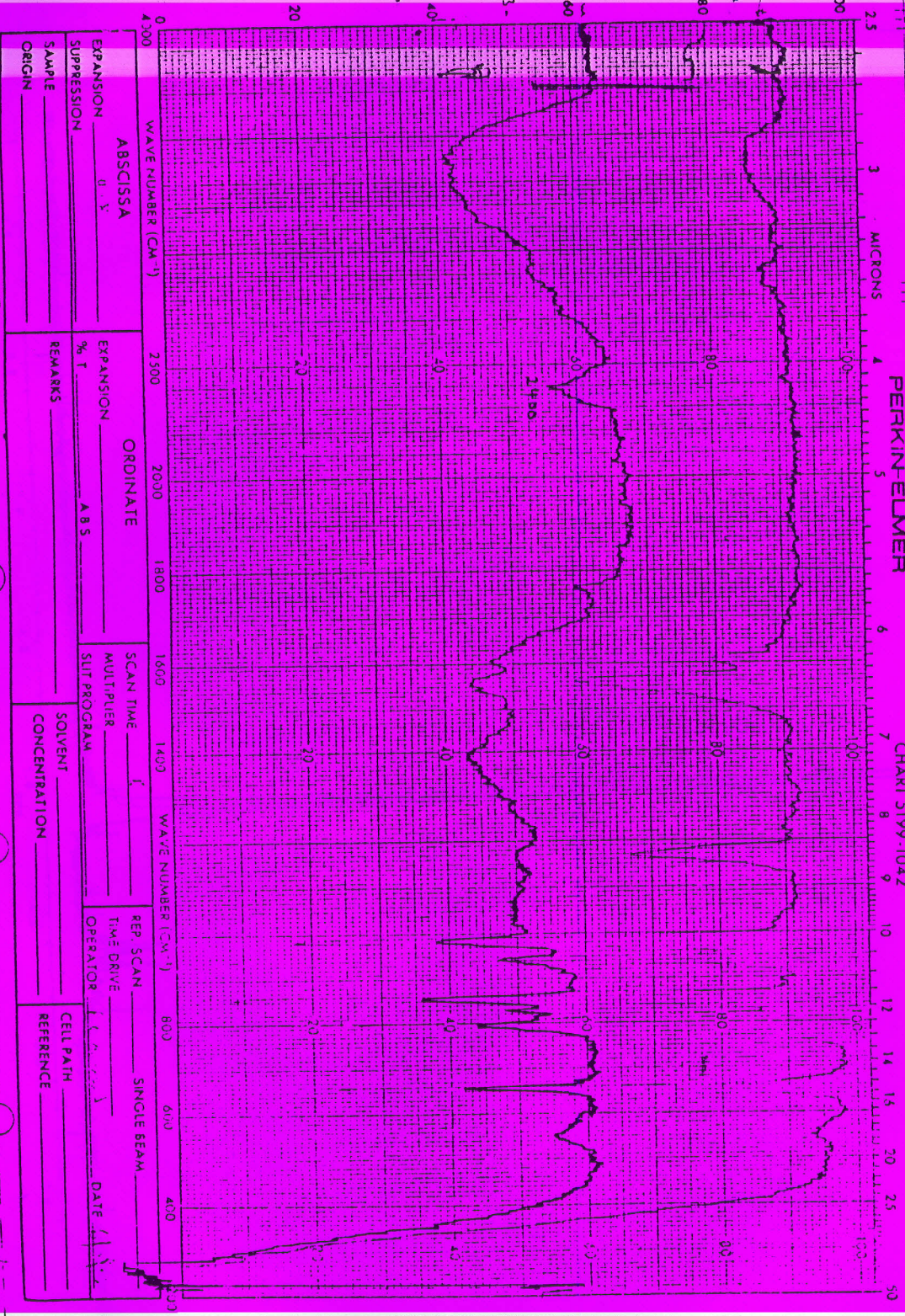


FIGURE 27: Mass spectrum of compound E

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WAVE NUMBER (CM ⁻¹)		ORDINATE		WAVE NUMBER (CM ⁻¹)		WAVE NUMBER (CM ⁻¹)		WAVE NUMBER (CM ⁻¹)	
4000	2500	2000	1800	1600	1400	800	600	400	200
ABSCISSA		EXPANSION		MULTIPLIER		SUIT PROGRAM		REP. SCAN	
EXPANSION _____		EXPANSION _____		MULTIPLIER _____		SUIT PROGRAM _____		TIME DRIVE _____	
SUPPRESSION _____		% T _____		SUIT PROGRAM _____		OPERATOR _____		SINGLE BEAM _____	
SAMPLE _____		REMARKS _____		SOLVENT _____		DATE _____		CELL PATH _____	
ORIGIN _____		CONCENTRATION _____		REFERENCE _____					

FIGURE 28: Ir spectrum of compound F

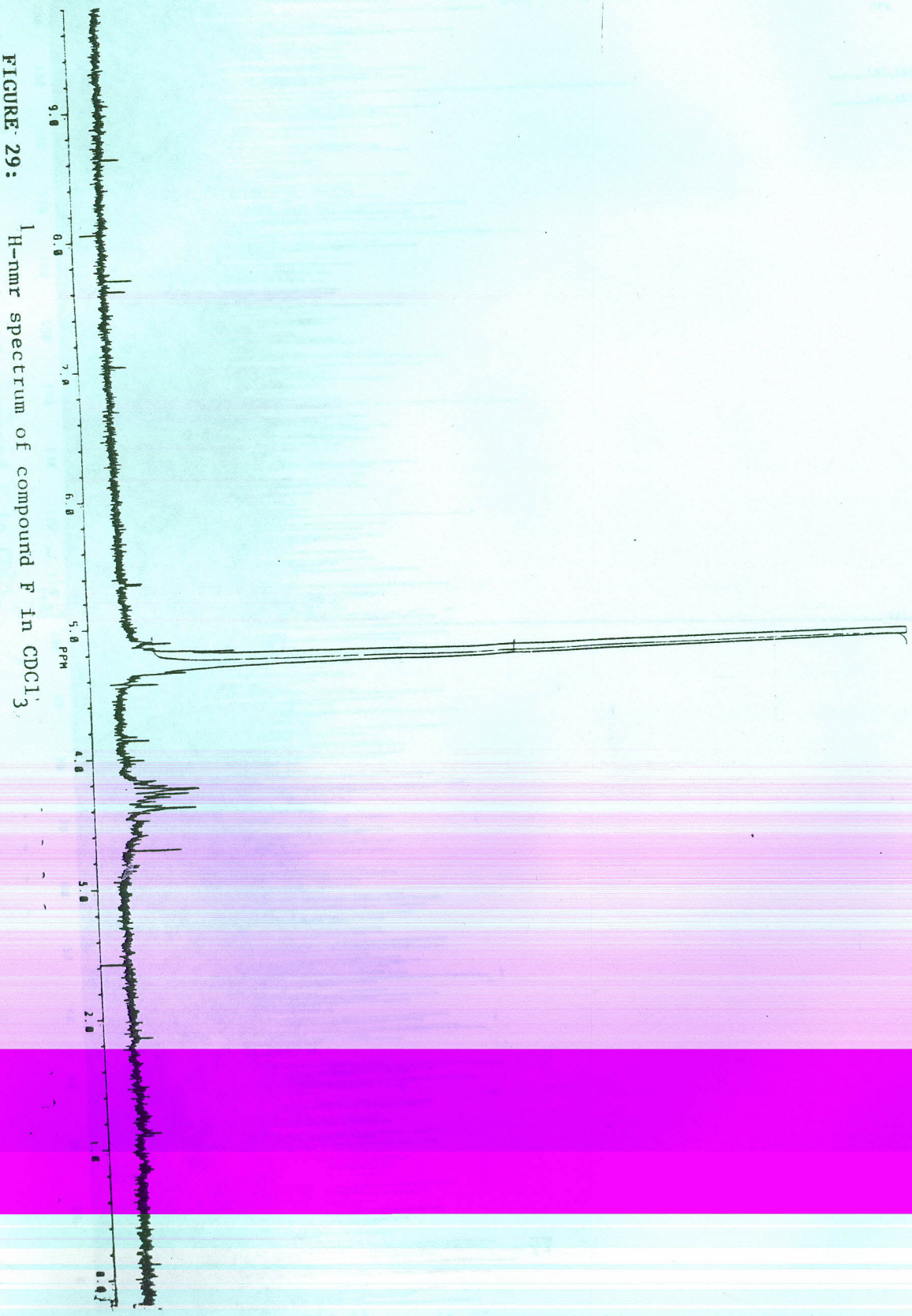


FIGURE 29: 1H-nmr spectrum of compound F in CDCl₃.

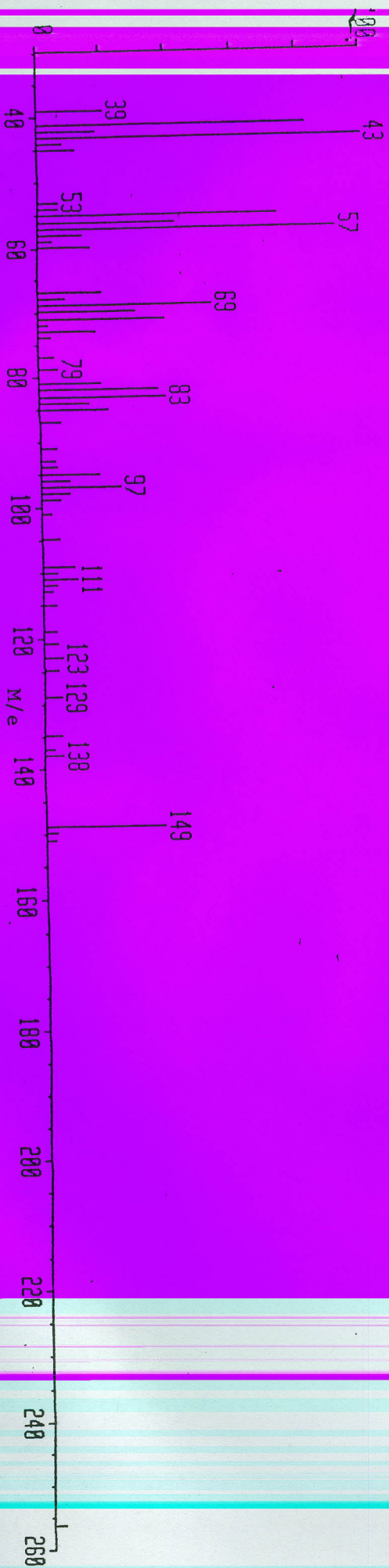


FIGURE 31: Mass spectrum of compound F