ASPECTS OF THE PRODUCTION OF ALKALOIDS IN *PENICILLIUM* AND *CLAVICEPS.*

A thesis submitted in fulfilment of the requirements of the University of London for the award, Ph.D.

JULY 1990

Jane Marina Boyes-Korkis

Department of Biochemistry

Imperial College of Science, Technology and Medicine, London. SW7.

ABSTRACT.

[^{14}C-carbonyl]-anthranilic acid has been used as a biosynthetic probe in a search for novel benzodiazepines. A novel benzodiazepine comprising anthranilic acid and leucine, which together form the benzodiazepine nucleus, and which is substituted by a cyclic glutarimide function has been isolated from liquid cultures of *Penicillium aurantiogriseum*. In addition a novel diketopiperazine comprising tryptophan and phenylalanine has been isolated. The novel diketopiperazine contains an inverted isoprene group and also exhibits rather rare N-acetylation of the indolic moiety. The structures of the novel compounds were determined by a combination of spectroscopic techniques, particularly ^{1}H- and ^{13}C-NMR spectroscopy. Confirmation of the participation of anthranilic acid, leucine and glutamate in the biosynthesis of the benzodiazepine and of the involvement of tryptophan in the formation of the diketopiperazine has been achieved by radiolabelled precursor feeding experiments. Process development of large-scale fermentation of *P. aurantiogriseum* was necessary to obtain a sufficient amount of each metabolite for structural determination and so the dynamics of the fermentation were also followed.

As a prelude to isolation of the enzyme catalysing an isoprenylation step in secondary biosynthesis of *P. aurantiogriseum* a model system, appertaining to the first enzyme in alkaloid biosynthesis in *Claviceps*, has been explored for experience, critical appraisal and development of the special techniques involved. Dimethylallyltryptophan-synthetase has been isolated from *C. fusiformis*. Enzyme activity in relevant protein fractions was followed by isoprenylation of ^{14}C-tryptophan by specially synthesised 4-dimethylallylpyrophosphate. The partially purified enzyme was not sufficiently pure to form the basis of a comparison with that of *C. purpurea*.

To my Family, with love.

TABLE OF CONTENTS.

	Page
Title Page	i
Abstract	ii
Table of Contents	iv
List of Figures	x
List of Tables	xiii
Index of Structures	xv
List of Abbreviations	xix
Acknowledgements	xx
1. INTRODUCTION	1
1.1 General Introduction	1
1.2 Microbial Secondary Metabolism	2
1.3 Stuctural Constituents Of Secondary Metabolites	4
1.4 Diketopiperazine Metabolites Of Fungi	13
1.5 Isoprenylated Diketopiperazine Metabolites	17
1.6 Possible Inter-Generic Relationships Between Fungi Producing Isoprenylated Indole Derivatives	21
1.7 Anthranilic Acid-Derived Fungal Secondary Metabolites	21
1.8 Ergot Fungi And Ergotism	29
1.9 Ergot Alkaloids: Lysergic Acid Derivatives And Clavines	31
1.9.1 Pharmacological Properties Of Ergot Alkaloids	37
1.9.2 Ergot Alkaloid Biosynthesis	38
1.9.3 Regulation And Control Of Alkaloid Biosynthesis	41
1.9.4 Enzymology Of Ergot Alkaloid Biosynthesis	41
1.9.4.1 Dimethylallyltryptophan-Synthetase	42
1.9.4.2 Cell-Free Conversion Of DMAT To Clavicipitic Acid	44
2. MATERIALS AND METHODS	46
2.1 Growth And Maintenance Of Fungi	46
2.1.1 *Penicillium aurantiogriseum*	46
2.1.1.1 Origin Of Strain	46
2.1.1.2 Culture Media	46
2.1.1.3 Sterilisation Of Media	46
2.1.1.4 Culture Maintenance	46
2.1.1.5 Axenic Culture Conditions	47
2.1.1.6 Inoculum Development For 60 Litre Fermentations	47
2.1.2 Ergot Fungi	48
2.1.2.1 Origin Of Strains	48
2.1.2.2 Culture Media	48
2.1.2.3 Culture Maintenance	48
2.1.2.4 Re-Isolation Of *Claviceps purpurea* Strain 12-2	49

2.1.2.5 Axenic Culture Conditions 49
2.1.2.6 Parasitic Cultivation Of *Claviceps* spp. 50
2.1.2.7 Liquid Nitrogen Storage Of *Claviceps* spp. 51

2.2 General Methods 51
2.2.1 Centrifugation 51
2.2.2 Separation Of Biomass From Culture Medium 51
2.2.3 Extraction Of Lyophilised Mycelium 51
2.2.4 Radiolabels 52
2.2.5 Scintillation Counting 52
2.2.6 Autoradiography 52
2.2.7 Mass Spectrometry 52
2.2.8 Spectroscopy 52
2.2.8.1 Nuclear Magnetic Resonance (NMR) Spectroscopy 52
2.2.8.2 Infra-Red (IR) Spectroscopy 53
2.2.8.3 Ultra-Violet (UV) Spectroscopy 53

2.3 Methods Relating To Experiments With *P. aurantiogriseum* 53
2.3.1 [^{14}C-carbonyl]-Anthranilic Acid Labelling Experiments 53
2.3.1.1 Administration Of Radiolabel 53
2.3.1.2 Extraction Of Lyophilised Cells 53
2.3.1.3 TLC Conditions For Resolving Mycelial Extracts 53
2.3.1.4 HPLC Conditions For Resolving The ^{14}C-Anthranilate-Labelled Benzodiazepine. 53
2.3.2 Protocol For Calcium Chloride Additions To CDYE Broth 54
2.3.3 Shaken Flask Fermentations Of *P. aurantiogriseum* 54
2.3.3.1 Culture Conditions 54
2.3.3.2 Sporulation 54
2.3.3.3 Biomass Measurement 54
2.3.3.4 Diketopiperazine Analysis 55
2.3.3.5 Analytical HPLC Conditions For The Diketopiperazine 55
2.3.4 Large-Scale Fermentation Of *P. aurantiogriseum* 55
2.3.4.1 Fermentation Conditions 55
2.3.4.2 Sporulation And Biomass Measurement 56
2.3.4.3 pH Measurement 57
2.3.4.4 Sugar Analysis 57
2.3.4.5 Benzodiazepine Assessment 58
2.3.5 Down-Stream Processing For Isolation Of Novel Metabolites 59
2.3.5.1 Silica Column Chromatography 60
2.3.5.2 Preparative-Layer Chromatography 61
2.3.5.3 Preparative HPLC In The Isolation Of The Benzodiazepine 61
2.3.5.4 Gradient Elution HPLC For Large-Scale Purification Of The Novel Diketopiperazine 61

2.3.6 Additional Biosynthetic Studies 61
2.3.6.1 Administration Of L-[U-^{14}C]-Leucine And L-[U-^{14}C]-Glutamate To Surface Cultures Of *P. aurantiogriseum* 61
2.3.6.2 Incorporation of [^{14}C-methylene]-Tryptophan Into The Novel Diketopiperazine 62

2.4 Methods Relating To Experiments With *Claviceps* Fungi 62
2.4.1 General Analytical Methods 62
2.4.1.1 Determination Of Ergot Alkaloid Titre By Colorimetric Assay 62
2.4.1.2 Extraction Of Basic Alkaloids From Culture Filtrate 63
2.4.1.3 Extraction Of Amphoteric Alkaloids From Culture Filtrate 63
2.4.1.4 Extraction Of Alkaloids From Sclerotia 63
2.4.1.5 Extraction Of Bases From Surface Cultures Of *Claviceps* 63
2.4.1.6 Thin- And Preparative- Layer Chromatography Of Ergot Alkaloids 65
2.4.1.7 HPLC Conditions For Resolving *Claviceps* Alkaloids 65
2.4.2 Alkaloid Content Of Sclerotia Of *C. purpurea* 12-2 66
2.4.3 Isolation Of DMAT From Ethionine-Blocked Cultures Of *C. fusiformis* 66
2.4.3.1 Ehrlich's Reagent For Localising Alkaloids 66
2.4.3.2 Preparative-Layer Chromatography Of DMAT 66
2.4.4. Production Of Chanoclavine In Axenic Culture By *C. purpurea* KL1 66
2.4.5 Administration Of Precursors Of The Ergoline Biosynthetic Pathway To Parasitic Tissue Preparations Of *Claviceps* spp. 67
2.4.5.1 Quantities Of Ergoline Precursors Added To Sclerotial Tissue Preparations. 67
2.4.5.2 Extraction Of Sclerotial Slices 67
2.4.5.3 Treatment Of Supernatants 68
2.4.6 Synthesis Of 3,3-Dimethylallylpyrophosphate 68
2.4.6.1 Preparation Of Bis-Triethylamine Phosphate 68
2.4.6.2 Esterification Of 3,3-Dimethylacrylic Acid 68
2.4.6.3 Isolation Of Methyl-3,3-Dimethylacrylate 70
2.4.6.4 Reduction Of Methyl-3,3-Dimethylacrylate 70
2.4.6.5 Pyrophosphorylation Of 3,3-Dimethylallyl Alcohol 71
2.4.6.6 Extraction Of Phosphorylated Products 71
2.4.6.7 Characterisation Of Phosphorylated Products 72
2.4.6.8 Silica Column Chromatography In The Purification Of 3,3-Dimethylallylpyrophosphate 73
2.4.6.9 Hanes-Isherwood Reagent For Localising Phosphates 74

2.4.7 Incorporation Of [^{14}C-methyl]-Methionine Into Agroclavine In Ethionine-Inhibited And Control Cultures Of *C. fusiformis* 74

2.4.8 Time-Course Of Incorporation Of [^{14}C-methyl]-Methionine Into Agroclavine And Amphoteric Intermediates Of The Clavine Biosynthetic Pathway In *C. fusiformis* 75

2.4.9 DMAT-Synthetase Activity In *C. fusiformis* 76
2.4.9.1 Buffer Solutions For Cell-Free Preparations 76
2.4.9.2 Methods Of Cell Disruption 76
2.4.9.3 Preparation Of A Protein Fraction Exhibiting DMAT-Synthetase Activity From *C. fusiformis* 77
2.4.9.4 Cell-Free Incubations 78
2.4.9.5 TLC Assay For DMAT-Synthetase Activity 79
2.4.9.6 HPLC Assay For DMAT-Synthetase Activity 79

3. RESULTS 80
3.1 [^{14}C-carbonyl]-Anthranilic Acid As A Probe For Benzodiazepine Metabolites Of *P. aurantiogriseum* 80

3.2 Biosynthetic Evidence For Incorporation Of [^{14}C-carbonyl]-Anthranilic Acid, L-[U-^{14}C]-Leucine And L-[^{14}C]-Glutamate Into The Novel Benzodiazepine In Surface Cultures Of *P. aurantiogriseum* 81

3.3 Large-Scale Fermentation Of *P. aurantiogriseum* 87
3.3.1 Biomass 87
3.3.2 pH 87
3.3.3 Sugar Analysis 90
3.3.4 Culture Morphology And Sporulation 90
3.3.5 Benzodiazepine Production 92

3.4 Selection Of A Suitable Calcium Chloride Concentration For Large-Scale Fermentation Of *P. aurantiogriseum* 93

3.5 Effect Of Calcium Chloride Addition On Diketopiperazine Production In Fermentations Of *P. aurantiogriseum* 93
3.5.1 Biomass 93
3.5.2 Diketopiperazine Production 95
3.5.3 Incorporation Of [^{14}C-methylene]-Tryptophan Into The Novel Diketopiperazine 96
3.5.4 Culture Morphology 96

3.6 Structure Determination Of The Novel Benzodiazepine 97
3.6.1 Ultra-Violet Spectroscopy 97
3.6.2 Infra-Red Spectroscopy 100
3.6.3 Mass Spectrometry (MS) 100
3.6.3.1 Fast Atom Bombardment MS 100
3.6.3.2 Electron Impact MS 100
3.6.4 ^{1}H-Nuclear Magnetic Resonance (NMR) Spectroscopy 106
3.6.5 ^{1}H-NMR Spectroscopy Of Cyclo-Anthranilyl-Leucine Dipeptide 117
3.6.6 ^{1}H-NMR Spectroscopy Of L-Pyroglutamide 117
3.6.7 ^{1}H-NMR Spectroscopy Of The Novel Benzodiazepine After Prolonged Exposure To Deutero-Chloroform 118
3.6.8 ^{13}C-NMR Spectroscopy Of The Novel Benzodiazepine 125

3.7 Stucture Elucidation Of The Novel Diketopiperazine 134
3.7.1 Ultra-Violet Spectroscopy 134
3.7.2 Mass Spectrometry 134
3.7.3 ^{1}H-NMR Spectroscopy Of The Novel Diketopiperazine 141
3.7.3.1 Structural Information Obtained By ^{1}H-^{1}H COSY 146
3.7.3.2 Proton Assignments 146
3.7.3.3 Nuclear Overhauser Effect Experiments 146
3.7.4 ^{13}C-NMR Spectoscopy Of The Novel Diketopiperazine 150
3.7.4.1 ^{13}C-^{1}H Heteronuclear Shift Correlation Experiment 150
3.7.4.2 Carbon Assignments Of The Novel Diketopiperazine 150

3.8 Isolation Of A Putative Oxidative Transformation Product Of The Novel Diketopiperazine 156

3.9 Aspects Of Alkaloid Production In *Claviceps* Fungi 159
3.9.1 Biosynthesis Of Alkaloids In Parasitic Tissue Of *C. purpurea* Strains KL1 And 12-2 159
3.9.1.1 Alkaloid Content Of Sclerotia Of *C. purpurea* Strains Kl1 And 12-2 159
3.9.1.2 Incubation Of Parasitic Tissue Preparations Of *C. purpurea* Strain 12-2 With Precursors Of The Ergoline Alkaloid Biosynthetic Pathway 160
3.9.1.3 Incubation Of A Parasitic Tissue Preparation Of *C. purpurea* Strain KL1 With [^{14}C-methylene]-Tryptophan 165
3.9.2 Alkaloid Production In Axenic Culture Of *C. purpurea* Strain KL1 167
3.9.3 Alkaloid Production As An Indicator Of DMAT-Synthetase Activity In *C. fusiformis* 170

3.9.3.1 Fate Of [^{14}C-methyl]-Methionine In Ethionine-Treated And Control Cultures Of *C. fusiformis* 170

3.9.3.2 Time-Course Of Incorporation Of [^{14}C-methyl]-Methionine Into Agroclavine And Amphoteric Intermediates Of *C. fusiformis* 175

3.9.4 Optimisation Of Cell-Free Enzyme Preparations Of *C. fusiformis* 182

4. DISCUSSION 186

4.1 Studies With *Penicillium aurantiogriseum* 186

4.2 Studies With *Claviceps* Fungi 195

5. APPENDICES 198

5.1 Appendix I 198

5.2 Appendix II 199

5.3 Appendix III 200

5.4 Appendix IV 201

6. REFERENCES 202

List of Figures.

Fig. 1 Shikimic acid-derived fungal metabolites.

Fig. 2 Examples of fungal metabolites of mixed biosynthetic origin.

Fig. 3 Examples of isoprenylated fungal metabolites.

Fig. 4 Fungal metabolites of mixed biosynthetic origin, exhibiting tremorgenic properties.

Fig. 5 Formation of the benzodiazepine nucleus from anthranilic acid and an α-amino acid.

Fig. 6 Benzodiazepine metabolites of *Penicillium* and *Aspergillus*

Fig. 7 Anthranilic acid-containing fungal metabolites (non-benzodiazepine structures).

Fig. 8 Structure of the synthetic benzodiazepine, diazepam.

Fig. 9 Structure of ergoline and D-lysergic acid.

Fig. 10 Cyclol-type peptide ergot alkaloids.

Fig. 11 Naturally occurring lysergic acid derivatives other than peptides.

Fig. 12 Clavine alkaloids.

Fig. 13 6,7-Seco-ergoline alkaloidsand other cycloclavines.

Fig. 14 Biosynthetic relationships between clavine alkaloids.

Fig. 15 Steps in the synthesis of 3,3-dimethylallylpyrophosphate.

Fig. 16 Autoradiograph of TLC-resolved mycelial extract of a culture of *P. aurantiogriseum* fed with [^{14}C-carbonyl]-anthranilate.

Fig. 17 Structures of novel compounds isolated from *P. aurantiogriseum.*

Fig. 18 HPLC profile of an eluted region (R_f 0.25, Fig. 16) of TLC-resolved mycelial extract of a culture of *P. aurantiogriseum* fed with [^{14}C-carbonyl]-anthranilic acid (50 μCi)

Fig. 19 Autoradiograph showing incorporation of L-[U-^{14}C]-leucine and L-[U-^{14}C]-glutamic acid into the native benzodiazepine.

Fig. 20 Progress of a stirred 60 litre fermentation of *P. aurantiogriseum* in calcium chloride supplemented medium

Fig. 21 Distribution of sucrose and constituent monosaccharides during the sugar utilisation phase of a stirred 60 litre fermentation of *P. aurantiogriseum.*

Fig. 22 Mycelial colours of *P. aurantiogriseum* observed during progress of a 60 litre fermentation.

Fig. 23 Biomass accumulation in cultures of *P. aurantiogriseum* and dynamics of diketopiperazine production in calcium chloride supplemented and unsupplemented media.

Fig. 24 Autoradiograph of HPLC-purified diketopiperazine isolated from a culture of *P. aurantiogriseum* fed with [^{14}C-methylene]-tryptophan.

Fig. 25A UV spectrum of the native benzodiazepine.
Fig. 25B IR spectrum of the native benzodiazepine.
Fig. 26 Positive Fast-atom bombardment mass spectrometry of the native benzodiazepine (Fig. 17, S.111).
Fig. 27 Positive Fast-atom bombardment mass spectrometry of the diastereoisomeric benzodiazepine (Fig. 17, S.112).
Fig. 28 Electron impact mass spectrum of the native benzodiazepine (Fig. 17, S.111).
Fig. 29 Electron impact mass spectrum of the diastereoisomer of the benzodiazepine (Fig. 17, S.112).
Fig. 30A ^{1}H-NMR spectrum of the native benzodiazepine in $CDCl_3$.
Fig. 30B Scale expanded spectrum of certain signals in Fig. 30A.
Fig. 31A ^{1}H-NMR spectrum of the native benzodiazepine in D_6-DMSO.
Fig. 31B Scale expanded spectrum of certain signals in Fig. 31A.
Fig. 32 ^{1}H-NMR spectrum in D_6-DMSO of a pyrolysed sample of the benzodiazepine (1:1 mixture of diastereoisomers).
Fig. 33 HPLC profile of a pyrolysed sample of the benzodiazepine (1:1 mixture of diastereoisomers).
Fig. 34A ^{1}H-^{1}H COSY NMR spectrum of the benzodiazepine (1:1 mixture of diastereoisomers) in $CDCl_3$.
Fig. 34B ^{1}H-^{1}H COSY NMR spectrum of the benzodiazepine (1:1 mixture of diastereoisomers) in D_6-DMSO.
Fig. 35A Structure of cyclo-anthranilyl-leucine dipeptide and L-pyroglutamide.
Fig. 35B Speculative formation of a pyroglutaminyl-derivative of the benzodiazepine.
Fig. 36 ^{1}H-NMR spectrum of cyclo-anthranilyl-leucine dipeptide (Fig. 35A, S.116) in D_6-DMSO.
Fig. 37 HPLC profile of cyclo-anthranilyl-leucine dipeptide.
Fig. 38 ^{1}H-NMR spectrum of L-pyroglutamide (Fig. 35A, S.117) in D_6-DMSO.
Fig. 39 ^{1}H-NMR spectrum of a sample of the native benzodiazepine which had been exposed to chloroform for three days.
Fig. 40 ^{13}C-NMR spectrum of the native benzodiazepine in D_6-DMSO.
Fig. 41 ^{13}C-NMR spectrum of the native benzodiazepine in $CDCl_3$.
Fig. 42A Scale expanded spectrum of signal at 126.7 ppm in Fig. 40.
Fig. 42B Scale expanded spectrum of signal at 54.9 ppm in Fig. 40.
Fig. 43 ^{13}C-NMR spectrum of 1:1 mixture of diastereoisomers of the benzodiazepine in D_6-DMSO.
Fig. 44 Scale expanded spectrum of certain signals in Fig. 43.
Fig. 45 UV spectrum of the novel diketopiperazine.
Fig. 46 Electron impact mass spectrum of the novel diketopiperazine.

Fig. 47 Summary of key ions in the fragmentation of link-scanned mass spectometry of the novel diketopiperazine.

Fig. 48A Structure of the novel diketopiperazine showing mass spectrometric cleavages of the molecule.

Fig. 48B Adopted numbering system of the novel diketopiperazine.

Fig. 49 ^{1}H-NMR of the novel diketopiperazine in D_6-DMSO.

Fig. 50 Compounds bearing structural analogies with the novel diketopiperazine.

Fig. 51 Comparison of chemical shift positions in ^{1}H-NMR spectroscopy of α and β protons of the tryptophan moiety of tryptophan-containing metabolites.

Fig. 52 ^{1}H-^{1}H COSY NMR spectrum of the novel diketopiperazine in D_6-DMSO.

Fig. 53 ^{13}C-NMR spectrum of the novel diketopiperazine in D_6-DMSO.

Fig. 54 Factorised ^{13}C-spectrum of the novel diketopiperazine.

Fig. 55 ^{1}H-^{13}C heteronuclear shift correlation of the novel diketopiperazine.

Fig. 56 Electron impact mass spectrum of a compound (S.115), related to the novel diketopiperazine, isolated from a 60 litre fermentation of *P. aurantiogriseum*.

Fig. 57 Autoradiograph of TLC-resolved methanol extract of 54 day old sclerotial tissue of *C. purpurea* 12-2 which had been incubated with [^{14}C-methylene]-tryptophan.

Fig. 58 Autoradiograph of TLC-resolved methanol extract of 64 day old sclerotial tissue of *C. purpurea* KL1 which had been incubated with [^{14}C-methylene]-tryptophan.

Fig. 59 Low electron voltage electron impact mass spectrum of chanoclavine isolated from culture filtrate of *C. purpurea* KL1.

Fig. 60 Alkaloid production in ethionine-inhibited and control cultures of *C. fusiformis*.

Fig. 61 Autoradiograph of TLC-resolved bases from [^{14}C-methyl]-methionine-fed cultures of *C. fusiformis*, with and without ethionine addition.

Fig. 62A ^{14}C-activity in culture filtrate after [^{14}C-methyl]-methionine addition to ethionine-treated and control cultures of *C. fusiformis*.

Fig. 62B Scale expansion of the first 7 hours of the graph in Fig. 62A.

Fig. 63 Chromatogram showing alkaloid bases extracted from culture supernatants of ethionine-treated and control cultures od *C. fusiformis* (corresponding to autoradiograph in Fig. 64).

Fig. 64 Autoradiograph of TLC-resolved alkaloid bases extracted from culture supernatants of [^{14}C-methyl]-methionine-fed control and ethionine-treated cultures of *C. fusiformis*.
Fig. 65 ^{14}C-activity in chloroform-extracted culture filtrate of ethionine-treated and control cultures of *C. fusiformis*.
Fig. 66 Distribution of ^{14}C-radiolabel in control and ethionine-treated cultures of *C. fusiformis*.
Fig. 67 HPLC resolution of cationic compounds in the cell-free incubation mixture.
Fig. 68 Autoradiograph showing conversion of [^{14}C-methylene]-tryptophan to ^{14}C-DMAT by a partially purified enzyme preparation of *C. fusiformis*.
Fig. 69 Structure of anthramycin.
Fig. 70 1,4-Benzodiazepines in clinical use.
Fig. 71 Structures of glutarimide-containing compounds.

List of Tables.

Table 1 Building blocks used in secondary biosynthesis.
Table 2 Types of secondary metabolite derived from amino acid metabolism in *Penicillium* fungi.
Table 3 Types of diketopiperazine isolated from fungi.
Table 4 Composition of eluting solvent used in flash chromatography of compounds in culture extract of a 60 litre fermentation of *P. aurantiogriseum*.
Table 5 Colour response of fractions in column chromatographic separation of compounds in the pyrophosphorylation of dimethylallyl alcohol.
Table 6 Incorporation of [^{14}C-carbonyl]-anthranilic acid into the two diastereoisomers of the novel benzodiazepine.
Table 7 Incorporation of L-[U-^{14}C]-leucine and L-[U-^{14}C]-glutamate into the native benzodiazepine (Fig. 17, S.111)
Table 8 Mass measurements of the native benzodiazepine.
Table 9 ^{1}H-NMR spectroscopic assignments of the native benzodiazepine (Fig. 17, S.111) in D_6-DMSO.
Table 10 ^{1}H-NMR spectroscopic assignments of the diastereoisomer of the benzodiazepine (Fig. 17, S.112) in D_6-DMSO.
Table 11 ^{1}H-NMR spectroscopic assignments of cyclo-anthranilyl-leucine dipeptide (S.116), molecular formula $C_{13}H_{16}N_2O_2$.

Table 12 ^{13}C-NMR spectroscopic assignments of the native benzodiazepine in D_6-DMSO.

Table 13 Chemical shift values of the native benzodiazepine in $CDCl_3$ and shift differences between the two diastereoisomers in D_6-DMSO in ^{13}C-NMR spectroscopy.

Table 14 Accurate mass measurements of the novel diketopiperazine.

Table 15 Metastable ion analysis of the novel diketopiperazine.

Table 16 Mass spectrometric fragmentations of the novel diketopiperazine.

Table 17 Proton assignments of the novel diketopiperazine.

Table 18 Nuclear Overhauser effect experiments on the novel diketopiperazine.

Table 19 Carbon assignments of the novel diketopiperazine.

Table 20 Accurate mass measurements of a compound (S.115), related to the novel diketopiperazine and isolated from a 60 litre fermentation of *P. aurantiogriseum*.

Table 21 Administration of precursors of the ergoline biosynthetic pathway to parasitic tissue preparations of *C. purpurea* strains 12-2 and KL1.

Table 22 Administration of agroclavine and elymoclavine to parasitic tissue preparations of *C. purpurea* 12-2.

Table 23 Alkaloid production in surface cultures of *C. purpurea* KL1.

Table 24 Addition and fate of [^{14}C-methyl]-methionine in ethionine-treated and control cultures of *C. fusiformis*, assessed on day 8.

Table 25 Transformation of [^{14}C-methylene]-tryptophan to ^{14}C-DMAT by cell-free preparations of *C. fusiformis*.

Index of Structures.

		Page
S.1	Citrinin	6
S.2	Chlorogentisyl alcohol	6
S.3	Deoxynivalenol	6
S.4	G-L-glutaminyl-4-hydroxybenzene	6
S.5	Cyclopenin	6
S.6	Viridamine	6
S.7	*p*-Hydroxybenzoic acid	8
S.8	Pyrogallol	8
S.9	Methyl-5-shikimate lactone	8
S.10	G-L-glutaminyl-3,4-benzoquinone	8
S.11	Hadacidin	9
S.12	Cyclopiazonic acid	9
S.13	Mycelianamide	9
S.14	Phenylalanine anhydride	9
S.15	Asperphenamate	9
S.16	Brevigellin	9
S.17	Mycophenolic acid	12
S.18	Ochratoxin A	12
S.19	Gliotoxin, gliotoxin acetate	12
S.20	LL-S490β	12
S.21	Verrucofortine	12
S.22	L-phenylalanine anhydride	14
S.23	Aspergillic acid	14
S.24	Peramine	14
S.25	Gliotoxin	12
S.26	Sporidesmin	14
S.27	Viridicatin	14
S.28	Echinulin	16
S.29	Cryptoechinulin G	16
S.30	Brevianamide E	16
S.31	Verruculogen	16
S.32	Fumitremorgen A	16
S.33	Fumitremorgen B	16

S.34	Roquefortine	16
S.35	Oxaline	16
S.36	Nigrifortine	16
S.37	Verrucofortine	16
S.38	Asterriquinone	16
S.39	Aflatrem	20
S.40	Penitrem F	20
S.41	Paspalinine A	20
S.42	Janthitrem A	20
S.43	Paxilline	20
S.44	Cyclopenin	23
S.45	Cyclopenol	23
S.46	Cyclopeptine	23
S.47	Auranthine	23
S.48	Asperlicin	23
S.49	Anthglutin	24
S.50	2-Pyruvoylaminobenzamide	24
S.51	Tryptanthrin	24
S.52	o-Aminophenol	24
S.53	Questiomycin A	24
S.54	Chrysogine	24
S.55	Austamide	24
S.56	Aspercolorin	24
S.57	Tryptoquivaline	24
S.58	Tryptoquivalone	24
S.59	Diazepam	28
S.60	Ergoline	32
S.61	D-lysergic acid	32
S.62	Ergotamine	32
S.63	Ergosine	32
S.64	Ergocristine	32
S.65	α-Ergokryptine	32
S.66	β-Ergokryptine	32
S.67	Ergocornine	32
S.68	Ergostine	32
S.69	Ergohexine	32
S.70	Ergoheptine	32

S.71	Ergometrine	33
S.72	Lysergic acid α-hydroxyethylamide	33
S.73	Lysergic acid amide	33
S.74	Paspalic acid	33
S.75	Agroclavine	35
S.76	Elymoclavine	35
S.77	Molliclavine	35
S.78	Lysergine	35
S.79	Lysergol	35
S.80	Isolysergol	35
S.81	Lysergene	35
S.82	Setoclavine	35
S.83	Isosetoclavine	35
S.84	Norsetoclavine	35
S.85	Penniclavine	35
S.86	Isopenniclavine	35
S.87	Festuclavine	35
S.88	Costaclavine	35
S.89	Pyroclavine	35
S.90	Dihydrolysergol I	35
S.91	Dihydrosetoclavine	35
S.92	Fumigaclavine A	35
S.93	Fumigaclavine B	35
S.94	Isofumigaclavine A	35
S.95	Isofumigaclavine B	35
S.96	Chanoclavine I	36
S.97	Chanoclavine II	36
S.98	Isochanoclavine I	36
S.99	Norchanoclavine I	36
S.100	Norchanoclavine II	36
S.101	Chanoclavine I acid	36
S.102	Paliclavine	36
S.103	Paspaclavine	36
S.104	Rugulovasine A	36
S.105	8-Chlororugulovasine A	36
S.106	Rugulovasine B	36
S.107	8-Chlororugulovasine B	36
S.108	Clavicipitic acid	36
S.109	Dihydrochanoclavine I	36
S.110	Isodihydrochanoclavine I	36

S.111	Novel benzodiazepine (S,S-stereochemistry)	83
S.112	Novel benzodiazepine (R,S-stereochemistry)	83
S.113	Benzodiazepine tautomer	83
S.114	Novel diketopiperazine	83
S.115	Putative oxidative transformation product of the novel diketopiperazine.	83
S.116	Cyclo-anthranilyl-leucine dipeptide	119
S.117	L-pyroglutamide	119
S.118	Speculative pyroglutaminyl-derivative of the novel benzodiazepine.	119
S.119	Ditryptophenaline	144
S.120	Anthramycin	188
S.121	Cycloheximide	190
S.122	*Aspergillus* glutarimide	190
S.123	Streptovitacin A	190
S.124	Streptimidone	190
S.125	Inactone	190
S.126	Actiphenol	190
S.127	Bemegride	190

List of Abbreviations.

Ac	Acetate
Ala	Alanine
BZD	Benzodiazepine
CDYE	Czapek Dox yeast extract
COSY	Correlated spectroscopy
DEPT	Distortionless enhancement by polarization transfer
DKP	Diketopiperazine
DMAPP	Dimethylallylpyrophosphate
DMAT	4-Dimethylallyltryptophan
DMSO	Dimethylsulphoxide
EI	Electron impact
FAB	Fast atom bombardment
Glu	Glutamate
Gly	Glycine
HPLC	High performance liquid chromatography
IBMK	Iso-butylmethyl ketone
IPP	Isopentenylpyrophosphate
IR	Infra-red
LAA	Lysergic acid amide
Leu	Leucine
LH	Logged hours
LSD	Lysergic acid diethylamide
m/z	Mass to charge ratio
MED T	Medium T
MTA	Medium T agar
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
PAGE	Polyacrylamide gel-electrophoresis
PDA	Potato dextrose agar
Phe	Phenylalanine
R_f	Relative mobility
RBF	Round-bottomed flask
RMM	Relative molecular mass
RT	Retention time
SCSL	Sucrose corn-steep liquor
SDS	Sodium dodecyl sulphate
TA	Tartaric acid
TLC/PLC	Thin/preparative-layer chromatography
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultra-violet

ACKNOWLEDGEMENTS.

I tender most sincere thanks to my supervisor and friend, Dr. P.G. Mantle who has guided me supremely through this research period; for his advice, wise counsel, encouragement, unfailing interest and for his dedication in constructive criticism of the manuscript, without which this task would have been impossible. It is an honour to have had the privilege of working with him.

I am extremely grateful to Dr. C.M. Weedon, who was undertaking his Ph.D. when I first started at Imperial College, for numerous discussions on Life, The Universe and Everything. I would also like to sincerely thank my current laboratory colleagues, Miss R. Adatia, Dr. D.E. Frederickson, Dr. K. Gurney, Mrs. K. McHugh (née Macgeorge) and Miss J. Penn for creating such an amiable working environment, for their gifts of friendship and for providing me with special memories of my time at Imperial.

Sincere thanks are due to Mr. J.N. Bilton of the Chemistry Department for performing mass spectrometry, for his encouragement, for all his help with spectral interpretation and for many stimulating discussions. I express sincere thanks to Mr. R.N. Sheppard for his invaluable NMR spectroscopic work and I would also like to extend my gratitude to Mr. G. Millhouse for black and white photography. I would like to record my thanks, in advance, to the examiners of this thesis, Dr. A.G. Dickerson of Imperial College and Dr. M.O. Moss of the University of Surrey.

I gratefully acknowledge receipt of a Science and Engineering Research Council Studentship.

Lastly, but by no means least, I wish to express heartfelt thanks to my mother and step-father, Ben, for generously supporting me throughout many years of study and for their continual love and care, to Julian, my step-brother, and to my sister, Susie, who has selflessly endured living with me through many ordeals.

The year of grace 1990.

In memory of my late father:

F. Boyes-Korkis, M.B., Ch.B., D.L.O., F.R.C.S.

What is it to be wise ?
'Tis but to know how little can be known,
To see all others' faults, and feel our own.

Alexander Pope (1688 - 1744).

1. INTRODUCTION.

1.1 GENERAL INTRODUCTION.

The rôle of alkaloids in the history of mankind is most interesting and it is not at all difficult to appreciate their current global importance. Ancient Man used crude plant extracts as remedies and palliatives for various illnesses long before the active ingredients were characterised. Mann (1978) has indicated that the early nineteenth century chemists isolated more than twenty of these natural products including quinine, which was found as a component of the bark of the cinchona tree. Thomas Sydenham (1624-89), a physician, prescribed finely powdered bark from the cinchona tree as a remedy for malaria. Ephedrine, the basis of an ancient Chinese remedy for respiratory ailments, is now used in the treatment of asthma and hay-fever. Curare, a plant extract containing several toxic alkaloids, was used by South American Indians as an arrow poison. A constituent of curare, tubocurarine, is now used as a muscle relaxant in surgery. Numerous pure compounds of plant and, relatively more recently, microbial origin have since been found to possess biological activity.

An increasing knowledge of natural products was generated through new ideas of molecular structure and chemical reactivity. Total synthesis of these compounds to confirm their structure became possible, drawing on the expertise founded mainly in the German dye industry for manipulating obdurate chemical reactions. Improvements in isolation methods and refinements in physical techniques, particularly mass spectrometry, nuclear magnetic resonance spectroscopy and X-ray crystallography, facilitated structural elucidation of natural products.

The alkaloid class of natural products constitutes a large group of frequently pharmacologically-active substances. Alkaloids are now

regarded as organic nitrogen-containing compounds (usually deriving the nitrogen atom from an amino acid) despite their initial broader definition as nitrogenous basic substances. Since the active principles of a vast array of crude medicinal plant extracts were found to be alkaloids, these compounds might erroneously be acknowledged as originating solely from higher plants. However, an increasing number of similar and novel alkaloids are elaborated by secondary metabolic pathways of micro-organisms.

1.2 MICROBIAL SECONDARY METABOLISM.

Microbial secondary metabolism is a relatively neglected area of modern Biochemistry but nevertheless contains a wealth of commercial potential as well as offering fascinating intellectual challenges. Secondary metabolism is perhaps most well known in connection with commercial products derived from such processes, notably Penicillin and Cephalosporin antibiotics from filamentous fungi and polyketide antibiotics, such as tetracycline, representative of the Actinomycete procaryotes. Although it is important to appreciate the considerable range of secondary metabolites produced by bacteria, this review will focus on the broad diversity of structural types elaborated by fungi.

What are secondary metabolites and why are they produced? An answer to these questions is best appreciated if one is clear about the metabolic processes occurring in a living cell. There are three kinds of biochemical process. First , there are the basal processes which provide energy and raw materials for all other cell functions. The second group may be sub-divided to consist of anabolic and catabolic pathways, the former of which involves energy-dependent utilisation of freely available substrates in a balanced manner, such as occurs during replicatory growth. Catabolism is the converse situation where synthesised compounds are broken down into simple molecules with a concomitant release of energy. These processes are

encompassed within primary metabolism and are essential for continued life of the cell. Primary metabolism is usually controlled by very specific mechanisms and tends to be the same over very large taxonomic groups. The third kind of process is secondary biosynthesis which rarely produces "normal" cell components, like triglycerides, but more readily produces entirely different, structurally diverse metabolites. These secondary products tend to be specific to one type of micro-organism and close relatives, and are responsible for some expressions of individuality in the evolution of fungal species.

Secondary metabolites are often produced, in laboratory fermentations at least, when the organism is in a state of unbalanced growth *i.e.* when a substance other than carbon becomes growth-limiting (Bu'Lock, 1961). In laboratory and industrial fermentations there is a tendency for an initial phase of proliferation, in which replicatory growth predominates, to be followed by a differentiation phase in which secondary biosynthesis is most active. The terms trophophase and idiophase respectively describe these patterns (Bu'Lock, 1967). Another characteristic of secondary metabolites is that they are phenotypically specific, *i.e.* their production is extremely sensitive to culture conditions and previous history (Bu'Lock, 1975). Secondary and primary metabolism may be further distinguished in that secondary processes are usually catalysed by non-specific enzymes and products derived from them usually "appear" to be of no use to the producing organism (Bu'Lock, 1975).

Enzymes in primary metabolism normally accept only one substrate and catalyse the formation of only one product. Such enzymes have clearly evolved because the precise nature of the product is a matter of real consequence for the organism. It is noteworthy that in the realm of secondary biosynthesis the equivalent degree of specificity is not always encountered. This fact is of technical importance since

it may allow us to use a system to produce novel structures simply by providing unnatural substrates (Bu'Lock, 1975). The lack of absolute specificity in some secondary biosynthetic sequences may enable both the product and the substrate of a particular reaction to function as substrates for another enzyme. The precise order of steps in a sequence may be of no great consequence and a multiplicity of pathways and intermediates may result to create a "metabolic grid" effect (Bu'Lock, 1975).

Bu'Lock (1961) has provided a plausible explanation of the importance of secondary metabolites. He was aware of the need of an explanation to account for the chemical diversity of secondary metabolites elaborated by micro-organisms to extend the theory, put forward by Foster (1949), that secondary metabolites are shunt products. Foster proposed that secondary metabolites were produced as reserve food supplies for when the conditions for growth became unfavourable. This was based on the observation that most secondary metabolites of laboratory cultures of fungi are consumed upon exhaustion of carbohydrate nutrients. However, Bu'Lock pointed out that many secondary metabolites are excreted into the surrounding medium; a seemingly inefficient act! Bu'Lock (1961) therefore suggested that it was not the secondary metabolites themselves that were important but that it was the process of secondary metabolism which was of prime significance. He reasoned that the operation of secondary metabolic pathways enabled continued functioning of primary pathways by removal of intermediates which would otherwise feed-back inhibit and close down pathways essential for growth.

1.3 STRUCTURAL CONSTITUENTS OF SECONDARY METABOLITES.

Although secondary metabolites have a remarkable chemical diversity they are built up from a relatively small group of compounds which are usually, though not exclusively, intermediates of

primary metabolism. There are three principal building blocks for secondary metabolites, namely acetate, mevalonate and amino acids (Table 1). Amino acids, prime constituents of alkaloids, are most often considered to represent an end-point in primary metabolism but they could justifiably be regarded as intermediates in the biosynthesis of proteins.

One of the principal intermediates of primary metabolism that is incorporated into secondary products is acetate. Acetate can be diverted to secondary metabolites by two routes: either directly as multiple C_2 units to form a polyketide chain, in a manner that is similar to the primary metabolic process of fatty acid biosynthesis, or indirectly *via* mevalonic acid. Acetate is incorporated *via* mevalonic acid into the five-carbon (C_5) compounds, dimethylallyl-pyrophosphate (DMAPP) and isopentenyl-pyrophosphate (IPP). It is through condensation of these two isomers and subsequent chain elongation, by repeated addition of C_5 units, that the terpenoid metabolites are elaborated.

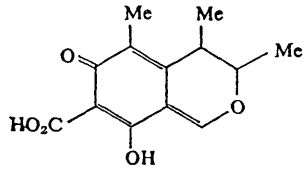
The polyketide citrinin (Table 1, S.1) is a common mould metabolite, first isolated from *Penicillium citrinum* in 1931, and exemplifies direct incorporation of acetate into secondary metabolites. Citrinin has subsequently been determined in *P. citrinum* isolated from yellowed rice which was shown to produce a nephropathy in mice (Saito *et al.*, 1971). Chlorogentisyl alcohol (Table 1, S.2) is another acetate-derived metabolite and contains a substituent chlorine atom, illustrating halogenation as a biological modification to the basic tetraketide structure (in addition to loss of one carbon atom). The presence of a chlorine atom in some compounds may confer a greater biological activity than that of the non-chlorinated counterpart.

TABLE 1 Building blocks used in secondary biosynthesis.

Key intermediate	Fate in primary metabolism	Examples of products formed in diversion to secondary metabolism
1. Acetate	Fatty acids	Citrinin (S.1) Chlorogentisyl alcohol (S.2)
↓		
Mevalonate	Sterols	Tricothecanes *e.g.* Deoxynivalenol (S.3)
2. Shikimate	Lignins (in higher plants)	γ-L-glutaminyl-4-hydroxybenzene (S.4)
↓		
Anthranilate	Tryptophan	Benzodiazepine alkaloids *e.g.* cyclopenin (S.5)
3. Aromatic and aliphatic amino acids	Proteins	Diketopiperazine alkaloids *e.g.* viridamine (S.6)

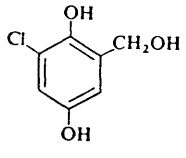
Structure of compounds in Table 1.

S.1



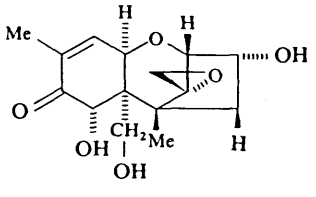
(Penicillium citrinum)[a]

S.2



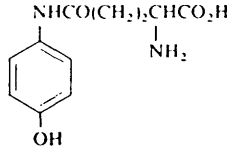
(P. canadense)[b]

S.3



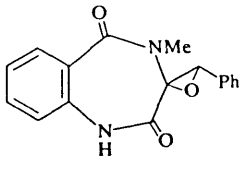
(*Fusarium* spp.)[c]

S.4



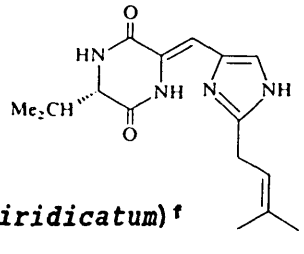
(*Agaricus bisporus*)[d]

S.5



(*P. cyclopium*)[e]

S.6



(*P. viridicatum*)[f]

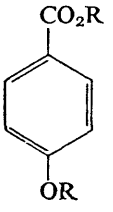
[a] Brown *et al.*, (1949)
[b] McCorkindale *et al.*, (1967)
[c] Ishii *et al.*, (1975)
[d] Stüssi and Rast, (1981)
[e] Birkinshaw *et al.*, (1963)
[f] Holzapfel and Marsh, (1977)

Tricothecanes result from terpenoid biosynthesis in several fungal genera. Such compounds have attracted much attention in their likely involvement in mycotoxicoses in farm animals. For example, deoxynivalenol (Table 1, S.3) has been identified as the "vomiting factor" of *Fusarium*-infected mouldy corn (Ishii *et al.*, 1975). Terpenoid biosynthesis was generally considered to be a eukaryotic capability exclusively until the discovery of undecaprenol in *Lactobacillus plantarum* (Gough *et al.*, 1970).

The shikimate pathway, as a primary process, is of great importance in higher plants for the production of lignins. Shikimic acid is frequently assimilated by non-primary processes which accounts for the abundance of shikimic acid-derived natural products in higher plants. In primary metabolism of fungi the shikimate pathway is involved rather exclusively in aromatic amino acid biosynthesis. Shikimate, moreover, plays a smaller part in fungal secondary metabolism and is rarely used by the Fungi Imperfecti. In 1958, *p*-hydroxybenzoic acid (Fig. 1, S.7) and pyrogallol (Fig. 1, S.8) were isolated from *Penicillium viridicatum* (Bassett and Tanenbaum, 1958). A more recent example is methyl-5-lactyl-shikimate lactone (Fig. 1, S.9), isolated from a *Penicillium* sp. (Isogai *et al.*, 1985). Basidiomycete fungi may also divert shikimate to secondary products. For example, *Agaricus bisporus* transforms shikimic acid to γ-L-glutaminyl-4-hydroxybenzene (Table 1, S.4) which is a precursor of the red pigment, γ-L-glutaminyl-3,4-benzoquinone (Fig. 1, S.10), an inhibitor of mitochondrial respiration. In the biosynthesis of the aromatic amino acid tryptophan, shikimate is converted to anthranilic acid which may itself act as a precursor of fungal secondary metabolites, in particular the benzodiazepine group of compounds. Cyclopenin (Table 1, S.5) is a benzodiazepine elaborated by *P. cyclopium* (Nover and Luckner, 1974).

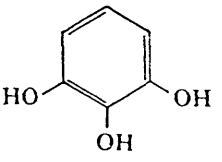
Fig. 1 Shikimic acid-derived fungal metabolites.

S.7



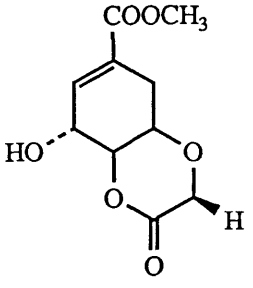
p-hydroxybenzoic acid; R=H

S.8



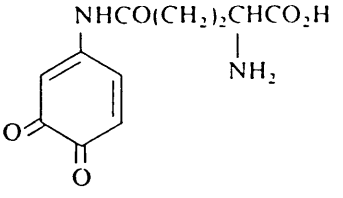
pyrogallol

S.9



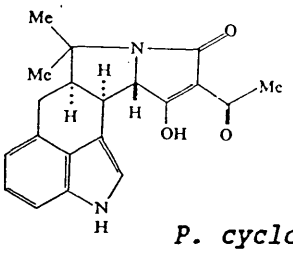
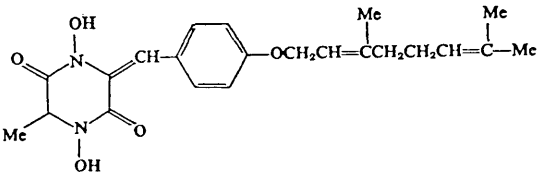
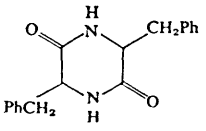
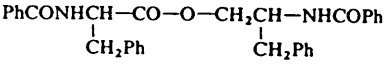
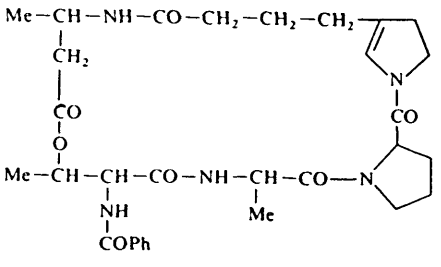
methyl-5-lactyl-shikimate lactone

S.10



γ-L-glutaminyl-3,4-benzoquinone

TABLE 2 Types of secondary metabolite derived from amino acid metabolism in *Penicillium* fungi.

Metabolite Type	Examples
Single aliphatic amino acid	S.11 Hadacidin (from gly) $OHCN(OH)CH_2CO_2H$ *P. purpurescens* (Gray *et al.*,1964)
Single aromatic amino acid	S.12 Cyclopiazonic acid (from trp)  *P. cyclopium* (Holzapfel, 1968)
Diketopiperazine (two amino acids: aliphatic + aromatic)	S.13 Mycelianamide (from tyr and ala)  *P. griseofulvum* (Birch *et al.*, 1962)
Diketopiperazine (two aromatic amino acids)	S.14 Phenylalanine anhydride (phe + phe)  *P. nigricans* (Birkinshaw and Mohammed, 1962)
Two amino acids but without DKP formation	S.15 Asperphenamate (phe and phenylalinol)  *P. brevicompactum* (Bird and Campbell, 1982)
Several amino acids	S.16 Brevigellin  *P. brevicompactum* (McCorkindale and Baxter, 1981)

Diversion of amino acids to secondary biosynthesis results notably in the formation of diketopiperazine alkaloids such as viridamine (Table 1, S.6). However, several different alkaloid types are produced as a result of amino acid metabolism by micro-organisms (Table 2). Alkaloids may be derived from either a single aliphatic or aromatic amino acid as illustrated by hadacidin (S.11) and cyclopiazonic acid (S.12) respectively (Table 2). Two amino acids may condense by cyclic anhydride formation to create a diketopiperazine, such as mycelianamide (S.13) and phenylalanine anhydride (S.14), or the two residues may react to produce quite a different structure as exemplified by asperphenamate (Table 2, S.15). Frequently more than two amino acids participate in secondary biosynthesis and peptides may be formed such as brevigellin (S.16), isolated from *Penicillium brevicompactum* (McCorkindale and Baxter, 1981). The cyclosporins, fairly large peptidic metabolites of *Trichoderma polysporum* (Dreyfuss *et al.*, 1976), are currently used as immuno-suppressant agents. The β-lactams are smaller cyclic tripeptides, elaborated notably by *Penicillium chrysogenum* and are widely used antibiotics. It is interesting to note that peptide secondary metabolites seem rather frequently to exhibit antibiotic activity.

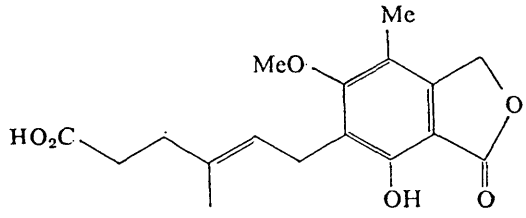
As a slight diversion (the relevance of which may become apparent later) it may be argued philosophically in the case of antibiotics that a selective advantage is conferred on the producing organism in its natural habitat. It would make sense for fungi to produce agents to inhibit the growth of bacteria that would otherwise compete directly with them for nutrients. However, antibiotics are only a small group of secondary metabolites and for the majority of micro-organisms no such selective advantage is apparent. Alkaloids formed by minor chemical modifications of the diketopiperazine and benzodiazepine nuclei may, moreover, exhibit other biological activities different from antibiosis.

In addition to simple products derived from the few key intermediates of primary metabolism is a vast array of secondary metabolites of mixed biosynthetic origin. A classical example of a compound of mixed biogenetic origin is provided by mycophenolic acid (Fig. 2, S.17), isolated from *Penicillium brevicompactum* (Birkinshaw *et al.*, 1952), which contains a polyketide ring system and a farnesyl isoprenoid moiety. Mycophenolic acid is the result of unified biosynthesis, encompassing both distinct routes of acetate diversion to secondary products. Ochratoxin A (Fig. 2, S.18), containing a chlorine atom, is a secondary metabolite of mixed biosynthetic origin, variously reported to be produced by a few *Penicillium* spp. including, within the sub-genus *Penicillium*, *P.viridicatum* (Scott *et al.*, 1970; Northolt *et al.*, 1979) and *P. cyclopium* (Northolt *et al.*, 1979). Following rationalisation within the Penicillia, however, production of ochratoxin A seems now to be restricted to *P. verrucosum* (Pitt, 1985). *Aspergillus ochraceus*, now subject to possible change of name to *A. alutaceus* (Kozakiewicz, 1989), was isolated during screening of cereals for toxigenic fungi and was shown also to produce ochratoxin A (van der Merwe *et al.*, 1965). As well as being hepatotoxic, ochratoxin A was found to be primarily a nephrotoxin in the rat (Purchase and Theron, 1968) and also an inhibitor of protein synthesis (Heller and Röschenthaler, 1978). A moiety resembling the polyketide citrinin (Table 1, S.1) is clearly recognisable as a component of the ochratoxin A structure with phenylalanine comprising the other part of the molecule.

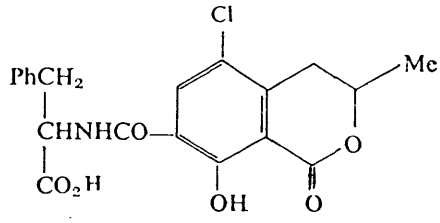
Some alkaloids are produced as a result of mixed metabolism of aromatic amino acids and mevalonate and thus contain one or more C_5 units in the molecule. Dimethylallyl-pyrophosphate (DMAPP), formed *via* acetate, is the C_5 unit most frequently incorporated. Acetate alternatively may be incorporated directly into an alkaloid, for example as an acetoxy unit, which is evident in the biosynthesis of

Fig. 2 Examples of fungal metabolites of mixed biosynthetic origin.

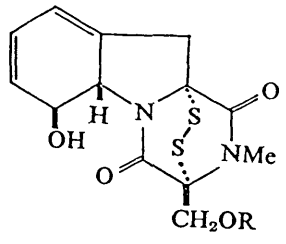
S.17 Mycophenolic acid



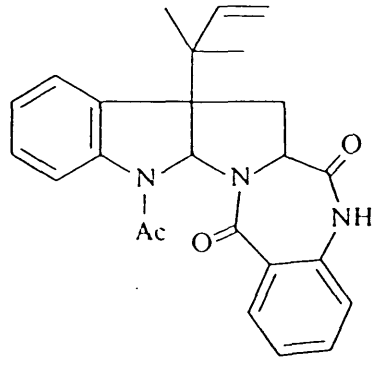
S.18 Ochratoxin A



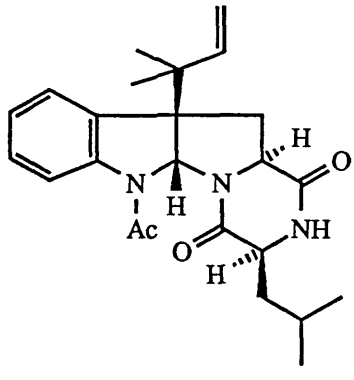
S.19 Gliotoxin acetate; R=Ac
Gliotoxin; R=H



S.20 LL-S490β



S.21 Verrucofortine



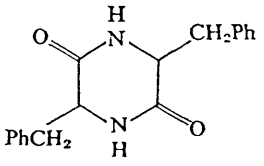
gliotoxin acetate (Fig. 2, S.19) in *Penicillium terlikowski* (Johnson *et al.*, 1953). There are few examples of secondary metabolites of fungi, amongst diketopiperazines at least, containing both an isoprene (C_5) and a non-polyketide C_2 unit within the molecule. An unidentified *Aspergillus* sp. was found to produce a benzodiazepine metabolite, LL-S490β (Fig. 2, S.20), containing an isoprene unit at C-3 and an indolic N-acetyl group (Ellestad *et al.*, 1973). Until the isolation of verrucofortine (Fig. 2, S.21) from *Penicillium verrucosum* (Hodge *et al.*, 1988), there were apparently no examples of *Penicillium* secondary products containing both an acetyl and an isoprene group. Incorporation of both an acetyl and a C-5 unit into a molecule raises interesting questions about the regulation of the competing biosynthetic rôles of acetate. Isomerisation of the C_5 unit is evident in LL-S490β and verrucofortine in the form of an inverted isoprene unit, which creates additional structural diversity amongst secondary metabolites. Turner (1971) and Turner and Aldridge (1983) have provided the authoritative texts for rationalisation of the structural diversity of fungal secondary metabolites.

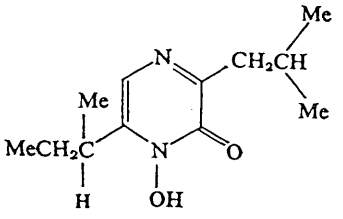
1.4 DIKETOPIPERAZINE METABOLITES OF FUNGI.

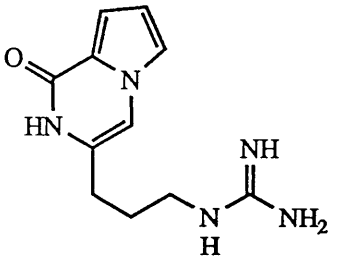
Fungal alkaloid biosynthesis often incorporates cyclisation of two amino acids to form a diketopiperazine. Table 3 illustrates the main groups of diketopiperazine metabolites: (i) simple diketopiperazines in which the ring is unchanged, (ii) modified diketopiperazines such as aspergillic acid in which the ring system has been oxidised at a nitrogen atom and has lost one of the carbonyl oxygens, (iii) the sporidesmins and related compounds in which the diketopiperazine ring is bridged by a dithio group and (iv) compounds such as viridicatin in which the ring has contracted, in this case from a 7-membered benzodiazepine nucleus. Diketopiperazines from each group have been comprehensively reviewed (Turner, 1971; Turner and Aldridge, 1983).

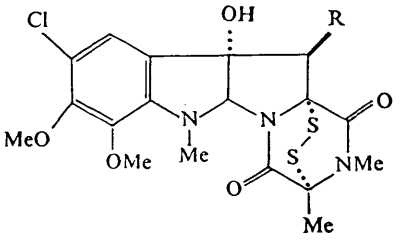
Table 3 Types of diketopiperazine (DKP) produced by fungi.

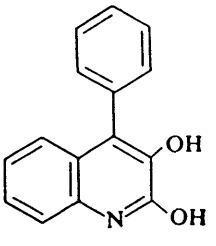
Modification to DKP ring		Examples
1. Unchanged ring	S.22	L-phenylalanine anhydride
2. Loss of one carbonyl oxygen atom and/or oxidation at a nitrogen atom	S.23	Aspergillic acid
	S.24	Peramine
3. Bridged by a dithio group	S.25	Gliotoxin; see Fig. 2, S.19
	S.26	Sporidesmin; R=OH
4. Ring contraction	S.27	Viridicatin











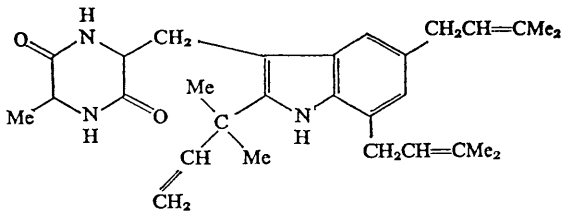
A simple diketopiperazine may be produced by condensation of two similar or different aromatic amino acids. L-phenylalanine anhydride (Table 3, S.22), isolated from *P. nigricans* (Birkinshaw and Mohammed, 1962), exemplifies the former situation as a phenylalanine dimer. Modification of the diketopiperazine ring, such as observed in aspergillic acid, gives rise to metabolites chemically known as pyrazine-1-oxides. Aspergillic acid (S.23) was reported to be biosynthesised from leucine and isoleucine on the basis of degradation studies (Dunn *et al.*, 1949a,b). Later, supportive evidence was obtained from the work of MacDonald (1961) which showed incorporation of radiolabelled leucine into the isolated molecule. Aspergillic acid, produced by *Aspergillus flavus*, was first isolated as an antibiotic. White and Hill (1943) suggested that it caused convulsions in animals. Peramine (S.24) is an *Acremonium* metabolite (Rowan *et al.*, 1986) and appears to be derived as a cyclic anhydride of proline and arginine with loss of one carbonyl oxygen atom. It has insect anti-feedant properties and is of potential interest as an agrochemical.

Sporidesmin (Table 3, S.26) is a dithio-bridged diketopiperazine, exhibiting methylation at several positions in the molecule. The sporidesmins are of economic concern in that they cause facial eczema in sheep and cattle, notably in New Zealand (Leigh and Taylor, 1976). Gliotoxin (Fig.2, S.19) is derived from phenylalanine and serine and is another example of a dithio-bridged diketopiperazine. Gliotoxin has anti-viral properties but is too toxic for clinical use. It also exhibits anti-bacterial and anti-fungal properties.

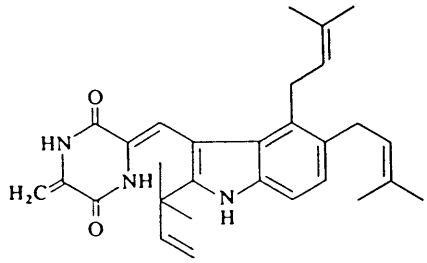
Viridicatin (S.27) exemplifies ring contraction as a further modification of the basic diketopiperazine nucleus. This molecule contains two fused aromatic ring systems and is biosynthesised from cyclopenin (Table 1, S.5), a process which involves contraction of the 7-membered benzodiazepine ring.

Fig. 3 Examples of isoprenylated fungal metabolites.

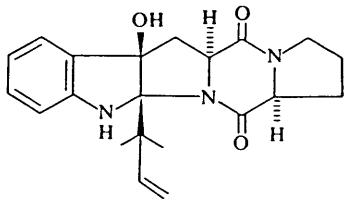
S.28 Echinulin



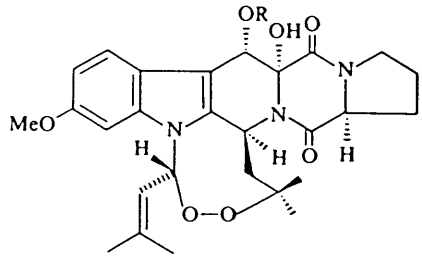
S.29 Cryptoechinulin G



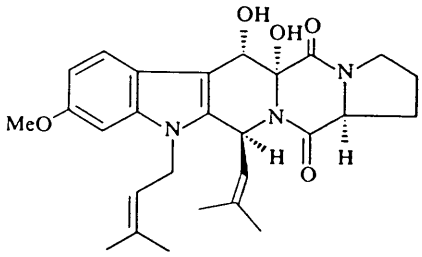
S.30 Brevianamide E



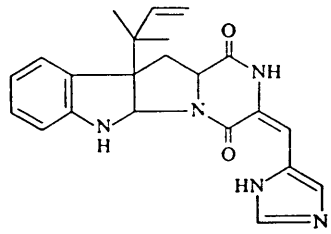
S.31 Verruculogen; R=H
S.32 Fumitremorgen A; R=$(Me)_2$-C=CH-CH_2-



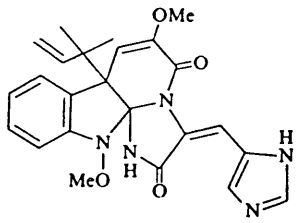
S.33 Fumitremorgen B



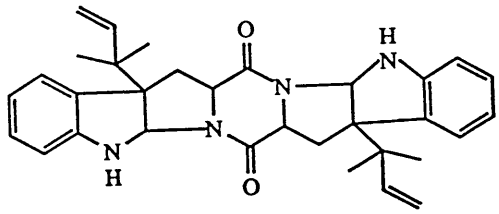
S.34 Roquefortine



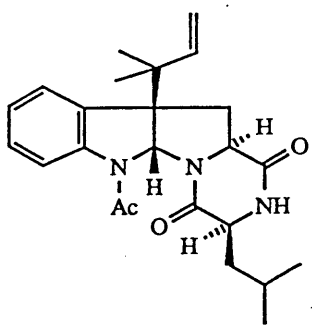
S.35 Oxaline



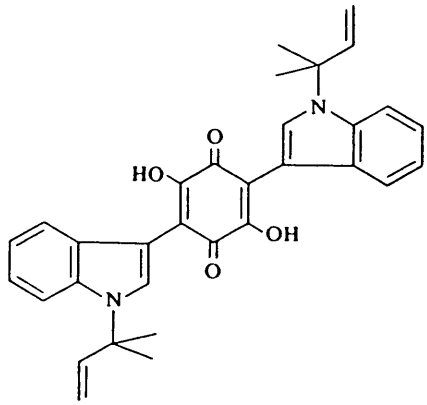
S.36 Nigrifortine



S.37 Verrucofortine



S.38 Asterriquinone



1.5 ISOPRENYLATED DIKETOPIPERAZINE METABOLITES.

The amino acid tryptophan frequently participates in the formation of the diketopiperazine nucleus together with both aliphatic and aromatic amino acids. The echinulin series of metabolites, from *Aspergillus echinulatus*, *A. amstelodami* and *A. ruber*, are formed from tryptophan and alanine (Quilico and Panizzi, 1943; Birch *et al.*, 1961; Birch and Farrar, 1963; MacDonald and Slater, 1966; Allen, 1972). Echinulin (Fig. 3, S.28) serves as an example of a metabolite of mixed biosynthetic origin, integrating both mevalonic acid and amino acid biosyntheses. The tryptophan moiety of echinulin has three isoprene units attached at positions C-2, C-5 and C-7.

It is important to note the orientation of the isoprene units attached to the indole nucleus of tryptophan-containing metabolites. An increasing number of fungal metabolites have been isolated which contain one or more inverted isoprene groups.

Cryptoechinulin G (Fig. 3, S.29), isolated from *A. ruber* (Gatti *et al.*, 1978), contains in addition to the inverted isoprene at C-2, two "normal" 1,1-dimethylallyl groups at C-4 and C-5 of the indole ring. The large group of brevianamide metabolites, consisting of tryptophan and proline, are structural variants of brevianamide E (S.30), isolated by Birch and Wright (1969). The single inverted isoprene unit in brevianamide E is attached to C-2 of the indole group in a way similar to that of one of the isoprene units in echinulin. The brevianamide group now includes the mycotoxins verruculogen (S.31) and fumitremorgens A and B (S.32, S.33), which demonstrate attachment of normal 1,1-dimethylallyl groups.

Other isoprenylated compounds include roquefortine (Fig. 3, S.34) and oxaline (S.35), biogenetically similar mycotoxins derived from tryptophan and histidine. During the biosynthesis of oxaline, the diketopiperazine ring apparently undergoes an oxidative cleavage

which can lead to the uncommon linking of the tryptophan and histidine moieties in this molecule (Nagel *et al.*, 1976). Oxaline, isolated from *P. oxalicum* (Nagel *et al.*, 1976), has an N-methoxy group in the tryptophan moiety, adding to the diverse structural repertoire. In roquefortine and oxaline the isoprene unit is present in an inverted configuration at a novel position on C-3 of the indole moiety. Nigrifortine (Fig. 3, S.36), a symmetrical molecule resulting from cyclic anhydride formation between two tryptophan entities, has inverted isoprene units similarly at the C-3 position in both halves of the dimeric species (Laws and Mantle, 1985). Verrucofortine (S.37) also has an inverted isoprene unit at C-3.

Attachment of the isoprene unit to the indole nitrogen atom is observed in the fumitremorgen metabolites and verruculogen and also occurs in the tryptophan-tryptophan cyclic anhydride, asterriquinone (Fig. 3, S.38) isolated from *Aspergillus terreus* (Yamamoto *et al.*, 1976). Various research groups have sought to identify the mechanism of isoprenylation. In one study of a model reaction an acid catalysed rearrangement of the isoprene chain was observed from position 1 to 2 of the indole nucleus together with a partial rearrangement of the alkyl chain (Casnati and Ponchini, 1970). Treatment of the model compound, 3-methyl-N-(1,1-dimethylallyl)indole, with trifluoroacetic acid gave two isoprenylated compounds, one having an inverted and the other a normal type isoprene unit at C-2. This finding indicated the possible participation of an initial prenylation at the nitrogen atom of the indole ring followed by a rearrangement to position 2 with an inversion of the migrating isoprene group in the biosynthesis of echinulin type metabolites. The occurrence of a normal dimethylallyl group at C-2 of one compound in the reaction indicated a possible mechanism for the biosynthesis of fumitremorgen-type metabolites (Casnati *et al.*, 1974). The reason for the normal linkage of the isoprene group solely in the fumitremorgen-related metabolites

remains obscure (Sammes, 1975).

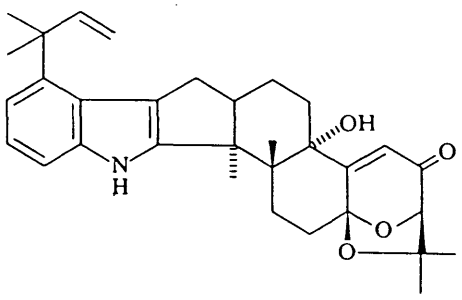
The indole-containing metabolites fumitremorgen A and B (S.32, S.33) were identified in the fungal extract of *Aspergillus fumigatus*. Certain strains of *A. fumigatus* were found to cause tremor and convulsions in experimental animals (Yamazaki *et al.* 1971). Verruculogen (S.31) was obtained from a strain of *Penicillium verruculosum* isolated in 1972 from peanuts and found to be a tremorgenic mycotoxin. Administration of verruculogen by mouth to mice caused tremor and convulsions, but intra-peritoneal injection was more effective (Cole et al., 1972).

Roquefortine (S.34) was isolated from the mycelium of *P. roquefortii* (Scott *et al.*, 1976). This fungus is of interest to food scientists owing to its use in the production of roquefort cheese. Roquefortine was found to possess neurotoxic properties and to cause convulsions in animals (Scott *et al.*, 1976). However, nigrifortine (S.36), structurally similar to roquefortine, had no apparent effect when administered intra-peritoneally to mice (Laws and Mantle, 1985).

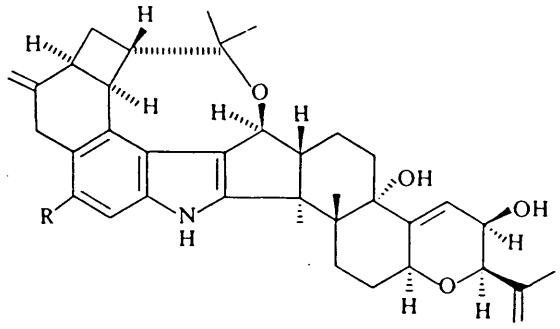
Specific structural features of fungal metabolites are sometimes indicative of a certain biological activity. Indole-isoprenoid metabolites containing a tertiary hydroxyl group, for example, have a tendency to be tremorgenic in experimental animals. Such tremorgenic mycotoxins are shown in Fig. 4 and include aflatrem (Cole *et al.*, 1981), penitrems (De Jesus *et al.*, 1983), paspalinines (Cole *et al.*, 1977), janthitrems (De Jesus *et al.*, 1984) and paxilline (Springer *et al.*, 1975).

Fig. 4 Fungal metabolites of mixed biosynthetic origin, exhibiting tremorgenic properties.

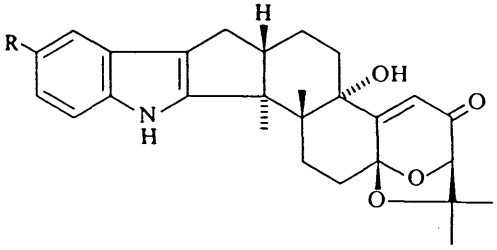
S.39 Aflatrem



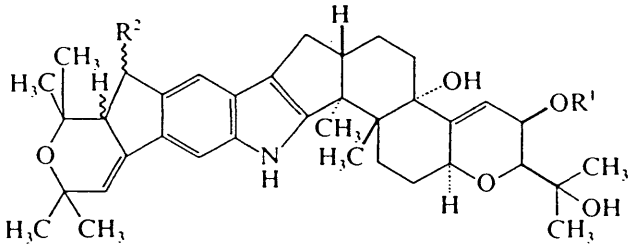
S.40 Penitrem F; R=Cl



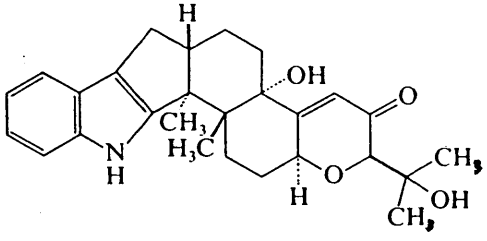
S.41 Paspalinine A; R=H



S.42 Janthitrem A; R^1=H, R^2=OH
Janthitrem B; R^1=$COCH_3$, R^2=OH
Janthitrem C; R^1=$COCH_3$, R^2=H



S.43 Paxilline



1.6 POSSIBLE INTER-GENERIC RELATIONSHIPS BETWEEN FUNGI, PRODUCING ISOPRENYLATED INDOLE DERIVATIVES.

Isoprenylated indole derivatives are not extensively produced in nature, being primarily biosynthesised by fungi. A large proportion of indole-isoprenoid metabolites are derived from species of *Penicillium*, as exemplified above. However, the ergot alkaloids (section 1.9) derived from *Claviceps* spp. constitute a major group of metabolites encompassing biosynthesis of 4-dimethylallyltryptophan. An intergeneric relationship with regard to alkaloid biosynthesis between the two groups of fungi may be implied. Such a link is further substantiated by the fact that agroclavine, a conventional intermediate in the biosynthesis of lysergic acid derivatives (see section 1.9 - Ergot alkaloids) in *Claviceps* spp., is also a *Penicillium* metabolite (Abe *et al.*, 1967).

Substitution of tryptophan by isoprene units occurs in cyclopiazonic acid (Table 2, S.12) which especially bears a biogenetic relationship to ergoline alkaloids. Holzapfel (1968) was the first to report the isolation of this major toxic metabolite from *P. cyclopium*. The possibility that 3,3-dimethylallyltryptophan, the direct precursor of ergot alkaloids, was involved in biosynthesis of cyclopiazonic acid was eliminated, however, by biosynthetic studies (McGrath *et al.*, 1976). A phylogenetic connection between *Claviceps* and *Penicillium* may be further implied by the production of the indole-isoprenoid paspalinines by *C. paspali* (Cole *et al.*, 1977). The main skeleton of these compounds is identical to paxilline, the penitrems and janthitrem metabolites from *Penicillium* species.

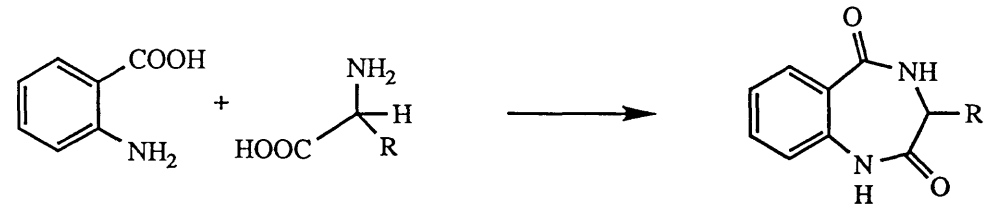
1.7 ANTHRANILIC ACID-DERIVED FUNGAL SECONDARY METABOLITES.

Anthranilic acid is ordinarily an intermediate in the biosynthesis of tryptophan but it may also be diverted to secondary biosynthesis, in which case, if it reacts with an α-amino acid to form a cyclic

anhydride, the benzodiazepine nucleus is created (Fig. 5). The first benzodiazepine to be characterised was cyclopenin (Fig. 6, S.44) which appears to be biosynthesised from anthranilic acid and phenylalanine (Luckner and Mothes, 1962) although quantitative evidence for the incorporation of ^{14}C-radiolabelled precursors seems not to have been published. Cyclopenol and cyclopeptine (Fig. 6, S.45, S.46) were isolated at about the same time (Birkinshaw *et al.*, 1963; Mohammed and Luckner, 1963). The benzodiazepines auranthine (S.47) and asperlicin (S.48) are discussed later.

Other biosynthetic combinations, apart from the formation of benzodiazepines, are possible. The metabolite anthglutin (Fig. 7, S.49) demonstrates a hydrazine linkage between, presumably, glutamine and anthranilic acid. Anthglutin was isolated from *P. oxalicum* and was found to inhibit γ-glutamyl transpeptidase (Minato, 1979), which catalyses the degradation of glutathione and transfers the glutamyl moiety to amino acid and peptide acceptors. 2-Pyruvoylaminobenzamide (S.50) was identified as the anti-auxin produced by *Colletotrichum lagenarium* (Kimura *et al.*, 1973). *Candida lipolytica* converts tryptophan and anthranilic acid into tryptanthrin (S.51) and substituted precursors into substituted tryptanthrins (Fiedler *et al.*, 1976), indicating that the enzyme(s) involved are relatively non-specific. An isophenoxazine synthase, catalysing the conversion of o-aminophenol (S.52) to questiomycin A (S.53), was isolated from *Pycnoporus coccineus* (Nair and Vining, 1964). Since the enzyme is specific for o-aminophenol and since the pigments of *P. coccineus* are all substituted phenoxazinones, the natural significance of the enzyme is not clear (Nair and Vining, 1965). Other examples of anthranilate-containing fungal metabolites are provided by chrysogine (S.54), austamide (S.55) and aspercolorin (S.56). Some anthranilate-containing metabolites exhibit toxicity to animals. Examples are provided by tryptoquivaline (S.57) and tryptoquivalone (S.58).

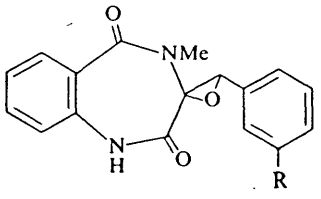
Fig. 5 Formation of the benzodiazepine nucleus from anthranilic acid and an α-amino acid.



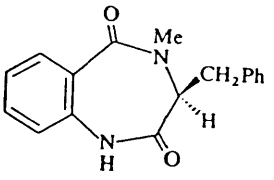
Anthranilic acid α-amino acid Benzodiazepine nucleus

Fig. 6 Benzodiazepine metabolites of *Penicillium* and *Aspergillus* fungi.

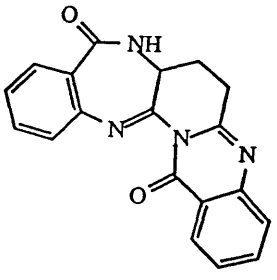
S.44 Cyclopenin; R=H
S.45 Cyclopenol; R=OH



S.46 Cyclopeptine



S.47 Auranthine



S.48 Asperlicin

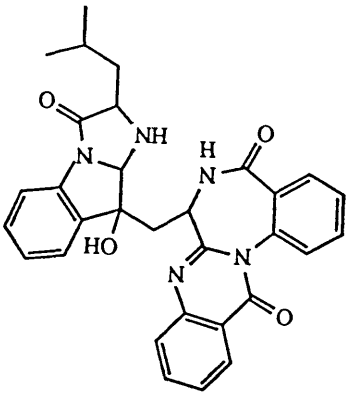
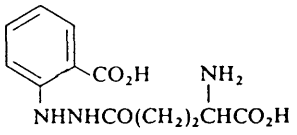
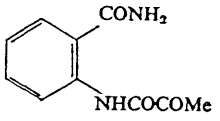


Fig. 7 Anthranilic acid-containing fungal metabolites (non-benzodiazepine structures)

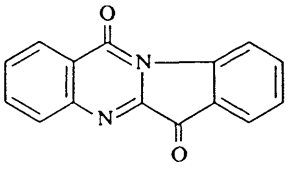
S.49 Anthglutin
(*Penicillium oxalicum*)



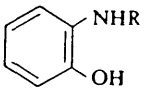
S.50 2-pyruvoylaminobenzamide
(*P. chrysogenum*)



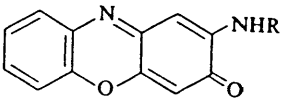
S.51 Tryptanthrin
(*Candida lipolytica*)



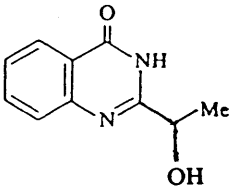
S.52 *o*-aminophenol; R=H
(*P. notatum*)



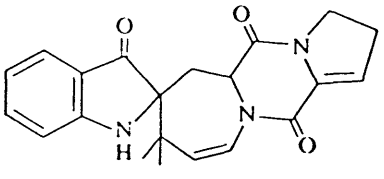
S.53 Questiomycin A; R=H
(*P. notatum*)



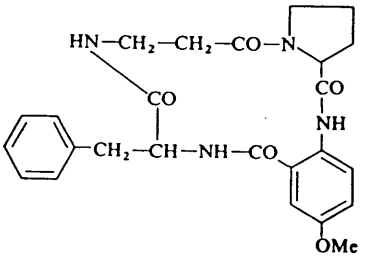
S.54 Chrysogine
(*P. chrysogenum*)



S.55 Austamide
(*Aspergillus ustus*)

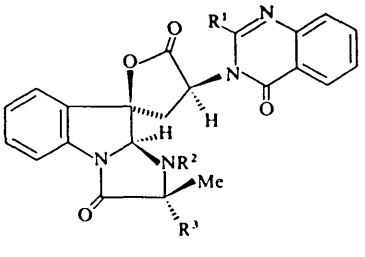


S.56 Aspercolorin
(*A. versicolor*)



S.57 Tryptoquivaline; R^1 =  R^2=OH, R^3=Me

S.58 Tryptoquivalone; R^1 =  R^2=OH, R^3=H
(*A. clavatus*)



Tryptoquivaline and tryptoquivalone were isolated by Clardy *et al.* (1975) from a toxigenic strain of *Aspergillus clavatus* and have been reported to be tremorgenic (Büchi *et al.*, 1973).

Penicillium fungi, certain species of which are well renowned for their production of antibiotics, may also elaborate toxic secondary metabolites as described earlier, or they may produce compounds possessing a different biological activity of potential therapeutic application. However, since about half the Penicillia remain unexplored for elaboration of biologically active compounds (Mantle, 1987), let alone other fungal genera, there awaits a large source of untapped potential. Man has been able to exploit antibiotic-producing organisms for commercial gain and the elimination of much potential suffering has been possible by the discovery of novel antibiotics. Changes in the rationale behind screening techniques have been largely responsible for the discovery of the so-called "second generation" antibiotics *e.g.* carbapenems and monobactams. Improved methods in organic chemistry have enabled the development of semi-synthetic antibiotics, with an enhanced or wider spectrum of activity, and have contributed greatly to a clearer understanding of structure-activity relationships. Structural considerations may therefore allow tentative perception of potential biological activity. Antibiosis is not, however, the only clinically useful property of secondary metabolites and, moreover, several fungal metabolites are toxic to humans and animals.

The old, empirical approach of screening fungi involves isolation from the wild, careful identification and cultivation of morphologically different types and final evaluation of their ability to produce potential therapeutic compounds. This approach, as well as being extremely tedious, does not take into account the fact that, although some organisms might share the same phenotype, they may have different genotypes and therefore might produce a different array of

secondary metabolites. Some organisms might be genotypically-silent, also, when grown under laboratory conditions.

Novel genotype-specific screening methods are desperately required to capitalise on this area of microbial biochemistry. Methods for predicting fungal species that contain hidden biosynthetic potential are needed as well as selective techniques for isolating specific structural types of secondary metabolites. Mantle (1987) ingeniously proposed the use of a biosynthetic probe as an aid to selecting benzodiazepine metabolites from laboratory cultures of micro-organisms. This perceptive proposal was based on the predicted biosynthesis of the benzodiazepine nucleus, from anthranilic acid and an α-amino acid (Fig. 5).

The administration of radiolabelled anthranilic acid to a fungal culture is usually most effective after the period of rapid growth when there is reduced competition for the precursor by primary processes. The rationale behind this approach, using a specific radiolabel, is best appreciated by considering the normal fate of the carboxyl group of anthranilic acid in tryptophan biosynthesis. In this way, [^{14}C-carbonyl]-anthranilic acid will be decarboxylated by the normal mechanism in primary metabolism and end up in protein or in a tryptophan-containing secondary metabolite, in which case the product does not retain the radiolabel. If, however, part of the anthranilate pool is directed into a secondary pathway so that the carbonyl carbon atom is retained, there is a chance for the molecule to form a cyclic anhydride with an α-amino acid and thereby create a labelled benzodiazepine. Therefore, there is a distinct advantage in having the radiolabel in the carbonyl position as against the aromatic carbons of the probe; selection of benzodiazepines from all other tryptophan-containing metabolites is ensured. Complete specificity for the benzodiazepine nucleus cannot be guaranteed because the anthranilic acid molecule, with an intact carbonyl carbon

atom, may react to form other structures such as anthglutin, for example.

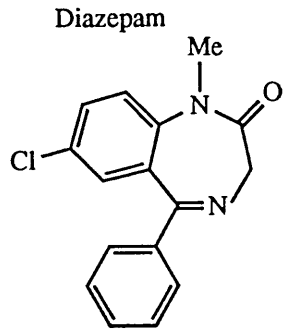
The effective use of [^{14}C-carbonyl]-anthranilic acid as a biosynthetic probe was demonstrated by the discovery of the novel benzodiazepine auranthine (Yeulet and Mantle, 1987). Auranthine (Fig. 6, S.47) is structurally similar to asperlicin (S.48), a benzodiazepine fungal metabolite isolated in the Merck, Sharp and Dohme laboratories (Goetz *et al.*, 1985), which exhibits cholecystokinin antagonism. Cholecystokinin is a peptidal gastro-intestinal transmitter involved in the control of pancreatic and gastric secretion, contraction of the gall bladder and gut movements. There is an obvious market for non-peptidal antagonists in the design of orally effective drugs since they will be less likely to be degraded by digestive enzymes. Auranthine, however, did not show significant competitive activity in receptor binding assays (Merck, Sharp and Dohme, personal communication).

The benzodiazepine nucleus was found to be a crucial element in the binding of non-peptidal ligands to peptide receptors (Evans *et al.*,1986) and is also an important feature of the synthetic anti-anxiety drug diazepam (Fig. 8, S.59). Diazepam also contains a chlorine atom which, as indicated earlier, is a structural feature of other biologically active fungal metabolites.

Evans *et al.* (1986) enhanced the activity of asperlicin by creating structural analogues that contained the benzodiazepine nucleus but which had a prenyl group substituted at C-5 of the ring, as in diazepam. The 5-phenyl-1,4-benzodiazepine ring system is thought to be a very effective peptide receptor ligand. There is evidence that the natural ligand for the diazepam receptor is in fact a peptide (Guidotti *et al.*, 1983; Alho *et al.*, 1985).

Fig. 8 Structure of diazepam, a synthetic benzodiazepine.

S.59



Natural products, therefore, may provide useful leads to therapeutic agents. There is an obvious need to isolate novel fungal secondary metabolites, to explore their biological activity and to modify the structure accordingly (such as synthesis of a 5-phenyl derivative). Chemical modification is currently used successfully in creating semi-synthetic antibiotics. However, the extent to which novel molecules might be accepted by appropriate fungal enzymes *in vivo* to effect aromatic chlorination, for example, could be explored in fungi naturally producing such substituted aromatic natural products. The use of cell-free enzyme systems might prove to be a feasible method for achieving specific structural modifications.

1.8 ERGOT FUNGI AND ERGOTISM.

The ergot alkaloids are the largest class of metabolites biosynthesised from tryptophan and mevalonate. These metabolites are produced by various species of *Claviceps* fungi and some were responsible for outbreaks of ergotism, a recurrent pestilence of the Middle Ages. The ergot fungi (*Claviceps* spp.) are plant parasites and contain the toxic alkaloids within the ergot-body (sclerotium), which is a hard, frequently pigmented structure that protrudes from the seedhead of various grasses. The sclerotium falls to the ground and, if undisturbed, it overwinters and germinates the following Spring to produce a stroma consisting of a stipe and a capitulum, containing the ascogenous perithecia. The resultant ascospores are released into the air and alight on stigmas of flowers of the appropriate host plant to initiate the infection process. The invading hyphae grow through the ovary which, approximately ten days after initial infection, is totally replaced by white fungal tissue (sphacelial stage). Asexual spores are formed which are spread to other flowers by honey-dew produced by the host plant. In a later stage of parasitism, about three weeks after initial infection, the hyphae become differentiated, filled with lipid and so are more densely packed, to produce the sclerotium. In axenic culture sclerotial hyphae appear morphologically different from sphacelial tissue. Sphacelial hyphae are long, thin and infrequently branching whereas sclerotial hyphae are short, lipid-containing structures.

There are several species of *Claviceps* which differ in morphology and host plant; for example, *C. purpurea* readily infects rye but also infects barley, wheat and more than one hundred temperate grasses. The tropical small grain crop, millet, is parasitised by *C. fusiformis*, the morphology of which is quite distinctive and in which the ergot alkaloid biosynthetic pathway of *C. purpurea* is restricted to the clavine alkaloid intermediates.

Classical ergotism in humans, caused by consumption of bread made from ergot-contaminated rye, may be the earliest and most formidable example of mycotoxicosis in Man. Two types of epidemic have been recorded, gangrenous and convulsive. Gangrenous ergotism was prevalent from the Middle Ages to the nineteenth century. The symptoms included violent burning pains in the hands and feet which resulted in this type of ergotism being known also as St. Anthony's Fire. Often feet but sometimes hands became swollen and inflamed and gradually became numb, turned black, shrank and finally became mummified and dry. The gangrenous part often separated from the limb at a joint.

Convulsive ergotism is the name given to describe a syndrome which was manifest between 1581 and the last large outbreak in Russia in 1928. An early symptom of convulsive ergotism was a tingling sensation such as experienced when one's hands and feet have "Pins and Needles". The entire body was racked by spasms and severe contortions of the body ensued. There is some doubt as to whether ergot was responsible for outbreaks of this kind because the symptom of muscle spasms does not correlate with the pharmacology of the ergot alkaloids (P.G. Mantle, personal communication). However, in the last Russian epidemic it was reported that death occurred when flour containing 7% ergot was ingested. It might be, perhaps, that ergot was a contributary factor in the aetiology of convulsive ergotism. Since the Russian incident, most European countries have set limits of 0.1-0.2% ergot in flour (Floss and Anderson, 1980).

The occurrence of ergotism has declined as the diet has become more varied. Use of clean seed, crop rotation, cutting of wild grasses near the fields, deep planting and selection of varieties of plants which all flower at the same time are cultural practices which have reduced infection in the field. A flotation method in brine can be used to remove ergot from grain before milling.

1.9 ERGOT ALKALOIDS: LYSERGIC ACID DERIVATIVES AND CLAVINES.

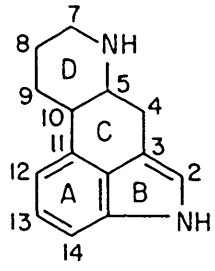
The ergot alkaloids are mostly derivatives of the four-ring structure of ergoline (Fig. 9, S.60). They may be grouped on account of structural features into (i) clavine alkaloids and (ii) lysergic acid derivatives. The ergot alkaloids proper are amides of lysergic acid (Fig. 9, S.61) and occur as interconvertible pairs, epimeric at C-8. The amide part of the molecule may be simple such as a short chain alkylamide or it may be quite a complex cyclic tri-peptide, giving rise to the cyclol-type peptide alkaloids (Fig. 10). Hydrolysis of the peptide alkaloids yields D-lysergic acid, L-proline, another α-amino acid and an α-keto acid. The α-keto acid is produced from the amino acid moiety adjacent to D-lysergic acid.

In addition to the peptide alkaloids, non-peptide amides of lysergic acid occur in ergot fungi and such alkaloids include ergometrine (S.71), lysergic acid α-hydroxyethylamide (S.72), lysergic acid amide (S.73, ergine) and paspalic acid (S.74) which are illustrated in Fig. 11. Ergometrine (ergobasine, ergonovine) was isolated simultaneously in several laboratories in the early part of this century (*e.g.* Thompson, 1935). Lysergic acid α-hydroxyethylamide spontaneously decomposes to lysergic acid amide. Paspalic acid is produced by *Claviceps paspali* and differs from lysergic acid in having a double bond in the 8,9 position rather than between C-9 and C-10.

The first crystalline alkaloid from ergot, which was later found to be a mixture of three alkaloids, was obtained by Tanret (1875). These three alkaloids, ergocornine, ergokryptine and ergocristine were also obtained in a crystalline form in 1907 and the mixture was called ergotoxine (Barger and Carr, 1907). Ergotamine (S.62), one of the most important alkaloids, was purified from ergot by Stoll (1918, 1945), and its structure was proved by synthesis in 1961 (Hofmann *et al.*, 1963).

Fig. 9 Structure of ergoline and D-lysergic acid.

S.60 Ergoline



S.61 D-lyse rgic acid

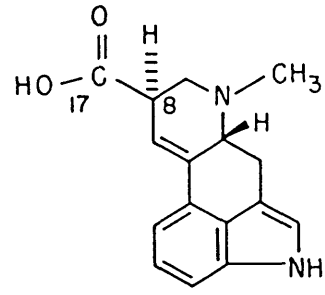
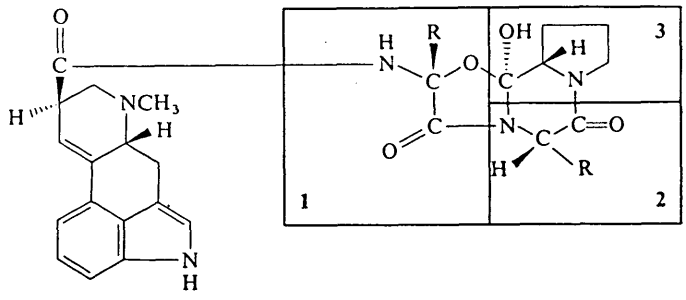
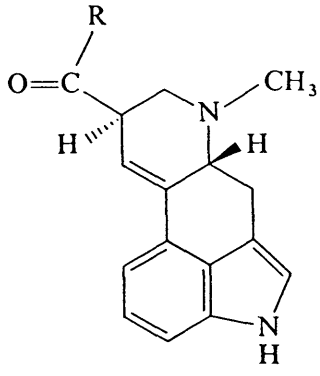


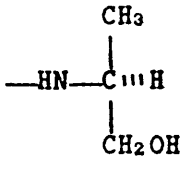
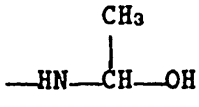
Fig. 10 Cyclol-type peptide ergot alkaloids.



	Alkaloid	α-Hydroxy-L-amino acid 1	L-amino acid 2
S.62	Ergotamine	Alanine	Phenylalanine
S.63	Ergosine	Alanine	Leucine
S.64	Ergocristine	Valine	Phenylalanine
S.65	α-Ergokryptine	Valine	Leucine
S.66	β-Ergokryptine	Valine	Isoleucine
S.67	Ergocornine	Valine	Valine
S.68	Ergostine	α-Aminobutyric acid	Phenylalanine
S.69	Ergohexine	Alanine	Homoleucine
S.70	Ergoheptine	Valine	Homoleucine

Fig. 11 Naturally occurring lysergic acid derivatives other than peptides.



	Alkaloid Name	R
S.61	Lysergic acid	—OH
S.71	Ergometrine (ergonovine, ergobasine)	
S.72	Lysergic acid α-hydroxyethylamide	
S.73	Lysergic acid amide	—NH_2
S.74	Paspalic acid (C-8,9 double bond)	—OH

The clavine class of alkaloids (Fig. 12) developed after agroclavine was identified in saprophytic cultures of an ergot fungus (Abe, 1951). Later, small amounts of clavine alkaloids were found accompanying the lysergic acid alkaloids in parasitic tissues (*e.g.* Voight, 1962). The clavine alkaloids do not contain the carboxyl group at position 17 but instead a group of lower oxidation state *e.g.* methyl or hydroxy methyl. As is the case for the cyclic tripeptide alkaloids (*e.g.* ergotamine/ergotaminine) clavines occur as pairs of C-8 epimers.

A further class of related structures exists, the chanoclavines or 6,7-secoergolenes (Fig. 13), which are tricyclic alkaloids since ring D of the ergoline nucleus is not closed. There are two series of chanoclavines, I and II which respectively have hydrogen *trans* or *cis* at positions 6 and 10. Until 1960 ergot sclerotia were thought to be the sole source of lysergic acid derivatives. However, lysergic acid amide, isolysergic acid amide and chanoclavine were isolated from seeds of higher plants belonging to the Convolulaceae family (Hofmann and Tscherter, 1960). Lysergic acid derivatives have also been found in endophytic Clavicipitaceous fungi, *Balansia epichloe* and *B. henningsiana* and in an Ascomycete, closely related to *Claviceps, Epichloe typhina* (Porter, 1979). Interestingly, many of the clavine alkaloids have been found in other fungal genera. Spilsbury and Wilkinson (1961) were the first to isolate the clavine alkaloids, festuclavine (S.87) and fumigaclavines A and B (S.92, S.93) from *Aspergillus fumigatus*. Agurell (1964) isolated costaclavine (S.88) from *Penicillium chermesinum* and further evidence emerged from Biourge and Abe's group which demonstrated clavines in species of *Penicillium* and *Aspergillus*.

Fig. 12 Clavine alkaloids.

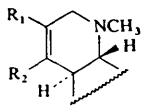
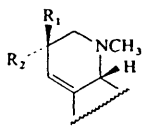
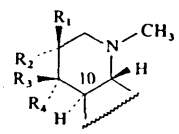
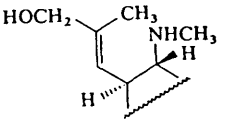
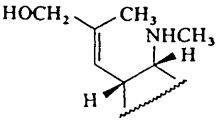
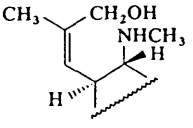
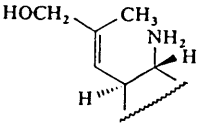
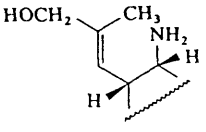
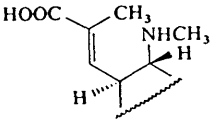
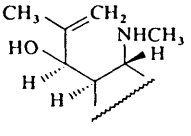
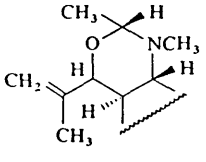
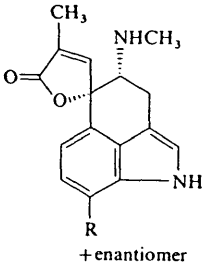
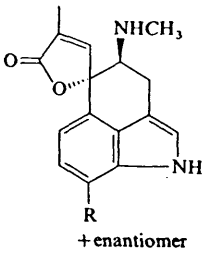
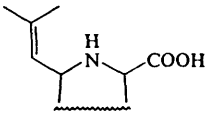
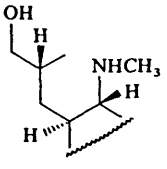
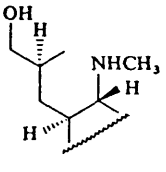
Ergolines	Alkaloid	R_1	R_2	R_3	R_4
$\Delta^{8,9}$-Ergolines					
	S.75 Agroclavine	CH_3	H		
	S.76 Elymoclavine	CH_2OH	H		
	S.77 Molliclavine	CH_2OH	OH		
$\Delta^{9,10}$-Ergolenes					
	S.78 Lysergine	CH_3	H		
	S.79 Lysergol	CH_2OH	H		
	S.80 Isolysergol	H	CH_2OH		
	S.81 Lysergene	$=CH_2$			
	S.82 Setoclavine	CH_3	OH		
	S.83 Isosetoclavine	OH	CH_3		
	S.84 Norsetoclavine (NH instead of NCH_3)	CH_3	OH		
	S.85 Penniclavine	CH_2OH	OH		
	S.86 Isopenniclavine	OH	CH_2OH		
	S.87 Festuclavine	CH_3	H	H	H
	S.88 Costaclavine [epimeric at C(10)]	CH_3	H	H	H
	S.89 Pyroclavine	H	CH_3	H	H
	S.90 Dihydrolysergol I	CH_2OH	H	H	H
	S.91 Dihydrosetoclavine	CH_3	OH	H	H
	S.92 Fumigaclavine A	H	CH_3	CH_3COO	H
	S.93 Fumigaclavine B	H	CH_3	OH	H
	S.94 Isofumigaclavine A (roquefortine A)	CH_3	H	H	CH_3COO
	S.95 Isofumigaclavine B (roquefortine B)	CH_3	H	H	H

Fig. 13 6,7-Secoergolenes and other cycloclavine alkaloids.

Alkaloid		Structure
6,7-Secoergolenes		
S.96	Chanoclavine I	
S.97	Chanoclavine II	 + enantiomer
S.98	Isochanoclavine I	
S.99	Norchanoclavine I	
S.100	Norchanoclavine II	 and/or enantiomer
S.101	Chanoclavine I acid	
S.102	Paliclavine	
S.103	Paspaclavine	
S.104	Rugulovasine A; R=H	 +enantiomer
S.105	8-Chlororugulovasine A; R=Cl	
S.106	Rugulovasine B; R=H	 +enantiomer
S.107	8-Chlororugulovasine B; R=Cl	
Other Cycloclavines		
S.108	Clavicipitic acid	
S.109	Dihydrochanoclavine I	
S.110	Isodihydrochanoclavine I	

1.9.1 Pharmacological Properties Of Ergot Alkaloids.

Although some ergot alkaloids were infamous for their toxic effects, these are often only an expression of pharmacological properties which at appropriate doses can be therapeutic. A major effect of ergot alkaloids is contraction of smooth muscle including, for example, that lining peripheral blood vessels and muscles of the uterus. The use of crude extracts of *C. purpurea* to help contractions of the uterus at birth was reported in a German herbal in 1582. Methylergometrine and ergometrine are used to induce uterine contractions and reduce post-partum haemorrhage. Migraine headaches have for many years been treatable with ergotamine and dihydroergotamine. These ergot alkaloids antagonise the action of the neurohormones serotonin and adrenalin.

Although ergot alkaloids cause vasoconstriction they may also exert a vasodilatory effect by blocking the action of adrenalin. The dihydro derivative of ergotoxine *i.e.* the 1:1:1 mixture of dihydroergocornine, dihydroergocristine and dihydroergokryptine (Hydergine) causes vasodilation and is used in the treatment of high blood pressure and cerebral circulatory disorders.

The ergot alkaloids also affect the central nervous system. Effects on the *medulla oblongata* cause decreased heart-rate, vomiting and reduction of vascular tone. Action on the mid-brain causes hyperthermia, hyperglycemia, rapid breathing, dilation of the pupils of the eye and exaggerated tendon reflexes. The most pharmacologically active components of ergot are amides of D-lysergic acid. The corresponding L-isomer is essentially inactive. LSD, the diethylamide derivative of lysergic acid, obtained by semi-synthesis, has the greatest effect on the mid-brain. In doses of 20-30 µg, LSD is a potent hallucinogen and has had a limited use in psychotherapy. The clavine alkaloids show negligible vasopressor or oxytocic activity by comparison with the lysergic acid derivatives but

agroclavine has been shown to stimulate CNS activity in animals (Mantle, 1975).

1.9.2 Ergot Alkaloid Biosynthesis.

The biosynthesis of ergot alkaloids includes prenylation of tryptophan followed by decarboxylation, cyclisation and oxidation to produce lysergic acid derivatives like ergotamine. The ergoline ring was found to be derived from tryptophan (Gröger *et al.*, 1959; Taber and Vining, 1959) and mevalonic acid (Birch *et al.*, 1960; Gröger *et al.*, 1960; Taylor and Ramstad, 1960). Both D- and L-tryptophan can be incorporated into the ergoline alkaloids (Taber and Vining, 1959), although L-tryptophan is the immediate precursor (Floss *et al.*, 1964) and the D-isomer is presumably incorporated indirectly *via* L-tryptophan. Isoprenylation of tryptophan, at position 4 of the indole ring, to form 4-dimethylallyltryptophan (DMAT) is the first step in the biosynthesis of ergoline alkaloids (Baxter *et al.*, 1961; Floss and Gröger, 1963; 1964). DMAT was synthesised by Plieninger *et al.* (1963) and was shown to be incorporated into ergoline alkaloids with up to 35% efficiency (Plieninger *et al.*, 1964).

It has been shown that DMAT accumulates under anaerobic conditions (Robbers and Floss, 1968). This was the first demonstration that *Claviceps* spp. were capable of synthesising the first intermediate, DMAT, of the alkaloid biosynthetic pathway. Addition of ethionine to cultures of *Claviceps* also caused the accumulation of DMAT (Agurell and Lindgren, 1968). Another amphoteric metabolite, clavicipitic acid (Fig. 13, S.108), was isolated from *Claviceps* cultures to which ethionine had been added (Robbers and Floss, 1968) and was also found to be present in small amounts in uninhibited cultures. King *et al.* (1973) drew attention to the possibility that clavicipitic acid was an artefact of the method of isolation. However, studies of Bajwa *et al.* (1975) confirm that clavicipitic acid is a true metabolite of

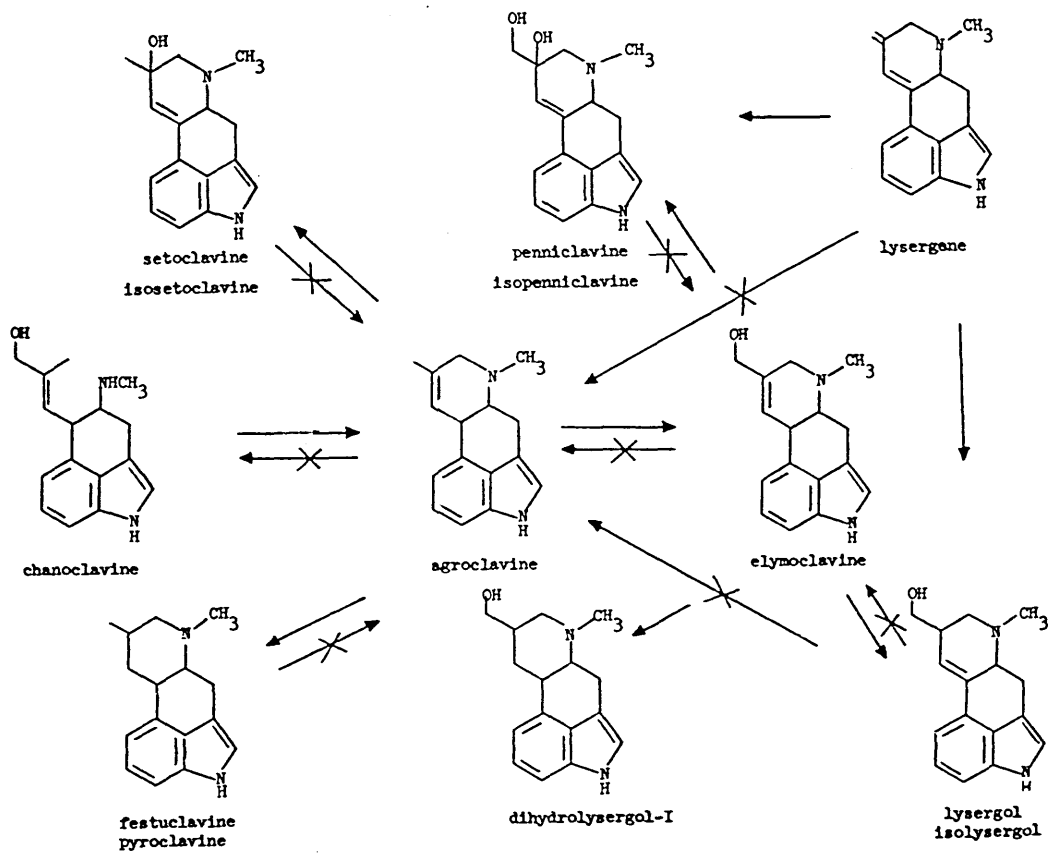
DMAT. Bajwa and Anderson (1975) have demonstrated the conversion of DMAT to clavicipitic acid. The first described structure of clavicipitic acid was revised by King *et al.* (1977) and accepted by Robbers *et al.* (1980). Clavicipitic acid is not a precursor of elymoclavine (Bajwa *et al.*, 1975; Robbers *et al.*, 1980) and its formation seems to represent a derailment of the metabolism leading to the tetracyclic ergolines between the first and second pathway-specific steps *i.e.* the formation and the N-methylation of DMAT (Barrow and Quigley, 1975; Otsuka *et al.*, 1979).

Clavine alkaloids form the end-point of alkaloid biosynthesis in *C. fusiformis* but *C. purpurea* transforms clavine alkaloids to lysergic acid derivatives. Rochelmeyer (1958) proposed the biosynthetic sequence:

chanoclavine ⟶ agroclavine ⟶ elymoclavine ⟶ lysergic acid derivatives.

Fig. 14 shows the biogenetic relationships between clavine alkaloids. Agroclavine is a precursor of setoclavine and isosetoclavine, and elymoclavine is a precursor of penniclavine and isopenniclavine (Agurell and Ramstad, 1962; Agurell, 1966). Radioactive incorporation experiments by Agurell and Ramstad established the conversion of agroclavine to elymoclavine and the failure of the reverse reaction (Agurell and Ramstad, 1962; Agurell, 1966). The conversion of chanoclavine I to elymoclavine is described in Floss *et al.* (1974). The detailed mode of closure of ring C *i.e.* the conversion of N-methyl-dimethylallyltryptophan to chanoclavine I was quite obscure. However, Kozikowski *et al.* (1988) have proposed a reaction sequence for C-ring formation in ergot alkaloid biosynthesis. A comprehensive general review of ergot alkaloid biosynthesis is provided by Floss and Anderson (1980).

Fig. 14 Biosynthetic relationships between clavine alkaloids. (Taken from Floss, 1976).



X Indicates failure of conversion in radioisotope incorporation studies.

1.9.3 Regulation And Control Of Alkaloid Biosynthesis.

Mothes *et al.* (1958) demonstrated the incorporation of tryptophan into ergometrine in parasitic tissue of *C. purpurea*. Since then its rôle as a central precursor of the ergoline ring system has been extensively exploited using labelling techniques (Floss and Anderson, 1980). Teuscher (1964) was the first to observe a correlation between active uptake of tryptophan from the medium by saprophytic cultures of *Claviceps*. Bu'Lock and Powell (1965) observed that excessive accumulation of tryptophan at the end of the growth phase effects cell regulation presumably by induction of various enzymes involved in secondary biosynthesis. During the growth phase tryptophan synthesis is controlled by normal feed-back mechanisms of catabolic repression (Horowitz, 1965; Marshall *et al.*, 1968). In saprophytic cultures when phosphate becomes limiting, there is a disproportionate increase in the tryptophan pool resulting from a change in the feed-back mechanisms (Robbers *et al.*, 1972). Thus the stimulation of alkaloid biosynthesis by tryptophan in sensitive strains is thought to be caused by a triggering effect of the accumulating amino acid, acting as an inducer of secondary enzyme synthesis. The induction effect can also be mimicked by non-metabolisable analogues of tryptophan, for example, thiotryptophan. The other alkaloid precursors, DMAPP and methionine are never rate limiting. Consequently, the induction and rate of alkaloid synthesis is governed by the enzyme catalysing the first pathway-specific step *i.e.* isoprenylation of tryptophan to DMAT, namely DMAT-synthetase.

1.9.4 Enzymology of ergot alkaloid biosynthesis.

Understanding of the enzymology of ergot alkaloid biosynthesis has developed rather slowly. Most knowledge concerning this area of ergot alkaloid biosynthesis has been obtained using cell-free enzyme preparations. However, crude cell-free mixtures represent only the

first stage towards purification of a particular enzyme activity.

1.9.4.1 DMAT-Synthetase.

Two research groups have obtained cell-free systems from *Claviceps* fungi which are capable of synthesising ergoline derivatives *de novo* from simple precursors. Cavender and Anderson (1970) found that the 60-80% ammonium sulphate precipitate from the supernatant of a centifuged homogenate of *Claviceps* strain PRL 1980 cells catalysed the formation of chanoclavine I and II, and to a lesser extent of agroclavine and elymoclavine, from ^{14}C-tryptophan and isopentenylpyrophosphate. This organism is Tyler's strain 47A (Abou-Chaar *et al.*, 1961) which is in fact *C. fusiformis*. The efficiency of conversion was low (0.15%) and required an incubation time of 12 hours. This reaction required the addition of a liver homogenate to the incubation mixture, presumably to provide add itional co-factors.

Abe (1971) and Ohashi *et al.* (1972) obtained cell-free systems from two *Claviceps* strains which catalysed the formation of setoclavine from ^{14}C-tryptophan, mevalonate and methionine. These systems were more efficient and resulted in a 4.4% efficiency of conversion and also required a shorter incubation time (3 hours) for optimal synthesis. The radioactive product was identified by co-crystallisation with authentic material (Ohashi *et al.*, 1972). This system also converted tritiated DMAT into setoclavine (Ohashi and Abe, 1970; Ohashi *et al.*, 1970; Abe, 1971; Ohashi *et al.*, 1972).

There has been some progress in purification of the enzyme DMAT-synthetase which catalyses the first step of ergot alkaloid biosynthesis. DMAT synthetase was isolated from 7 - 9 day old mycelia of *Claviceps fusiformis* strain SD 58 by Heinstein *et al.* (1971). Their work showed that the amount of enzyme activity present in the cells correlated with the amount of alkaloid formed by the same

culture during the fermentation period and their results indicated that little activity was present during the growth phase. The enzyme activity was shown to increase rapidly after about day 4, preceding the appearance of alkaloids in the medium by about one day, which is in accord with the view that alkaloid production in *Claviceps* is an induced process. The enzyme activity was also shown to decrease markedly after day 10 (Heinstein *et al.*, 1971).

DMAT-synthetase was purified to homogeneity by Lee *et al.* (1976). The enzyme was reported to be a single sub-unit protein with a molecular weight between 70,000* and 73,000* and with an isoelectric point at pH 5.8. It was shown to be activated by Fe^{2+}, Mg^{2+} and particularly Ca^{2+}. Cress *et al.* (1981) have reported the purification of DMAT synthetase from *Claviceps fusiformis* strain SD58. This research group have reported the enzyme to be pure as judged by polyacrylamide gel-electrophoresis (PAGE) and have determined the molecular weight to be 70,000*. However the enzyme was shown to run as a single band of molecular weight 34,000* under the denaturing conditions of SDS-PAGE. Cress *et al.* (1981) suggested that DMAT-synthetase was probably a dimeric protein with each sub-unit having a molecular weight of 34,000*. Lee *et al.* (1976) obtained multiple bands when they analysed their protein on SDS-polyacrylamide gels. They did not, however, subject the protein to strong denaturing conditions prior to electrophoresis. It is possible that the sub-units of DMAT-synthetase are linked by disulphide bridges which are cleaved only under strong denaturing conditions.

Cress *et al.* (1981) found that mature cultures of *Claviceps* as reported by Lee *et al.* (1976) were the best source of the enzyme. They have reported, in contrast to Lee *et al.* (1976), that submerged cultures of *Claviceps* strain SD58 did not contain detectable levels of the enzyme. They did not, however, record the morphology of their organism, which quite possibly could have been attributable to a

* Daltons

glucan-producing variant, a morphology which is not conducive to alkaloid formation.

Cress *et al.* (1981) attempted repeatedly to reproduce the enzyme purification scheme reported by Lee *et al.* (1976). They experienced a relatively poor yield using several of the purification techniques and moreover found that omission of calcium from the buffers stabilised the enzyme. The present study used a modification of the purification procedure employed by Lee *et al.* (1976).

DMAT-synthetase has also been isolated from two other alkaloid-producing *Claviceps* strains and partially characterised (Maier and Gröger, 1976).

1.9.4.2 Cell-Free Conversion Of DMAT To Clavicipitic Acid.

The conversion of DMAT into clavicipitic acid has been demonstrated in cell-free extracts of *Claviceps* (Bajwa *et al.*, 1975). The particle-bound enzyme responsible, termed DMAT-oxidase, was solubilised and further characterised (Saini *et al.*, 1976). It was found to require oxygen but no added reducing agent and to catalyse the following reaction with the stoichiometry shown:

$$\text{DMAT} + O_2 \longrightarrow \text{Clavicipitic acid} + H_2O_2$$

The formation of clavicipitic acid is a major alternative to the biosynthesis of clavine alkaloids in the utilisation of DMAT. There is apparently no feed-back control of clavicipitic acid formation by clavine alklaloids since agroclavine did not significantly inhibit DMAT-oxidase (Saini *et al.*, 1976). DMAT-oxidase and the ergot alkaloid biosynthetic pathway may have developed to remove DMAT under different conditions. This is reflected in the contrasting properties of the two pathways; DMAT-oxidase is membrane-bound, is produced during the rapid growth phase and has a high pH optimum. The components of the clavine alkaloid biosynthetic pathway are probably soluble (Cavender and Anderson, 1970), are produced during the phase

of slow or no growth and the pH optimum is near 7 (Cavender and Anderson, 1970; Hsu and Anderson, 1971). The production of clavicipitic acid rather than agroclavine or elymoclavine at higher pH would be advantageous since production of the basic alkaloids would tend to further increase the pH.

It was the aim of this study to examine aspects of the production of alkaloids in *Claviceps* and *Penicillium* fungi. The long-term aim was to characterise DMAT-synthetase from *C. purpurea* and to establish whether it would isoprenylate indolic precursor molecules from other fungal genera.

2. MATERIALS AND METHODS.

2.1 GROWTH AND MAINTENANCE OF FUNGI.

2.1.1 *Penicillium aurantiogriseum.*

2.1.1.1 Origin Of Strain.

The strain of *P. aurantiogriseum* was isolated in 1985 from mouldy maize in Kaniza, near Slavonski Brod., Yugoslavia by Dr. P.G. Mantle.

2.1.1.2 Culture Media.

(a) Potato Dextrose Agar (PDA).

Potato Dextrose Broth (Difco), 24g; distilled water to 1 litre; Oxoid agar no. 1 (2%).

(b) Czapek Dox Yeast Extract (CDYE).

(i) Seed Stage.

Czapek Dox Broth (Difco), 35g; yeast extract (Difco), 5g; distilled water to 1 litre; pH, 7.3 .

(ii) Production Stage (CDYE + 2% calcium chloride).

Czapek Dox Broth, 35g; yeast extract, 5g; $CaCl_2.2H_2O$, 20g; distilled water to 1 litre.

(iii) Large-Scale Fermentation Production Medium.

Sucrose, 1800g; yeast extract (Fould Springer powder), 300g; $NaNO_3$, 180g; K_2HPO_4, 60g; $MgSO_4.7H_2O$, 30g; KCl, 30g; $FeSO_4.7H_2O$, 0.6g; $CaCl_2$ (anhydrous), 1200g; polyglycol P-2000, 6 ml.

Pre-sterilisation pH: Adjusted to pH 5.9-6.1 with $NaOH/H_2SO_4$. Pre-sterilisation volume: 55 litres.

(c) Bran (for *Penicillium aurantiogriseum* spores).

Bran (Jordan's Breakfast Cereal), 10g; distilled water, 5 ml added before sterilisation. Bran medium was usually inoculated 2-3 days after preparation, before it had a chance to dry out.

2.1.1.3 Sterilisation Of Media.

All media including bran were sterilised at 15 psi for 20 min.

2.1.1.4 Culture Maintenance.

Cultures were maintained on slopes of Potato Dextrose Agar (PDA), grown at 27°C and sub-cultured every 1-2 months.

2.1.1.5 Axenic Culture Conditions.

(a) Bran Culture.

Spores from slope cultures were aseptically transferred to sterile bran (2.1.1.2 c) and cultivated for 4 days at 27°C. Profusely sporing bran cultures were used to establish surface mat cultures and primary seed stage submerged cultures of the fungus in CDYE medium.

(b) Surface Culture.

Some bran culture (*ca.* 10 cm^3) was aseptically transferred to an Erlenmeyer flask (500 ml) containing CDYE medium (100 ml) and was carefully scattered over the entire surface. The cultures were grown at 27°C for about 21 days.

(c) Submerged Culture.

A primary seed-stage culture was set up by transfer of some bran culture (*ca.* 10 cm^3) to a baffled Erlenmeyer flask (500 ml) containing CDYE medium (100 ml) which was grown for 24 hours at 27°C on an orbital shaker (200 rpm; 10 cm eccentric throw).

The production-stage culture was obtained by the aseptic transfer of seed-stage culture (5 ml) to an unbaffled Erlenmeyer flask (500 ml) containing CDYE medium, supplemented with 2% calcium chloride (composite medium volume, 100 ml). The culture was grown for 6-12 days at 27°C on an orbital shaker.

2.1.1.6 Inoculum Development For 60 Litre Fermentations.

(a) Primary Seed-Stage.

Primary seed-stage cultures (4 x 100 ml) were grown for 24 hours at 27°C.

(b) Secondary Seed-Stage.

Each entire primary culture (100ml) was aseptically transferred to an Erlenmeyer flask (4 l) containing 1 litre of CDYE medium, thus representing a 10% transfer. The cultures were grown for 24 hours at 27°C on an orbital shaker. Two fermenters (60 l) were each inoculated with secondary seed-stage cultures (2 x 1 l).

2.1.2 Ergot Fungi.

2.1.2.1 Origin Of Strains.

Claviceps fusiformis: strain CAS was a derivative of the glucan-autolysing strain 139/2/1G (Dickerson *et al.*, 1970) originally isolated from a sclerotium parasitic on Bullrush millet (Szczyrbak, 1972).

C. purpurea: strain 12-2 was originally isolated in 1960 from a sclerotium removed from rye in Tarporley, Cheshire. Sclerotial bodies from this isolate consistently fail to biosynthesise ergoline alkaloids (EA) during parasitic development (Corbett *et al.*, 1974).

C. purpurea: strain KL1 was isolated in Czechoslovakia and was a high yielding producer of ergotamine during parasitic growth.

2.1.2.2 Culture Media.

(a) Medium T (Mantle and Tonolo, 1968).

Sucrose, 100g; L-asparagine, 10g; $Ca(NO_3)_2.4H_2O$, 1g; KH_2PO_4, 0.25g; $MgSO_4.7H_2O$, 0.25g; KCl, 0.125g; $FeSO_4.7H_2O$, 0.033g; $ZnSO_4.7H_2O$, 0.027g; L-cysteine hydrochloride, 0.01g; yeast extract (Difco), 0.1g; distilled water to 1 litre. pH adjusted to 5.2 with NaOH. Dispensed as 100 ml in Erlenmeyer flasks (500 ml).

Solid media contained 2% agar (Oxoid no. 1).

(b) Sucrose Corn Steep Liquor (SCSL).

Sucrose, 200g; Corn Steep Liquor (50% solids), 5ml; Tri-ammonium citrate, 7.5g; $Ca(NO_3)_2.4H_2O$, 1.44g; $MgSO_4.7H_2O$, 0.5g; KH_2PO_4, 0.2g; distilled water to 1 litre. Dispensed as 200 ml in Erlenmeyer flasks (500 ml).

Pre-sterilisation pH, 6.8.

2.1.2.3 Culture Maintenance.

C. fusiformis CAS was maintained on slopes of Medium T Agar (MTA) at 27°C and sub-cultured every month.

C. purpurea 12-2 and *C. purpurea* KL1 were maintained on slopes of MTA at 24°C and sub-cultured every month.

2.1.2.4 Re-Isolation Of *C. purpurea* 12-2 From Sclerotia.

C. purpurea 12-2 sclerotia were scraped with a scalpel to remove invaginations and were surface sterilised in mercuric chloride (0.1%) for 5 min. The sterilisation procedure was immediately followed by washing the sclerotia three times in sterile distilled water and aseptically cutting them into small segments. Sclerotial segments were placed (cut face downwards) on PDA containing tetracycline (50 $\mu g\ ml^{-1}$) and incubated at 27°C. Pure cultures were obtained by transferring the emergent white mycelium to slopes of Medium T agar, usually 7 days after transfer to PDA. Large sclerotia were used as a source from which to re-select the organism for subsequent infection of rye. It was thought that a large size might correlate with increased vigour, thus providing ample sclerotial material for experiment.

2.1.2.5 Axenic Culture Conditions.

C. fusiformis CAS was generally cultivated in submerged fermentation. The seed-stage culture was prepared as follows:

A well-developed MTA slope culture (*ca.* 3 weeks old), being pigmented a dark purple colour, was aseptically transferred to an Erlenmeyer flask (500 ml) containing Medium T (100 ml). The mycelium was squashed against the inner surface of the flask to provide ample propagules for quick establishment of primary culture. The culture was grown at 27°C on an orbital shaker for 3-4 days.

The secondary culture was prepared by aseptic transfer of finely divided seed-stage culture (10 ml) to an Erlenmeyer flask (500 ml) containing Medium T (100 ml). If the seed-stage culture was not naturally finely divided then the culture was transferred to a sterile stainless steel homogeniser operated at high speed for 15-30 seconds. The secondary culture was grown at 27°C on an orbital shaker for an appropriate length of time.

C. purpurea 12-2 was cultivated as a surface mycelial mat from a slope culture prepared in the same way as for *C. fusiformis* seed-stage (2.1.2.5) except that the propagules were allowed to float on the surface of the medium. This strain, as well as being deficient in ergoline alkaloid production, is also not normally able to produce pigment in axenic culture (Corbett *et al.*, 1974). Slope cultures of this organism were cream in colour and partly consisted of conidia.

C. purpurea KL1 was cultivated as a surface mat on Sucrose Corn Steep Liquor (SCSL) and became heavily pigmented during growth on this medium. Since slope cultures of this organism did not appear to spore an alternative method of obtaining propagules was used. The most successful surface culture was established by first obtaining a submerged culture of the organism (as with *C. fusiformis* seed-stage), followed by homogenisation which provided a foam inoculum that floated on the surface of the medium.

2.1.2.6 Parasitic Cultivation Of *Claviceps* spp.

(a) *C. purpurea* 12-2.

Svalof's Fourex rye (Swedish Seed Association, Svalof, Sweden) was grown in open plots at The Chelsea Physic Garden, London. In May before the onset of anthesis, to pre-empt competition with pollen, rye ears were inoculated with a conidial suspension of *C. purpurea* 12-2 by injection with a hypodermic syringe into the floral cavity above the ovary. The inoculum had been prepared by macerating a mature slope culture (3 weeks old) of *C. purpurea* 12-2 with water (*ca.* 10 ml). The weather conditions at this time in 1988 (high rainfall and low evening temperatures) were not conducive to rapid fungal colonisation. Honeydew, an orange-amber, sweet-tasting exudate, was first evident 16 days after initial inoculation. At this stage one spikelet was removed and the floral parts were dissected to reveal green anthers at the top of a cream, spongy sphacelial tissue. Early infection of the ovary, before the anthers were fully mature, was thus indicated by their colour. On day 17 a heavily infected spike was macerated with water (*ca.* 15 ml) to provide a suspension of parasitically-produced spores as inoculum with which to spread the infection. The weather had improved and day-time temperatures between 20 and 25°C encouraged the formation of honeydew after 7 days. The exudate arising from natural infection, which appeared to be paler in colour than the originally-formed honeydew, became opaque within 2 days as spores were dislodged from the sphacelial tissue and carried out into the exudate. On day 25 there were signs of the florets being pushed apart and growth of the fungus continued to produce dark-purple pigmented sclerotia, of maximum size 1.2 cm x 0.5 cm, protruding from the rye spikelets in place of seed.

(b) *C. purpurea* KL1.

The inoculum was prepared by homogenisation (with the minimum amount of water) of a mycelial mat grown on SCSL medium. Honeydew, which was colourless in contrast with that arising from *C. purpurea* 12-2 infection, was first evident 13 days after injection of florets. By day 34 evidence of fungal development was apparent as several florets had been forced apart. Dissection of the floral parts at this time revealed a non-pigmented fungal mass. Further development of the fungus resulted in the formation of dark purple sclerotia which were more spherical and not so protuberant as those of *C. purpurea* 12-2.

2.1.2.7 Liquid Nitrogen Storage Of *Claviceps* spp.

Samples (1 ml) of submerged culture of *C. fusiformis* CAS and homogenised surface cultures of *C. purpurea* KL1 were stored in polypropylene ampoules with sterile glycerol/water (10 % V/V) in liquid nitrogen, after having been cooled slowly to -70°C.

2.2 GENERAL METHODS.

2.2.1 Centrifugation.

Centrifugation was performed in a Sorvall RC-5 Superspeed centrifuge. Fixed-angle rotors were used, accommodating 50 or 250 ml vessels.

2.2.2 Separation Of Biomass From Culture Medium.

Surface cultures of *Penicillium aurantiogriseum* and *Claviceps* spp. were separated from the culture medium by lifting the mycelial mat out of the flask as a single entity. The underside of the mycelial mat was washed with distilled water to remove broth solutes.

The cells from submerged cultures of *P. aurantiogriseum* and *C. fusiformis* were removed by vacuum filtration through a sintered glass funnel or by centrifugation at 10,000★ rpm for 15 minutes.

2.2.3 Extraction Of Lyophilised Mycelium.

Mycelial mats were homogenised with the minimum amount of water and lyophilised once the homogenate had been shelled around the inner surface of a round-bottomed flask by rotation in a freezing mixture of solid CO_2 and propan-2-ol.

★ Equivalent to 16,319 x g.

Cells obtained from submerged cultures were washed with distilled water, re-pelleted and lyophilised. Lyophilised mycelium was extracted with acetone (*ca.* 100 ml per g dry weight).

2.2.4 Radiolabels.

All radiolabels were purchased from Amersham International.

^{14}C-[carboxy]-anthranilic acid: specific activity, 10.5 mCi mmol^{-1}.

L-[methyl-^{14}C]-methionine: specific activity, 60.2 mCi mmol^{-1}.

DL-[methylene-^{14}C]-tryptophan: specific activity, 59 mCi mmol^{-1}.

L-[U-^{14}C]-leucine: specific activity, 342 mCi mmol^{-1}.

L-[U-^{14}C]-glutamic acid: specific activity, 275 mCi mmol^{-1}.

^{14}C-n-Hexadecane: specific activity, 0.872 x 10^6 dpm ml^{-1}.

2.2.5 Scintillation Counting.

Liquid scintillant (Scintillator 299™, Canberra-Packard; *ca.* 4 ml) was used and the samples (usually in 100-500 µl $CHCl_3$) were counted in a Kontron Intertechnique scintillation counter. ^{14}C-Hexadecane standard was used routinely to calculate the counting efficiency which was in the range 90-93%.

2.2.6 Autoradiography.

X-ray film (Fuji NIF-RX) was placed in contact with the chromatogram and was sandwiched between glass plates which were bound tightly together using adhesive tape. The package was protected from day-light and stored at -70°C to minimise blurring caused by stray radiation. Exposure of X-ray film to the ^{14}C-activity on the TLC plate was allowed for an appropriate length of time.

2.2.7 Mass Spectometry.

Mass spectrometry was performed on a VG Micromass 7070E instrument, operated at 70 eV with a 100 µA trap current. Low ionisation energy spectra were also usually obtained.

2.2.8 Spectroscopy.

2.2.8.1 Nuclear Magnetic Resonance (NMR) Spectroscopy.

^{1}H and ^{13}C NMR spectroscopy were performed using a Brüker AM 500 MH_z instrument. Chemical shift values were all relative to tetramethylsilane at 0.0 ppm.

2.2.8.2 Infra-Red (IR) Spectoscopy.

IR spectroscopy was performed using a Perkin-Elmer 881 infra-red spectrophotometer.

2.2.8.3 Ultra-Violet (UV) Spectroscopy.

UV spectroscopy was performed on a Varian CARY 210 spectrophotometer.

2.3 METHODS RELATING TO EXPERIMENTS WITH *P. AURANTIOGRISEUM.*

2.3.1 [^{14}C-carbonyl]-Anthranilic Acid Labelling Experiments.

2.3.1.1 Administration Of Radiolabel.

A surface culture of *P. aurantiogriseum* was grown on CDYE medium (100 ml). ^{14}C-anthranilic acid (50 µCi total) was administered in water (600 µl total) under the mycelial mat in equal portions 6, 8 and 10 days after inoculation. The culture was harvested on day 13 and lyophilised to yield a dry weight of 0.96g.

2.3.1.2 Extraction Of Lyophilised Cells.

150 ml of acetone was used over 3 hours to extract the cells. The filtered extract was taken to dryness by rotary evaporation and dissolved in chloroform (1 ml). A small amount was explored by TLC.

2.3.1.3 TLC Conditions For Resolving Mycelial Extracts.

Acetate-backed TLC plates (Camlab Silica G UV_{254}) were used. Mycelial extracts were resolved in solvent system A, chloroform: acetone (1:1), in which the R_f of the radiolabelled benzodiazepine was 0.2. Using solvent system B, chloroform:acetone (1:3), the benzodiazepine had an R_f of 0.5. The appropriate silica band was removed from preparative TLC plates and was eluted with chloroform: propan-2-ol (1:1).

2.3.1.4 HPLC Conditions For Resolving The ^{14}C-Anthranilate-Labelled Benzodiazepine.

A mobile phase of methanol: water (1:1) was used to resolve the compounds eluted from the silica band at R_f 0.2 and 0.5 respectively in solvent A and B (in 2.3.1.3). The sample was dissolved in methanol and resolved by HPLC using the following conditions:

Column, Spherisorb ODS 2, 5 µm particle size; column dimensions, 5 x 0.5 cm; wavelength, 226 nm; mobile phase, methanol: water (1:1); flow rate, 4 ml min^{-1}; chart speed, 5 mm min^{-1}; benzodiazepine (native isomer) retention time, 7 min; benzodiazepine (diastereoisomer) retention time, 13 min.

2.3.2 Protocol For Calcium Chloride Additions To CDYE Broth.

Calcium chloride was added to flasks of CDYE broth (100 ml) to a final concentration of 0.01, 0.1, 0.5 and 2% W/V. Replicate flasks at each concentration of calcium chloride were used. Two control flasks containing unsupplemented CDYE medium (100 ml) were used. Flasks were inoculated by 5% transfer from a primary culture of *P. aurantiogriseum* as described in 2.1.1.5 (c). Cultures were harvested on day 5.

2.3.3 Shaken Flask Fermentations Of *Penicillium aurantiogriseum.*

2.3.3.1 Culture Conditions.

A 5% transfer of a primary culture of *P. aurantiogriseum* as described in 2.1.1.5 (c) was made to each of 4 flasks containing CDYE (100 ml) and to each of 4 flasks containing CDYE supplemented with 2% calcium chloride.

2.3.3.2 Sporulation.

Whole culture was examined microscopically to assess sporophore differentiation. A small amount of culture (*ca.* 2 ml) was coarsely filtered through absorbent cotton-wool to remove hyphae and the spore concentration in the filtrate, suitably diluted where necessary, was measured using a haemocytometer.

2.3.3.3 Biomass Measurement.

The entire contents (*ca.* 100 ml) of one flask from each series was filtered immediately after inoculation to obtain a biomass value for the initial seed-culture. Culture aliquots (10 ml) were removed daily from another flask of each series up to and including day 6. Culture aliquots were filtered through a sintered glass funnel under vacuum and the culture filtrate was frozen and lyophilised. The mycelial cake from each sample was air-dried and weighed. Biomass values of the 10 ml samples were adjusted to correspond to 100 ml of culture.

2.3.3.4 Diketopiperazine Analysis.

Lyophilised broth and air-dried mycelia were extracted with acetone (25 ml) for 24 hours. Acetone extracts were filtered through a small cotton wool plug in a filter funnel and taken to dryness by rotary evaporation. Dried extracts were dissolved in methanol (500 µl) and 25 µl was injected to fill a 20 µl HPLC loop (Rheodyne). Each sample was analysed in triplicate.

2.3.3.5 Analytical HPLC Conditions For The Novel Diketopiperazine.

HPLC Conditions A: column, Novapak C-18, 5 µm particle size; column dimensions, 10 cm x 0.8 cm; wavelength, 250 nm; mobile phase, methanol: water (13:7); flow rate, 1 ml min^{-1}; chart speed, 5 mm min^{-1}; diketopiperazine retention time, 10-11 min.

HPLC Conditions B: column, Novapak C-18, as above; wavelength, 250 nm; flow rate, 4 ml min^{-1}; chart speed, 5 mm min^{-1}; diketopiperazine (DKP) retention time, 12 min; putative oxidation product of the DKP, retention time, 2-3 min; mobile phase, gradient elution as follows:

Time	Solvent composition (methanol: water)
0 - 4	2:3
7 - 10	1:1
11 - 15	2:3
17 - 25	methanol

2.3.4 Large-Scale Fermentation Of *Penicillium aurantiogriseum.*

2.3.4.1 Fermentation Conditions.

Two stainless steel 60 litre fermenters of conventional design (Banks *et al.*, 1974), coded 50/3 and 50/4, were used for analysis and production respectively. The fermenters and media therein were sterilised at 121°C for 20 min by live-steam injection. The pressure in the fermenters at this temperature was 15 psi. Allowance was made for the condensate formed during sterilisation, bringing the post-sterilisation volume to 60 litres. The post-sterilisation pH was between 5.5 and 6.5. The fermentation temperature was 27°C and temperature control was achieved automatically by the regulated flow

of cooling water through the fermenter jacket. Culture aeration was provided by sparging air through a ring-sparger at a rate of 30 l min^{-1} for the first 24 hours and thereafter at 60 l min^{-1}. A homogeneous mixture was maintained using a single disc turbine impeller operated at 367 rpm. Minimum amounts of polypropylene glycol antifoam (P-2000) were added as necessary during the fermentation. After inoculation the fermenters were monitored for contamination daily throughout the fermentation by microscopic examination of samples and plating out at 24, 30 and 37°C on media favourable for growth of bacterial or fungal contaminants.

Samples were taken from fermenter 50/3 as follows: 500 ml, 30 min after inoculation; 4 l at 6 hr; 2 l at 12 hr; 1.5 l at 18 hr; 1 l at 24 hr; 800 ml at 30 hr; 500 ml at 36, 42, 48 and 60 hrs; 300 ml at 72, 84, 96, 120 and 144 hrs.

Dry weight measurement was performed on the entire cell fraction obtained after filtration of the above sample aliquots and the values were adjusted to correspond to 100 ml of culture.

Although the culture in fermenter 50/4 was mainly for bulk production of secondary metabolites, the progress of product formation was monitored using samples removed from the fermenter as follows: 600 ml, 30 min after inoculation; 300 ml at 24, 48, 72, 96, 120 and 144 hrs of the fermentation.

2.3.4.2 Sporulation And Biomass Measurement.

Whole culture from each aliquot sampled from fermenter 50/3 was examined microscopically using Cotton Blue in lactophenol (0.1% W/V) to assess differentiation of sporophores.

The cell component of each aliquot was separated from culture broth by filtering through a sintered glass funnel under vacuum. The cell-paste was removed easily from the funnel by incorporating a filter-paper disc on the sinter. Mycelial samples were photographed using colour film (Fuji Color Super HR 100).

The mycelia were subsequently frozen and lyophilised to constant weight.

2.3.4.3 pH Measurement.

The pH of selected culture filtrates was measured with narrow range pH paper.

2.3.4.4 Sugar Analysis.

Preparation Of Fehling's Solution.

Solution A: powdered crystalline $CuSO_4.5H_2O$, 69.3 g in distilled water, 1 litre.

Solution B: crystalline sodium potassium tartrate, $C_4H_4O_6NaK.4H_2O$, 346 g; NaOH, 142 g; distilled water, 1 litre.

Fehling's solution was prepared by mixing equal volumes of solution A and solution B. Fehling's solution deteriorates slowly on keeping and so fresh solution was prepared when required.

(a) Free Reducing Sugar Determination.

Fehling's A (5 ml) was mixed with an equal volume of Fehling's B in a boiling tube. The appropriately diluted sample (10 ml) was added and the mixture boiled for 3 min and simmered for 3 min. Once the sample had cooled to a temperature comfortable to be hand-held, 2 M H_2SO_4 (10 ml) and 30% potassium iodide (5 ml) were added. The mixture was poured into a flask and a few drops starch solution (2%) were added as an indicator. Flask contents were titrated against 0.05 M sodium thiosulphate ($Na_2S_2O_3$) solution. The end-point was determined with starch solution (which gives a strong blue colour in the presence of free iodine) and was reached when the blue colour had just disappeared.

Reaction equations are:

$$2\ CuSO_4 + 4\ KI \longrightarrow Cu_2I_2 + 2\ K_2SO_4 + I_2$$

$$I_2 + 2\ Na_2S_2O_3 \longrightarrow 2\ NaI + Na_2S_4O_6$$

Since the reducing sugar reacts on an equimolar basis with copper sulphate, which in turn reacts with an equimolar amount of sodium thiosulphate, the amount of thiosulphate used corresponds to the amount of reducing sugar present. The titre of thiosulphate was read off a standard curve for an equimolar solution of glucose and fructose over the range 0-0.3% (see Appendix I).

(b) Total Reducing Sugar Determination (after hydrolysis).

The appropriately diluted sample (10 ml) was boiled with 1 M HCl (5 ml) for 5 min. Once the sample had cooled it was neutralised by the addition of 1 M NaOH (5 ml). The total sample (20 ml) was then added to Fehling's solution (10 ml) and the procedure for free-reducing sugars (2.3.4.4 a) was followed.

(c) Glucose Oxidase Assay.

To 1 ml of appropriately diluted culture filtrate was added glucose oxidase-peroxidase-dye reagent (2ml), prepared as follows:

Tris-hydroxymethylmethylamine, 7.266 g; conc. HCl until pH 7 ; glycerol, 76.5 ml; glucose oxidase (Boehringer), 25 mg; peroxidase (Boehringer), 2 mg; 1% o-dianisidine dihydrochloride (1 ml); distilled water to 200 ml. The reagent was stored at 4°C.

The mixture was incubated for 1 hr at 37°C. The reaction was terminated by the addition of 5 M H_2SO_4 (2 ml). The resultant pink colour was measured spectrophotometrically at 540 nm with reference to a standard curve for glucose in the range 0-50 µg ml^{-1} (see Appendix II).

2.3.4.5 Benzodiazepine Assessment.

The time-course samples from fermenter 50/3 as described in 2.3.4.1 were designated LH (logged hours) which represented the time (in hours) elapsed since inoculation. Each culture aliquot was filtered through a sintered glass funnel under vacuum.

The volume of culture filtrate extracted for each sample was as follows : LH ½, 300 ml; LH 6, 2 l; LH 12, 1.5 l; LH 18, 1 l; LH 24, 750 ml; LH 30, 700 ml; LH 36, LH 42, LH 48, LH 60, 400 ml; LH 72, LH 84, LH 96, LH 120, LH 144, 200 ml.

(a) Extraction Of Culture Filtrates.

The culture filtrates were extracted with a quarter volume of iso-butylmethyl ketone (IBMK). An emulsion was formed on shaking some of the samples with IBMK but it was successfully broken by centrifugation at 3,000 rpm for 10 minutes. The IBMK extracts were taken to dryness by rotary evaporation.

(b) Silica Sep-Pak Chromatography.

This step was employed to remove some of the least polar compounds of the broth extract by selective adsorption of the benzodiazepine to

a silica solid-phase whilst washing with a solvent which favoured solubilisation of the contaminating compounds. The dried IBMK-extracted material was dissolved in chloroform (1 ml) and a portion (200 µl) was loaded onto a silica Sep-Pak (Waters). The Sep-Pak was washed with 20 ml of a solvent comprising chloroform:acetone (5:1) and this was followed by elution of the benzodiazepine with methanol (5 ml). The methanol-eluted material was taken to dryness *in vacuo*.

(c) High Performance Liquid Chromatography (HPLC).

The dried sample, eluted from the silica Sep-Pak, was dissolved in HPLC-grade methanol (1 ml) and 25 µl injected (Hamilton syringe) to fill the 20 µl sample loop (Rheodyne). The HPLC conditions for analytical assessment of the benzodiazepine were as in 2.3.1.4.

2.3.5 Down-Stream Processing Of Fermenter 50/4 For Isolation Of Novel Metabolites.

The cells were separated from the broth by initial filtration through a mono-filament mesh under vacuum followed by filtration of the partially clarified suspension through a large scale filter pan covered with a sheet of filter paper. This was a slow operation because sufficient spores passed through the mesh to cause partial blockage of the filter paper. The cell paste was lyophilised to constant weight and 333.8 g of dried material was obtained. This value equates to 0.7 g dry weight per 100 ml (equivalent to a shake-flask culture). Dried cells were extracted with acetone (2.5 l), with occasional stirring for 48 hours. The acetone extract was dried by rotary evaporation.

The entire culture filtrate was partitioned against IBMK (12.5 l). On this occasion no emulsion was formed indicating, by the absence of particulate material, that the culture broth had been well filtered. The IBMK extract was dried by rotary evaporation to yield a brown oil.

Polyethylene glycol P-2000 had been inevitably extracted from the culture filtrate with IMBK but was selectively removed by the addition of n-hexane in which it was soluble but which caused the precipitation of the benzodiazepine and diketopiperazine metabolites. The flocculating sample was centrifuged at 10,000 rpm for 5 min to pellet the precipitated material. The pellet was washed with n-hexane (10 ml), resuspended in a further portion of n-hexane (10 ml) and

re-centrifuged as above. The pellet was dissolved in a 1:1 mixture of chloroform:methanol and the solution was transferred to a round-bottomed flask and concentrated by rotary evaporation. A salmon-pink solid (200 mg) was obtained.

2.3.5.1 Silica Column Chromatography.

Extracts of the mycelium and the culture filtrate were purified by preparative silica column chromatography, which is also known as flash chromatography. A slurry of silica (Kieselgel 60 Merck) was made by suspending 75 g silica in a 5:1 mixture of chloroform: acetone (300 ml) and was carefully poured into a glass column (i.d. 5.0 cm). The height of the settled silica bed was 7.9 cm.

Sample Preparation.

Broth and cell extracts were dissolved in a 1:1 mixture of chloroform:methanol (20 ml). About 6 g of silica (Kieselgel 60 Merck) was added and the suspension was rotary evaporated to adsorb the sample onto the silica until a damp powder consistency was achieved. The silica-adsorbed sample was layered carefully onto the surface of the silica column. The composition of the eluting solvent is shown in Table 4. Fraction volumes of 50 ml were collected.

Table 4 Composition of eluting solvent used in flash chromatography of culture extracts.

Fraction number (Vol = 50 ml)	Eluent composition
1 - 17	Chloroform:Acetone (5:1)
18 - 24	Chloroform:Acetone (1:1)
25 - 45	Methanol

The diketopiperazine was contained within fractions 4-8 and the benzodiazepine was located in fractions 34-36.

2.3.5.2 Preparative-Layer Chromatography.

Benzodiazepine Isolation.

Flash column fractions 34-36 inclusive were combined and dried by rotary evaporation. The sample was dissolved in a 1:1 mixture of chloroform: methanol (*ca.* 1 ml), loaded onto a preparative TLC plate and run in solvent system B (2.3.1.3). The band at R_f 0.5, corresponding to the benzodiazepine, was scraped off the glass plate and eluted with a mixture of chloroform: methanol (1:1).

The PLC step was omitted in the large-scale isolation of the diketopiperazine as gradient elution HPLC was employed as the final stage in the purification process (2.3.5.4.).

2.3.5.3 Preparative HPLC In The Isolation Of The Benzodiazepine.

Isocratic HPLC (as in 2.3.1.4) of the eluted band was employed as a final step in the purification of the benzodiazepine. 22.5 mg of HPLC-pure benzodiazepine were obtained.

2.3.5.4 Gradient Elution HPLC For Large-Scale Purification Of The Diketopiperazine.

Column, Separon SGX Strong reverse phase; Wavelength, 250 nm; Flow rate, 0.5 ml min^{-1}; Chart speed, 5 mm min^{-1}.

Mobile phase,	0 - 6 min	65:35 Methanol:water
	8 - 15 min	40:60 Methanol:water
	17 - 25 min	80:20 Methanol:water
	27 - 30 min	100:0 Methanol:water

Diketopiperazine retention time, 20-22 min. About 33 mg of HPLC-pure diketopiperazine were obtained.

2.3.6 Additional Biosynthetic Studies.

2.3.6.1 Administration Of L-[U-^{14}C]-Leucine and L-[U-^{14}C]-Glutamate To Surface Cultures Of *P. aurantiogriseum.*

Surface cultures (4 x 100 ml) of *Penicillium aurantiogriseum* were grown and a total of 15 µCi per flask of each radiolabel was added to duplicate flasks in the following way: 5 µCi added on day 7, 10 and 12. The cultures were harvested on day 17 and after lyophilisation a mean dry weight value of 1.1g per flask was obtained. The ^{14}C-activity of an aliquot (500 µl) of spent culture medium was

determined by scintillation counting.

Lyophilised mycelium was extracted with acetone (*ca.* 100 ml per g dry weight). The mycelial extract was resolved by preparative TLC in solvent system A (2.3.1.3). The benzodiazepine region was removed and eluted with a mixture of chloroform:propan-2-ol (1:1) and the eluate taken to dryness. The extract was dissolved in a suitable volume of methanol and a portion resolved by HPLC. Fractions were collected and the one containing the benzodiazepine was mixed with scintillant and counted.

2.3.6.2 Incorporation Of [^{14}C-methylene]-Tryptophan Into The Novel Diketopiperazine.

A production-stage culture of *Penicillium aurantiogriseum* (100 ml) was given 10 µCi of ^{14}C-tryptophan on the first day post-inoculation. The flask was harvested on the third day and an acetone extract of the lyophilised cells was prepared. The sample was processed by HPLC and the peak corresponding to the diketopiperazine was collected. The purity of the collected sample was assessed by TLC and the chromatogram was autoradiographed.

The ^{14}C-activity of the HPLC-purified diketopiperazine was quantified by scintillation counting.

2.4 METHODS RELATING TO *CLAVICEPS* FUNGI.

2.4.1 General Analytical Methods.

2.4.1.1 Determination Of Ergot Alkaloid Titre By Colorimetric Assay.

Quantitative determination of total alkaloid by colorimetric assay was performed directly on culture filtrate or on aqueous fractions after partitioning ether extracts with tartaric acid (2% W/V). A suitable dilution of test sample (containing a maximum alkaloid concentration of 50 µg ml^{-1}) was mixed with 2 volumes Van Urk's reagent: *p*-Dimethylaminobenzaldehyde, 0.625g; 65% V/V H_2SO_4, 500 ml; 5% $FeCl_3.6H_2O$, 0.5 ml. After 20 minutes the absorbance of the resultant blue colour was determined at 580 nm in a spectrophotometer (MSE Spectro-Plus). The absorbance of test samples was compared with that obtained for agroclavine in 2% tartaric acid from a standard curve in the range 0-60 µg ml^{-1} (see Appendix III).

2.4.1.2 Extraction Of Basic Alkaloids From Culture Filtrate.

Culture filtrate was adjusted to pH 8-9 with NH_4OH (0.88 specific gravity) and extracted with chloroform (2 x ½ vol.). The combined chloroform extracts were taken to dryness by rotary evaporation.

2.4.1.3 Extraction Of Amphoteric Alkaloids From Culture Filtrate.

The basic alkaloids were first extracted as in 2.4.1.2 and the extracted solution was adjusted to pH 5 with 2 M HCl. Cation-exchange resin (in the H^+ form) was added (*ca.* 10 cm^3 resin per 100 ml) to the solution. The pH of the solution was monitored to ensure that exchange of cations had occurred. The solution was left in contact with the resin for a minimum of two hours after which it was decanted and discarded. The resin was washed with distilled water (pH < 7) and adsorbed compounds were eluted with 2 M NH_4OH solution. The ammoniacal eluate was taken to dryness by rotary evaporation taking care as ammoniacal solutions had a tendency to boil vigorously.

2.4.1.4 Extraction Of Alkaloids From Sclerotia.

(i) Basic Alkaloids.

Sclerotia were ground to a fine powder in a coffee-mill and the sclerotial powder was mixed with $NaHCO_3$ (0.3g per 2.5g of powdered material) and a little water (until the mixture just began to adhere) and extracted exhaustively with diethyl ether (2 x 100 ml) for 2 hours. The ether extracts were combined and partitioned with 2% tartaric acid (2 x 100 ml). The tartaric acid layer was made alkaline (pH 8-9) with ammonia solution and partitioned with $CHCl_3$ (2 x 50 ml). The chloroform extracts were combined and taken to dryness *in vacuo*.

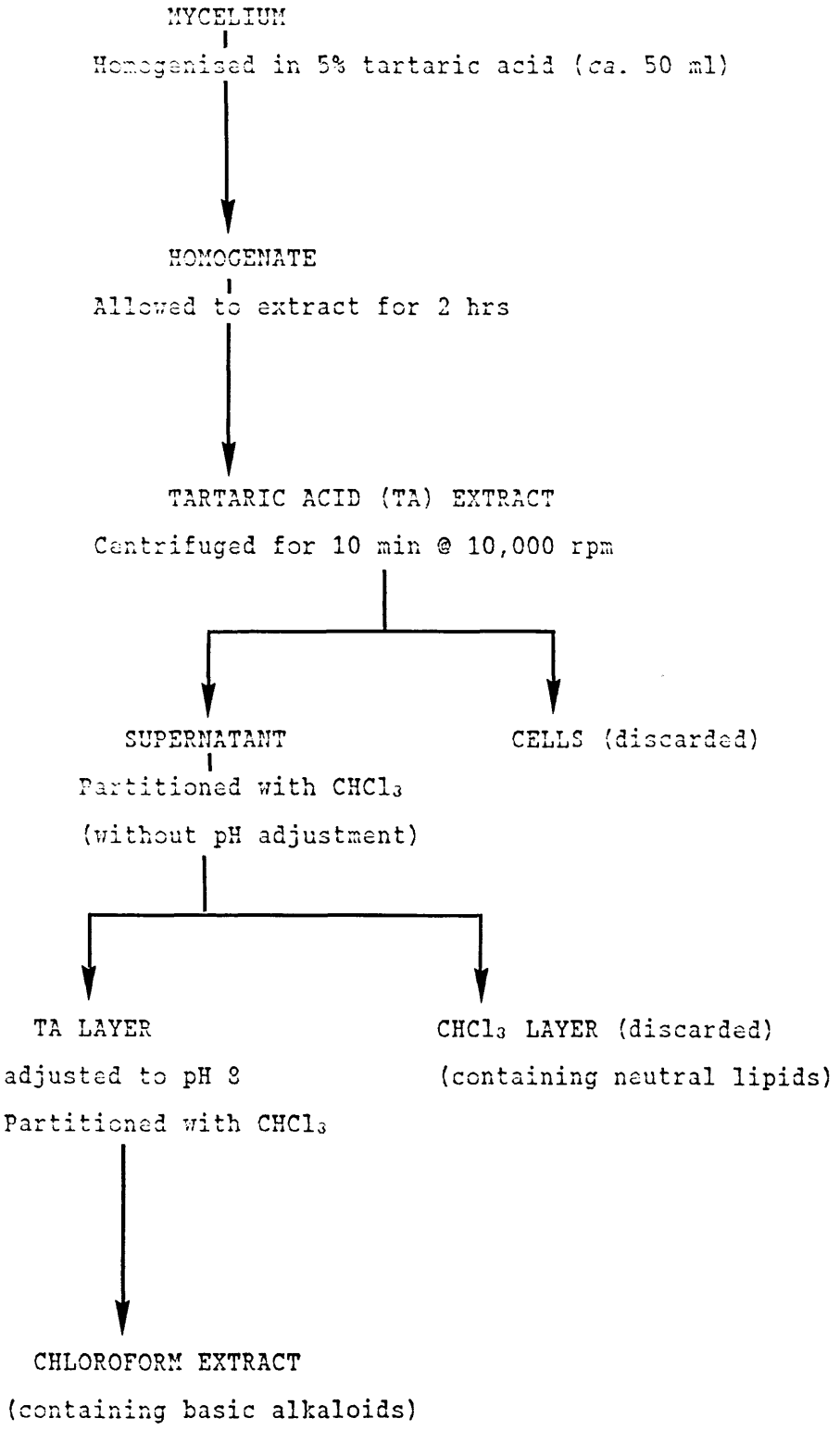
(ii) Amphoteric Alkaloids.

The tissue was rendered free of bases as in 2.4.1.4 (i). Methanol (30 ml) was added and the material was extracted for 18 hours on a Griffin flask shaker.

2.4.1.5 Extraction Of Bases From Surface Cultures Of *Claviceps* spp.

The mycelial mat was removed from the flask and the underside was washed with distilled water. The mycelium was extracted according to the following scheme:

Scheme For Extraction Of Basic Alkaloids From Surface Cultures.



2.4.1.6 Thin- And Preparative- Layer Chromatography Of Ergot Alkaloids.

Solvent System 1: for agroclavine and elymoclavine.

chloroform:methanol:ammonia (95:5:10).

Solvent System 2: for DMAT and tryptophan.

chloroform:methanol:ammonia (8:2:1).

Solvent System 3: for optimal resolution of DMAT and Trp.

chloroform:methanol:ammonia (7:3:1).

TLC Of Extracts Of Sclerotial Slices Incubated With Precursors Of Ergoline Alkaloids.

A solvent system of chloroform:methanol (5:1) was used. R_f values were as follows: agroclavine, 0.64; lysergic acid amide, 0.47; elymoclavine, 0.37.

2.4.1.7 HPLC Conditions For Resolving *Claviceps* Alkaloids.

In the course of clavine precursor incubation experiments a new HPLC assay system for agroclavine and elymoclavine was developed and represented a considerable improvement of the method published by Wurst *et al.* (1978). An HPLC assay system for DMAT was also developed in the present studies.

Agroclavine.

Column, Separon SGX, strong reverse phase; wavelength, 280 nm; mobile phase, methanol: 0.02 M ammonia (70:30); flow rate, 0.7 ml min^{-1}; chart speed, 5 mm min^{-1}; retention time, 10 min.

Elymoclavine.

Column, Separon SGX, strong reverse phase; wavelength, 280 nm; mobile phase, methanol: 0.02 M ammonia (60:40); flow rate, 0.5 ml min^{-1}; chart speed, 5 mm min^{-1}; retention time, 5 min.

N-methyl-DMAT.

Column, Separon SGX Strong Reverse Phase; wavelength, 250 nm; mobile phase (gradient elution),

0 - 4 min methanol: 0.02 M ammonia (3:7)

6 - 30 min methanol: 0.02 M ammonia (3:2);

flow rate, 0.5 ml min^{-1}; methionine retention time, 1-3 min; N-methyl-DMAT retention time, 13-14 min.

(A ^{14}C-methionine-labelled compound co-eluted with DMAT (RT 13-14 min) and was presumed to be N-methyl-DMAT).

2.4.2 Alkaloid Content Of Sclerotia Of *C. purpurea* 12-2.

Sclerotia (7.5 g) were powdered and extracted as in 2.4.1.4. The efficiency of extraction of the alkaloids into chloroform was checked by reacting some of the $CHCl_3$-extracted tartaric acid layer with Van Urk's reagent (2.4.1.1). Basic and amphoteric alkaloids were resolved by TLC in the appropriate solvent mixtures as described in 2.4.1.6.

2.4.3 Isolation Of DMAT From Ethionine-Blocked Cultures Of *Claviceps fusiformis.*

Secondary cultures of *C. fusiformis* CAS (10 x 100 ml) were prepared. On day 4 post-inoculation, sterile ethionine (10 ml containing 50 mg) was added to each flask. The cells were removed on day 7 by centrifugation (10,000 rpm for 15 min) and the basic alkaloids were extracted from the supernatant as detailed in 2.4.1.2. DMAT was extracted as an amphoteric alkaloid as in 2.4.1.3.

2.4.3.1 Ehrlich's Reagent For Localising Alkaloids.

p-Dimethylaminobenzaldehyde, 1 g in 100ml of a mixture of ethanol:concentrated HCl (13:7).

2.4.3.2 Preparative-Layer Chromatography Of DMAT.

The amphoteric fraction was dissolved in the minimum amount of methanol and applied to a preparative TLC plate and was resolved in solvent mixture 2 (2.4.1.6). The DMAT region was located by shield-spraying the edge of the plate with Ehrlich's reagent which became blue-green. The contiguous non-sprayed region was scraped off and eluted with methanol. DMAT was purified from a suitably concentrated methanolic solution of the eluate by HPLC (2.4.1.7). Identification of DMAT was confirmed by fast-atom bombardment (FAB) mass spectrometry which revealed an ion at m/z 273, indicative of $(M+H)^+$, verifying the molecular weight of the isolated compound to be 272.

2.4.4 Production Of Chanoclavine By *C. purpurea* KL1 In Axenic Culture.

A surface culture of *C. purpurea* KL1 was grown on SCSL medium for 20 days. The mycelial mat was extracted according to the protocol in 2.4.1.5. The chloroform extract was resolved by preparative TLC in solvent mixture 1 (2.4.1.6). Chanoclavine was located by spraying

only the edge of the TLC plate, otherwise shielded with aluminium foil, which became blue with Ehrlich's reagent. Chanoclavine was positively identified by FAB mass spectrometry.

2.4.5 [^{14}C-methylene]-Tryptophan, Agroclavine And Elymoclavine Administration To Parasitic Tissue Preparations Of *Claviceps* Fungi.

40 and 54 day old sclerotia of *C. purpurea* 12-2 and 64 day old sclerotia of *C. purpurea* KL1 were used. The proximal and distal 4 mm of each sclerotium were discarded and the remainder was sliced very finely, keeping the sections moist with 2 mM glucose solution (20 ml). Precursors (as detailed below) were added to the sclerotial slices and the flasks incubated on an orbital shaker at 24°C for 21 hours.

2.4.5.1 Quantities Of Precursors Of The Ergoline Biosynthetic Pathway Added To Parasitic Tissue Preparations Of *Claviceps* Fungi.

(a) [^{14}C-methylene]-Tryptophan.

Amounts of radiolabel added ranged from 1-5 µCi.

(b) Agroclavine And Elymoclavine.

6.5 mg of agroclavine and 6.0 mg of elymoclavine were added to the sclerotial slices.

2.4.5.2 Extraction Of Sclerotial Slices.

The incubates were centrifuged (15,000 rpm for 15 min) and the resultant pellets were washed with 2 mM glucose by repeated resuspension and centrifugation. The washed pellet was ground in a pestle and mortar with $NaHCO_3$ (0.6 g). Diethyl ether (100 ml) was added and the tissue was extracted for 1 hour on a Griffin flask shaker. Further aliquots of diethyl ether (3 x 25 ml) were added and the flask contents were extracted for ½ hr after each addition. The combined ether extract (175 ml) was partitioned with 2% tartaric acid (25 ml). The tartaric acid extract was adjusted to pH 8 with 20 M ammonia solution and the alkaline solution was partitioned with chloroform (15 ml). The chloroform extract was rotary evaporated to dryness and resolved by TLC (2.4.1.6) and HPLC (2.4.1.7).

2.4.5.3 Treatment Of Supernatants.

A portion of the supernatant was assessed for residual ^{14}C-activity and alkaloid content as appropriate.

2.4.6 Synthesis Of 3,3-Dimethylallylpyrophosphate.

The preparation of 3,3-dimethylallylpyrophosphate was accomplished by a three stage process (Fig. 15). The first step was esterification of 3,3-dimethylacrylic acid to make methyl-3,3-dimethylacrylate. Reduction of the ester formed in step 1 gave 3,3-dimethylallyl alcohol. The final stage was performed using a specifically prepared pyrophosphorylating agent, bis-triethylamine phosphate (2.4.6.1), to make 3,3-dimethylallylpyrophosphate from the 3,3-dimethylallyl alcohol.

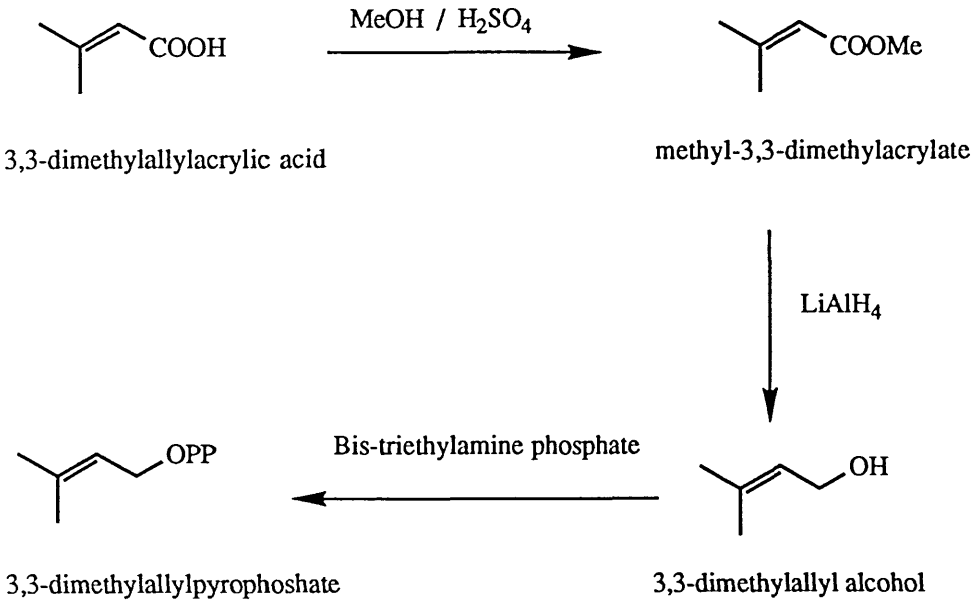
2.4.6.1 Preparation Of Bis-Triethylamine Phosphate.

To 100 ml of acetonitrile (HPLC grade), contained in a 500 ml round-bottomed flask (RBF), was added 20 g of orthophosphoric acid (98% W/V). Triethylamine (Sequenal) was introduced in small amounts, with swirling of the flask, until 41.3 g had been added. This was a mildly exothermic reaction. Once all the orthophosphoric acid had been added the flask was stoppered and immediately small, needle-shaped crystals were formed. Within 2 hours a network of long, pointed crystals had been formed around the inner surface of the flask. It was important to stopper the flask immediately after addition of the orthophosphoric acid as on occasions it had been extremely difficult to drive off excess moisture even under high vacuum.

2.4.6.2 STEP 1: Esterification Of 3,3-Dimethylacrylic Acid.

To 60 g of 3,3-dimethylacrylic acid (RMM 100), contained in a 500 ml RBF was added 100 ml of methanol (HPLC grade) which had been dried with anhydrous sodium sulphate. The mixing of these two components was an endothermic reaction. Concentrated sulphuric acid (3 ml) was added and a few anti-bumping granules. The reaction mixture was refluxed for 14 hours and became yellow in colour.

Fig. 15 Steps in the synthesis of 3,3-dimethylallylpyrophosphate.



2.4.6.3 Isolation Of Methyl-3,3-Dimethylacrylate.

The yellow solution obtained in 2.4.6.2 was poured into distilled water (500 ml), contained in a separating funnel. The contents of the separating funnel were allowed to settle out after having been shaken vigorously. The lower aqueous phase was run off into another separating funnel and partitioned against diethyl ether (100 ml). The upper ethereal layer became yellow in colour. A further 2 x 100 ml aliquots of diethyl ether were used to extract the aqueous phase. The lower layer was discarded and the ether extracts were combined and partitioned against saturated sodium hydrogen carbonate (about 60 ml) until effervescence ceased. The lower aqueous layer was discarded and the ether extract was washed with distilled water (3 x 70 ml). The ether extract was then transferred to a RBF (500 ml) and dried by rotary evaporation to yield a yellow oil, methyl-3,3-dimethylacrylate (RMM 114). The ester was redistilled to yield 44.3 g of a colourless oil (0.60 moles of 3,3-dimethylacrylic acid had been reacted to yield 0.39 moles of the ester). Assuming an efficiency of 100%, the theoretical yield of ester would be 0.60 moles. The esterification was therefore 65% efficient.

2.4.6.4 STEP 2: Reduction Of Methyl-3,3-Dimethylacrylate.

Reduction of the ester was accomplished by reaction with lithium aluminium hydride in a 2:1 (ester:$LiAlH_4$) molar ratio.

Method (for 44.3 g of ester).

Lithium aluminium hydride (7.4 g) was suspended in diethyl ether (150 ml) which had been dried with anhydrous sodium sulphate. The resultant grey suspension was refluxed gently for 1 hour, taking the normal safety precautions *i.e.* use of a safety screen. Dry diethyl ether (100 ml) was added to the methyl-3,3-dimethylacrylate (44.3 g). This mixture was added dropwise to the refluxed lithium aluminium hydride suspension. Vigorous bubbling of the liquid occurred upon addition and a white flocculating precipitate was formed initially but was obscured by a grey, sticky semi-solid mass once all the ester had been added. Diethyl ether (100 ml) was added and the mixture refluxed for 1 hour. The consistency of the mixture after refluxing had not changed. The flask was rotated in an ice-water bath to cool the contents before saturated ammonium chloride (100 ml) was added to remove the lithyl ion. The aqueous solution was well accepted by the

semi-solid grey mass. Diethyl ether (100 ml) was added and the flask was shaken vigorously. The ether layer remained immiscible with the grey mass. The ethereal layer was decanted and two further 100 ml portions of diethyl ether were added. Ether extracts were combined and shaken with 100 ml of saturated sodium hydrogen carbonate. The lower aqueous layer was discarded. The clarifed ether extract was dried over sodium sulphate, decanted into a RBF and subjected to gentle rotary evaporation so as not to distil the alcohol.

A colourless oil (37.0 g), was obtained. Electron impact mass spectrometry confirmed it to be 3,3-dimethylallyl alcohol (RMM 86).

2.4.6.5 STEP 3: Pyrophosphorylation Of 3,3-Dimethylallyl Alcohol.

Based on the method of Cornforth and Popjak (1969).

Bis-triethylamine phosphate (5.95 g), prepared as described in 2.4.6.1, was added to 170 ml of acetonitrile (HPLC grade). It was very slow to dissolve and even after 1 hour of continuous stirring, some crystals remained undissolved.

The partially dissolved bis-triethylamine phosphate solution was added, in small amounts, to a mixture of 3,3-dimethylallyl alcohol (1.9 g) and ice-cold trichloroacetonitrile (5.2 ml). The flask was stoppered and the contents stirred continuously for 5 hours. The bis-triethylamine phosphate had dissolved completely by this time and the solution had changed from a pale to a bright yellow in colour.

2.4.6.6 Extraction Of Phosphorylated Products.

A portion (50 ml) of the bright yellow solution (170 ml) of phosphorylated products was extracted with diethyl ether (250 ml). An emulsified solution resulted. 0.1 M ammonium hydroxide (50 ml) was added and, after thorough shaking in a separating funnel, the two phases were allowed to separate. The upper ethereal layer remained bright yellow and the lower aqueous phase became pale yellow. The aqueous layer was run off and the ether layer was extracted with a further 2 x 50 ml aliquots of 0.1 M ammonium hydroxide. The aqueous extracts were combined, transferred to another separating funnel and washed with diethyl ether (3 x 100 ml) to remove any ether-soluble compounds trapped in the aqueous phase. The aqueous phase was rotary evaporated until about 5 ml of liquid remained. The final solution was an amber-orange colour and had a neutral pH.

2.4.6.7 Characterisation Of Phosphorylated Products.

Paper chromatography as described in Anderson and Porter (1962) was used initially to separate the products of the pyrophosphorylation reaction. The resolution was not good and the bands were very diffuse. A thin-layer chromatography technique was developed which overcame these problems. Initially, phosphorylated products extracted from the reaction mixture were separated using a procedure similar to that of Plieninger and Immel (1965). This method involved preparative chromatography (20 cm x 20 cm glass plate) using Kieselgel H (Merck) as a stationary phase. An equal volume of 0.1 M ammonium hydroxide (5 ml) was added to the sample and a small proportion loaded alongside a solution of bis-triethylamine phosphate in 0.1 M ammonium hydroxide. The mobile phase comprised propan-1-ol: ammonium hydroxide (20 M): water (6:3:1). The plate was run until the solvent front had travelled 10.0 cm. After being thoroughly dried, the plate was sprayed with Hanes-Isherwood reagent (2.4.6.9). Initially the components appeared as white spots on a yellow background. After exposure to UV light (350 nm) for 10 minutes followed by a re-spray and further exposure to UV light the spot at R_f 0.5 was yellow tinged with blue and probably represented the mono-phosphorylated product. The band with an R_f value of 0.28 which became blue on exposure to UV light was probably the pyrophosphorylated product. R_f values cited in the literature of 0.43 and 0.18 for 3,3-dimethylallylmono- and pyro- phosphate, respectively (Plieninger and Immel, 1965), were used to aid identification. Although the cited R_f values were not the same as those obtained in the present study, they provided an idea of the relative positions. Under these conditions, bis-triethylamine phosphate had an R_f value of 0.05 and was initially yellow after spraying with Hanes-Isherwood reagent but became tinged with blue after exposure to UV light. Kieselgel H chromatography was unsatisfactory, however, on account of the puckering of the silica. In subsequent determinations, therefore, acetate-backed TLC plates were used (Camlab Silica G UV_{254}). Acetate-backed chromatograms, run up to 7.5 cm from the origin, gave R_f values of 0.4, 0.13-0.2 and 0.06, respectively, for the mono-, pyro- phoshorylated products and bis-triethylamine phosphate. Bis-triethylamine phosphate only became blue after *ca.* 1 hour exposure to UV light (350 nm) on this stationary phase.

2.4.6.8 Silica Column Chromatography In The Purification Of 3,3-Dimethylallylpyrophosphate.

A slurry of silica (Kieselgel 60, 230-400 mesh; 30 g) was made with a mixture of propan-1-ol:ammonia:water (6:3:1) and was poured into a sintered glass column (internal diameter 4.0 cm). The settled silica bed had a depth of 8.4 cm. The solvent was maintained 0.5 cm above the silica surface.

To the aqueous extract containing phosphorylated products were added 0.1 M ammonium hydroxide (20 ml) and silica (Kieselgel 60, 2g). The suspension was rotary evaporated to adsorb the sample onto the silica until a damp-powder consistency resulted. The silica-bound sample was layered carefully onto the column surface to form a band (4 mm). The column was eluted with several small volumes of propan-1-ol:ammonia:water (6:3:1), before applying a larger head of mobile phase. The flow of eluent was maintained with a hand-pump. Fractions were collected as indicated in Table 5 and each fraction was tested for colour reaction with Hanes-Isherwood reagent (2.4.6.9) and purity by TLC.

TABLE 5 Colour response of fractions of the pyrophosphorylation reaction mixture, resolved by flash chromatography.

Fraction number	Combined volume (ml)	Colour on chromatogram with Hanes-Isherwood reagent after exposure to UV (350 nm) light
1 - 11	50	None
12 - 22	33	Yellow
23 - 34	45	Greenish-blue
35 - 37	30	Blue
38 - 39	20	Deep-blue
40 - 80	410	Blue
81	10	None

Fractions 12-30 were pooled and dried by rotary evaporation. These mainly contained the monophosphorylated product. The sample

crystallised during storage at 4°C for two weeks. Although the pyrophosphorylated product was contained within fractions 23-80 only fractions 35-80 were pooled to obtain a sample which dried to a brown oil containing a rather amorphous semi-crystalline material (0.3 g), representing about a third of the chromatographed phosphorylated products. The pyrophosphate fraction was dissolved in 0.1 M ammonium hydroxide (5 ml) and its purity was assessed by TLC. A single spot (R_f 0.19) was revealed which did not stain immediately with Hanes-Isherwood reagent but which became blue only after exposure to UV light, indicating that it was a pyrophosphate. Attempts to form a more crystalline material by dissolving in acetone:ethanol (6:1) were unsuccessful.

2.4.6.9 Hanes-Isherwood Spray Reagent For Localising Mono- And Pyro-Phosphates.

The following ingredients were made up to 100 ml with distilled water: 60% perchloric acid (5 ml); concentrated HCl (1 ml); ammonium heptamolybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$ (1 g). The solution required gentle warming to dissolve the crystals.

Colour Responses (not well described in the literature).

The chromatograms were sprayed liberally with the reagent and the TLC plate was exposed to UV light (350 nm). It was necessary on occasions to warm the chromatogram in an oven before exposure to UV light. Monophosphates turned yellow before exposure to UV and become faintly tinged with blue after exposure.

3,3-dimethylallylpyrophosphate was not revealed immediately with the reagent but became blue after exposure to UV light for about 15 min. Residual bis-triethylamine phosphate only became blue after about 30 min exposure to UV.

2.4.7 Incorporation Of [^{14}C-methyl]-Methionine Into Agroclavine In Ethionine-Inhibited And Control Cultures Of *C. fusiformis* CAS.

Secondary cultures, as described in 2.1.2.5, of *C. fusiformis* CAS were used (4 x 100 ml). The fermentation was monitored for the presence of indolic alkaloids with Van Urk's reagent (2.4.1.1). On day 4 ethionine was added (50 mg in 10 ml water) to two cultures and sterile distilled water (10 ml) was added to each of the control

flasks. On day 5, [^{14}C-methyl]-methionine (10 µCi per flask) was added to one control and one ethionine-treated culture. The radiolabelled cultures were harvested on day 8 and it was evident that the ethionine-treated cultures had not become so prominently pigmented as the untreated cultures. The cells were separated from the broth by centrifugation and an aliquot (500 µl) of supernatant was examined for ^{14}C-activity by scintillation counting. The culture supernatant was adjusted to pH 8 and extracted with chloroform (5 ml). The ^{14}C-activity of an aliquot (100 µl) of the extract was determined. A small portion (20 µl) of the chloroform extract was back-extracted into 2% tar taric acid (5 ml). This solution was appropriately diluted and the alkaloid content determined with Van Urk's reagent (2.4.1.1). An aliquot (500 µl) of the base-extracted culture supernatant was scintillated and counted to assess the efficiency of extraction.

2.4.8 Time-Course Of The Incorporation Of [^{14}C-methyl]-Methionine Into Agroclavine And Amphoteric Intermediates Of The Clavine Biosynthetic Pathway In *C. fusiformis* CAS.

Secondary cultures (3 x 200 ml) of *C. fusiformis* CAS were grown. One culture was used to monitor the production of alkaloid and aliquots were removed from this flask over the entire fermentation period. Ethionine (100 mg in 20 ml water) was added to one flask on day 4 and sterile distilled water (20 ml) was added to the other flask which acted as a control. On day 5 *ca.* 10 µCi of [^{14}C-methyl]-methionine was added to both the ethionine-treated and the control culture. Aliquots (20 ml) of whole culture were removed from each flask at the following times: 0.2, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0 and 73 hours after addition of the radiolabel. The samples removed prior to and including 1 hr after addition of radiolabel were kept on ice. Each sample was centrifuged to obtain the supernatant which was made alkaline and extracted with chloroform (3 x 5 ml). The chloroform extracts were combined and taken to dryness by rotary evaporation. The extract was redissolved in chloroform (1.0 ml) and 200 µl was assessed by scintillation counting.

Selected base-extracted supernatants were assessed for ^{14}C-activity by scintillation counting of a small portion. The remaining solutions were acidified to pH 5 for extraction of amphoteric

compounds by batch cation-exchange chromatography (2.4.1.3). The alkaline eluates were taken to dryness by rotary evaporation and redissolved in methanol (500 µl). The compounds isolated by cation-exchange chromatography were resolved by HPLC (2.4.1.7). Various fractions were collected and their ^{14}C-activity assessed by scintillation counting.

2.4.9 DMAT-Synthetase Activity In *Claviceps fusiformis.*

C. fusiformis CAS was grown in submerged culture (100 ml) for an appropriate length of time. (Results from present studies indicated an optimal period of 2-3 days).

Cultures of *C. fusiformis* were harvested by centrifugation and the cells were generally kept frozen at -20°C until required but in initial experiments the cells were lyophilised.

2.4.9.1 Buffer Solutions For Cell-Free Preparations.

(a) Extraction Buffer.

10 mM Tris-HCl, pH 8.0; 20 mM sodium diethyldithiocarbamate, $(C_2H_5)_2NCS_2Na.3H_2O$; 20 mM 2-mercaptoethanol; 20 mM sodium thioglycollate, $HS.CH_2COONa$; 20 mM calcium chloride dihydrate; 10% V/V glycerol.

(b) Storage Buffer.

10 mM Tris-HCl, pH 8.0 containing: 20 mM $CaCl_2.2H_2O$; 20 mM 2-mercaptoethanol and 10% V/V glycerol.

2.4.9.2 Methods Of Cell Disruption.

(a) Perspex Homogeniser.

This homogeniser consisted of a perspex outer cylinder with a central channel into which a suspension of lyophilised cells was poured. The outer cylinder was filled with an ice/water mixture. A perspex-tipped plunger at the end of a metal shaft formed a small gap between itself and the walls of the inner chamber. The shaft was clamped in a rotary motor and the cell suspension was forced through the small gap by gently moving the sample-containing vessel in relation to the plunger for 1 minute.

(b) French-press.

The cell paste was transferred to the central channel of the press, which had been cooled to -70°C. Although the sample appeared to freeze immediately on contact with the cold surface the sample-containing press was returned to -70°C for 1 hour to obtain a solid plug of material. The frozen cells were forced through a small aperture at the bottom of the press under a pressure of 20 tons.

(c) Grinding With Sand.

Cells were thawed and ground with acid-washed sand in a pestle and mortar containing solid CO_2.

2.4.9.3 Preparation Of A Protein Fraction, Exhibiting DMAT-Synthetase Activity, From *C. fusiformis*.

All procedures were performed at 4°C unless otherwise stated. Finely ground lyophilised cells (40 g) of 5 day old cultures of *C. fusiformis* were extracted with *ca.* 200 ml extraction buffer and homogenised as in 2.4.9.2(a). The homogenate was centrifuged for 40 minutes at 23,000g. The supernatant (200 ml) was filtered through a glass-wool plug and 35.2 g of solid ammonium sulphate (Sigma, enzyme-grade) was added over half an hour to give 30% saturation (see standard tables in Appendix IV). The solution was maintained at pH 8 by addition of 2 M NH_4OH. Once all the ammonium sulphate had been added the mixture was stirred for half an hour to ensure complete "salting-out" of proteins at 30% ammonium sulphate saturation. Precipitated proteins were pelleted by centrifugation at 27,000 g for 30 min. The resultant supernatant (200 ml) was decanted and the percentage saturation of the ammonium salt was increased to 45% by addition of 18.4 g of ammonium sulphate (from standard tables in Appendix IV). The solution was maintained at pH 8 and the mixture was allowed to equilibrate for half an hour as before. The resultant pellet, after centrifugation at 27,000g for 30 min, was resuspended in storage buffer (60 ml). The solubilised proteins were frozen using a mixture of propan-2-ol and solid CO_2 and stored at -20°C until required.

2.4.9.4 Cell-Free Incubations.

Frozen cells (from 100 ml of culture) were thawed and a small amount of extraction buffer was added to form a paste. The cells were disrupted as in 2.4.9.2 (b). DMAT-synthetase activity was demonstrated in cell-free preparations at various stages of purification:

(a) Crude Homogenate.

The volume of crude homogenate was made up to 20 ml with extraction buffer.

(b) Supernatant Fraction.

Crude homogenates were centrifuged at 12,000 rpm for 15 min. The supernatant fraction was decanted and used in subsequent incubations.

(c) Ammonium Sulphate Precipitated Protein Fraction.

Supernatants as in (b) above were treated as described in 2.4.9.3, using appropriately smaller volumes of extraction buffer, to precipitate the protein fraction between 30-45% saturation of ammonium sulphate. Precipitated pellets were resuspended in extraction buffer (20 ml).

Incubation procedure.

Substrates of DMAT-synthetase, specially prepared DMAPP (*ca.* 5 mg) as in 2.4.6 and [^{14}C-methylene]-tryptophan (1 µCi) were added to various preparations of *C. fusiformis* as detailed in (a)-(c) above. Samples having a low endogenous tryptophan content were supplemented with unlabelled tryptophan (equimolar in amount to DMAPP) to ensure that the enzyme activity was not inhibited by low substrate concentration. Cell-free enzyme mixtures, with added ^{14}C-tryptophan and DMAPP, were incubated at 30°C on a rotary shaker for 3 or 18 hours.

Termination Of Incubation.

Cation-exchange resin (*ca.* 2 cm^3) in the H^+ form was added to the incubates and a drop in pH was noted as exchange occurred. The resin was left in contact with the solution overnight at 4°C. The solution was decanted and the resin was washed with distilled water (pH <7) and eluted with 3 M NH_4OH. The eluate was taken to dryness by rotary evaporation.

2.4.9.5 TLC Assay For DMAT-Synthetase Activity.

Dried eluted compounds were dissolved in methanol (*ca.* 1 ml) and applied to a TLC plate. The compounds were resolved in a mixture of chloroform: methanol: ammonia (8:2:1). R_f values for ^{14}C-tryptophan and ^{14}C-DMAT were 0.22 and 0.34 respectively.

2.4.9.6 HPLC Assay For DMAT-Synthetase Activity.

Mobile Phase (gradient elution).

0 - 4 min	methanol: 0.02 M ammonia (3:7)
6 - 12 min	methanol: 0.02 M ammonia (3:2)
12 - 22 min	methanol

Flow rate, 0.5 ml min^{-1}; chart speed, 5 mm min^{-1}; column, Separon SGX Strong Reverse Phase; wavelength, 267 nm; tryptophan retention time, 3 min; DMAT retention time, 13-14 min.

3. RESULTS.

3.1 [^{14}C-CARBONYL]-ANTHRANILIC ACID AS A PROBE FOR BENZODIAZEPINE METABOLITES OF *PENICILLIUM AURANTIOGRISEUM.*

Autoradiography of TLC-resolved mycelial extracts of ^{14}C-anthranilic acid-fed *P. aurantiogriseum* cultures revealed several bands, two of which were more prominently radiolabelled than the others (Fig. 16). The compound at R_f 0.25 was shown to be a novel benzodiazepine having the structure illustrated (Fig. 17, S.111). The radioactivity at R_f 0.25 was, however, not due to a single compound since the eluted band was further resolved by reverse phase HPLC into two distinct regions (Fig. 18). It was subsequently found that the two compounds were diastereoisomers co-chromatographing on a silica phase but differing in retention time (RT) by 6 minutes on a reverse phase (C_{18}). There was less of the later-eluting isomer (RT, 13 min) than the isomer eluting after 7 min (Fig. 18). Biosynthesis of the more abundant product from L-amino acids, demonstrated in section 3.2, implied that this compound (RT, 7 min) had S-stereochemistry at both α-centres (Fig. 17, S.111). The compound (S.111) is referred to as the native or S,S-benzodiazepine (BZD) in subsequent discussion. Spectroscopic evidence confirmed epimerisation solely at the leucine α-centre of the BZD. The compound eluting after 13 min, therefore, had R-stereochemistry at the leucine α-centre whilst retaining the S-configuration at the glutarimide α-centre (Fig. 17, S.112). S.112 is referred to as the diastereoisomer or the R,S-benzodiazepine.

In an attempt to purify the other prominently radiolabelled compound with an R_f value of 0.92 in Fig. 16, a non-labelled compound was mistakenly isolated. The isolated compound was more abundant than, and co-migrated with, a ^{14}C-labelled compound. However, the isolation was fortuitous as later it was shown to be a novel diketopiperazine (Fig. 17, S.114).

3.2 BIOSYNTHETIC EVIDENCE FOR THE INCORPORATION OF [^{14}C-CARBONYL]-ANTHRANILIIC ACID, L-[U-^{14}C]-LEUCINE AND L-[U-^{14}C]-GLUTAMATE INTO THE NOVEL BENZODIAZEPINE IN SURFACE CULTURE OF *P. AURANTIOGRISEUM.*

[^{14}C-carbonyl]-Anthranilic Acid.

Scintillation counting of fractions of HPLC-resolved mycelial extract (Fig. 18) revealed a 9-fold difference in the amount of ^{14}C-activity of the two isomeric forms of the benzodiazepine (BZD). The specific activities, however, were not that dissimilar for the two diastereoisomers as there was about nine times as much of the isomer eluting after 7 minutes (S,S-BZD, S.111) as the R,S-BZD (S.112) eluting after 13 minutes (Table 6). The presence of the R,S-BZD in the mycelial extract is probably not an artefact of the work-up procedure because this diastereoisomer was artificially generated only after exposure to extreme temperatures. It is probably more reasonable to expect initial biosynthesis of the S,S-BZD followed by epimerisation at the leucine α-centre than direct incorporation of D-leucine into the benzodiazepine molecule.

L-[U-^{14}C]-Leucine and L-[U-^{14}C]-Glutamate.

At least 85% of the added radiolabelled precursors was taken up by the culture. Incorporation of ^{14}C-leucine into the native benzodiazepine was 7- fold greater than that of ^{14}C-glutamate (Table 7). Autoradiographic evidence showing incorporation of ^{14}C-leucine and ^{14}C-glutamate into the BZD is presented in Fig. 19. Incorporation of ^{14}C-glutamate into the glutarimide moiety of the BZD reflects the intimate flux between glutamate and glutamine in primary metabolism. The greater incorporation of ^{14}C-leucine into the BZD possibly might reflect less metabolic demand for this precursor at the time of addition or it might indicate a temporal separation of the formation of the BZD moiety and its subsequent substitution with glutamine.

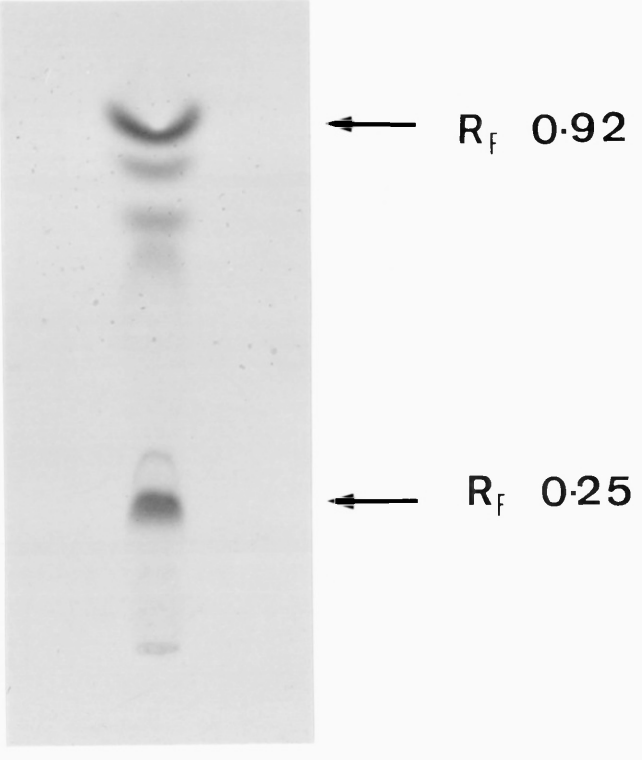


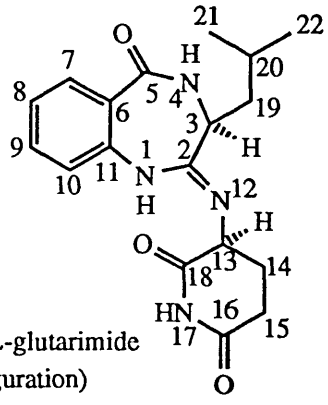
Fig. 16 Autoradiograph of TLC-resolved mycelial extract of a [^{14}C-carbonyl]-anthranilate fed culture of *P. aurantiogriseum*.

Solvent system; Chloroform: Acetone (1:1)

Autoradiograph exposure time; 2 weeks

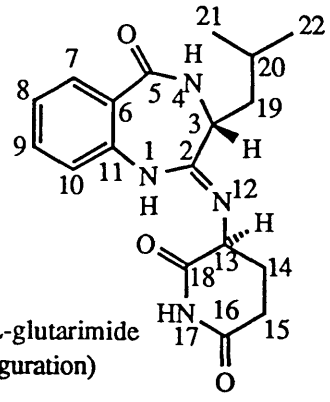
Fig. 17 Structures of novel compounds isolated from *P. aurantiogriseum*.

S.111 Native benzodiazepine



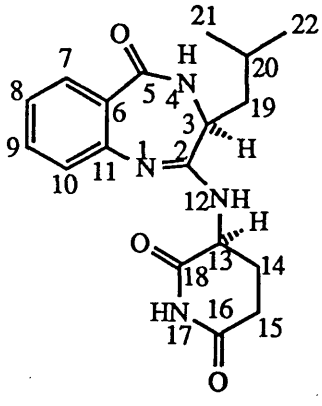
L-leucine, L-glutarimide
(S-S configuration)

S.112 Diastereoisomer

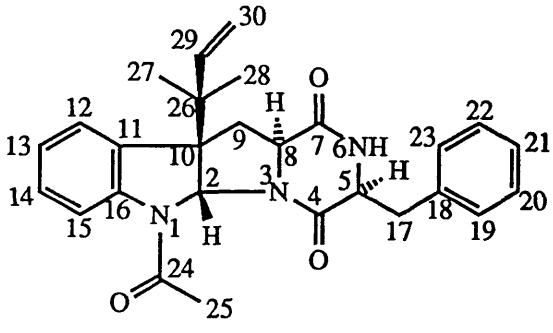


D-leucine, L-glutarimide
(R-S configuration)

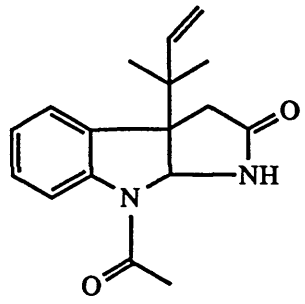
S.113 Benzodiazepine tautomer



S.114 Novel diketopiperazine



S.115 Putative oxidative transformation product of the diketopiperazine



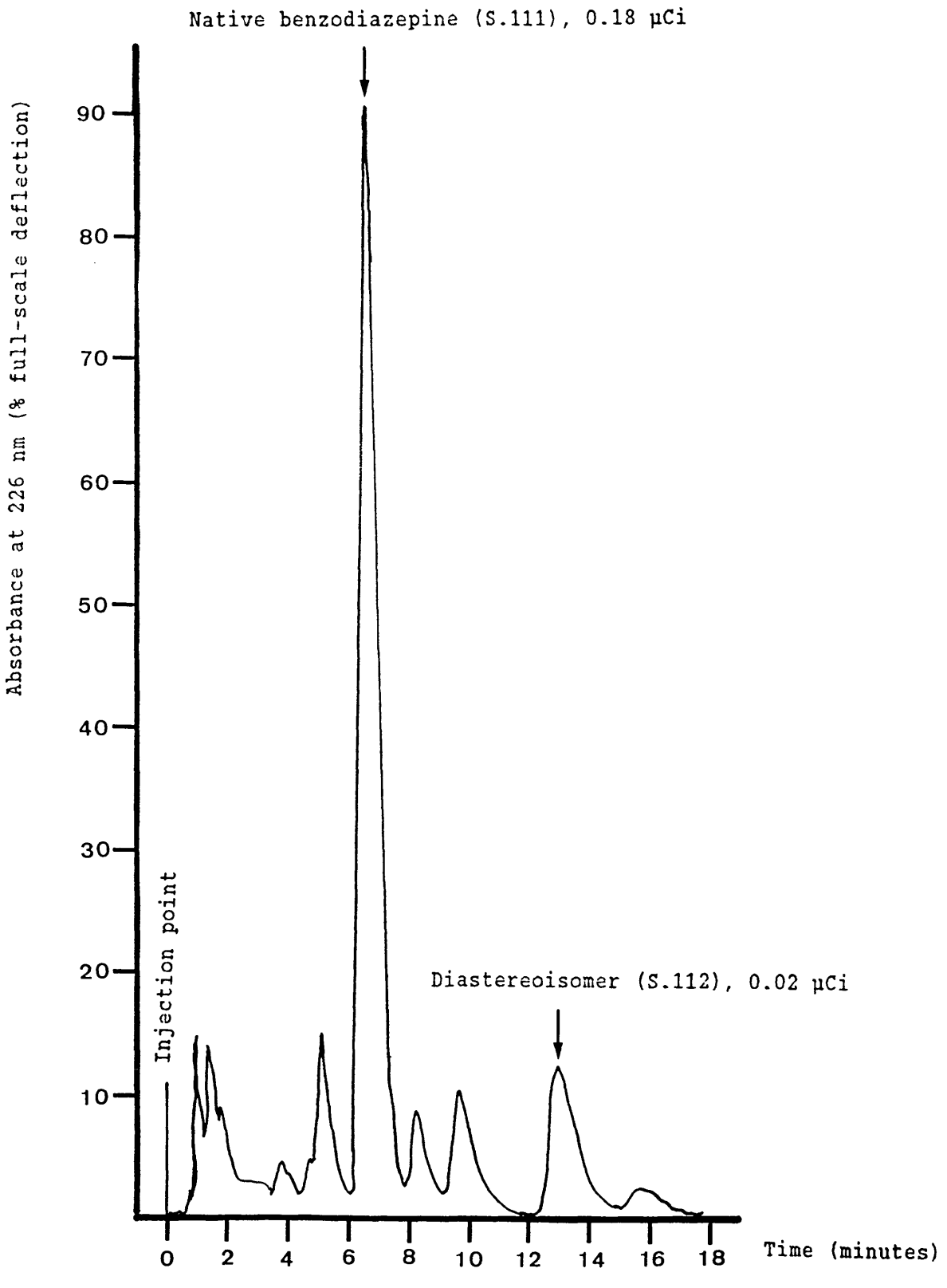


Fig. 18 HPLC profile of an eluted region (R_f 0.25; Fig. 16) of TLC-resolved mycelial extract of a culture of *P. aurantiogriseum* fed with [^{14}C-carbonyl]-anthranilate (50 µCi).

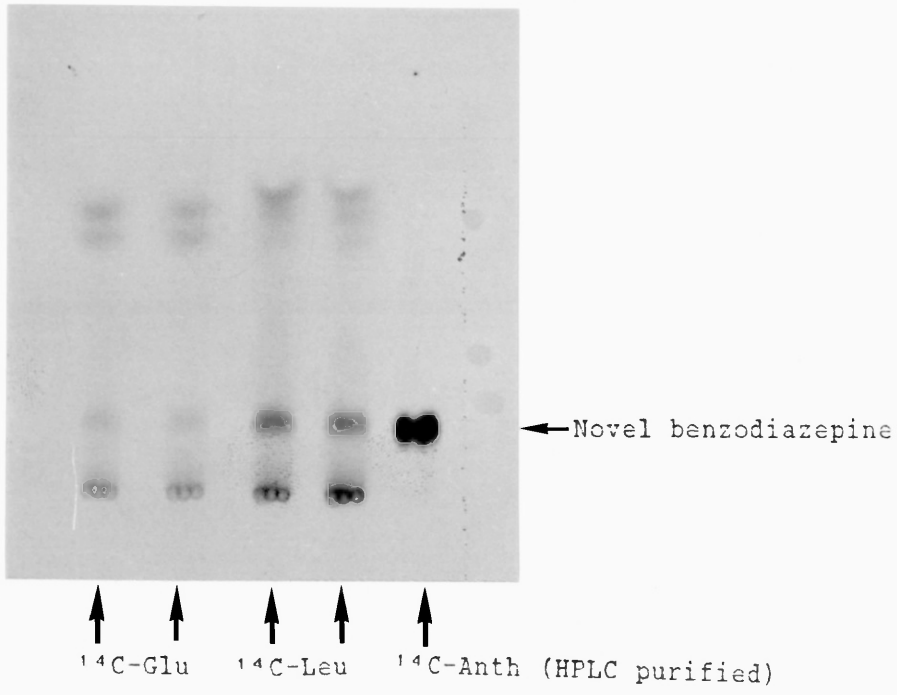
Table 6 Incorporation of [^{14}C-carbony]-anthranilic acid into the two diastereoisomers of the novel benzodiazepine.

Amount of ^{14}C-label added to culture (µCi)	^{14}C-activity in acetone extract of cells harvested on day 13 (µCi)	^{14}C-activity of HPLC-purified benzodiazepine (µCi) [a] (% incorporation of added radiolabel)
50	0.32	Isomer eluting after 7 min. 0.18 [a] (0.36%)
		Isomer eluting after 13 min. 0.02 [a] (0.04%)

Table 7 Incorporation of L-[U-^{14}C]-leucine and L-[U-^{14}C]-glutamate into the native benzodiazepine.

Amount of ^{14}C-label added (µCi)	^{14}C-activity in culture broth on day 17 (µCi)	Calculated ^{14}C-activity in cells on day 17 (µCi)	^{14}C-activity of HPLC-purified benzodiazepine (µCi) [a] (% incorporation of added radiolabel)
^{14}C-leucine.			
15	1.5	13.5	0.01 [a] (0.07%)
15	2.0	13.0	N.D.
^{14}C-glutamate.			
15	1.0	14.0	0.002 [a] (0.01%)
15	1.1	13.9	N.D.

N.D. = not determined.



Abbreviations: ^{14}C-Leu, L-[U-^{14}C]-Leucine

^{14}C-Glu, L-[U-^{14}C]-Glutamate

^{14}C-Anth, [^{14}C-carbonyl]-Anthranilic acid

Fig. 19 Autoradiograph showing incorporation of L-[U-^{14}C]-leucine and L-[U-^{14}C]-glutamate into the native benzodiazepine.

Solvent system, Chloroform: Acetone (1:1)

Autoradiograph exposure time; 2½ months

3.3 LARGE-SCALE FERMENTATION OF *PENICILLIUM AURANTIOGRISEUM.*

After ensuring that the metabolites of interest were produced in submerged culture, a 60 litre fermentation of *P. aurantiogriseum* was performed to provide a sufficient amount of each novel metabolite for structure determination. Some parameters of the fermentation were also monitored.

3.3.1 Biomass.

Maximum growth was achieved through smooth incremental dynamics within 42 hours (Fig. 20). Thereafter measured biomass values were somewhat unreliable on account of foaming, fermenter wall impaction of biomass and evaporation in the stirred vessel. Thus, a stable biomass component probably persisted throughout the latter part of the fermentation.

Most of each dry weight value prior to 24 hours related to vegetatively filamentous organism but thereafter spores contributed an increasing proportion of the dry weight. The acceleration in biomass accumulation during the first 24 hours was attributable to fragmentation of mycelium by impeller shear forces, giving a logarithmic growth component to the accumulated biomass values up to 30 hours (Fig. 20A).

3.3.2 pH

Within the first half-hour of the fermentation the pH value fell by approximately one-and-a-half units (Fig. 20), which may indicate extracellular formation of organic acids such as gluconic acid, or reflect uptake of ammonia released from amino-acids during sterilisation. However, the pH value returned to near neutrality by the end of the first day.

Fig. 20A Logarithmic plot of biomass against time over the first 30 hours of the fermentation.

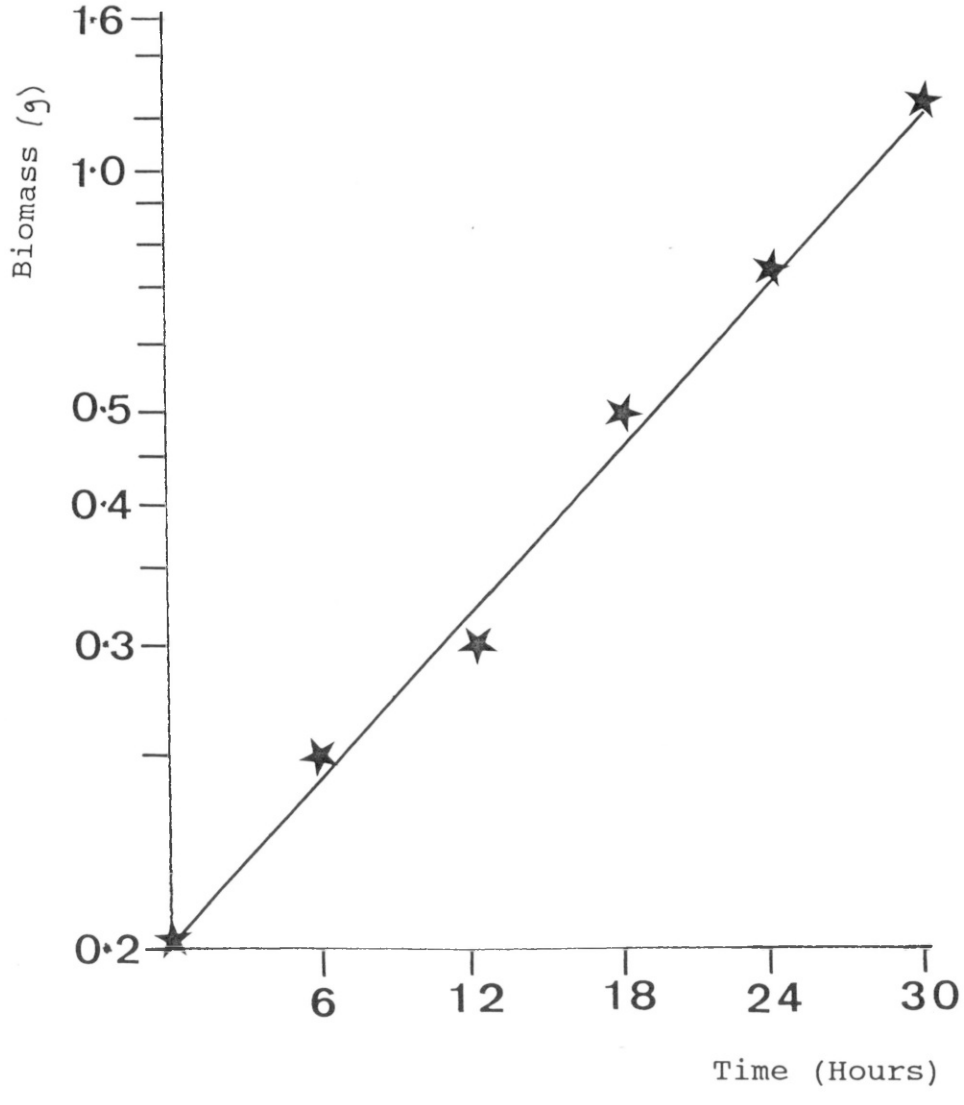


Fig. 20A Logarithmic plot of biomass against time over the first 30 hours of the fermentation.

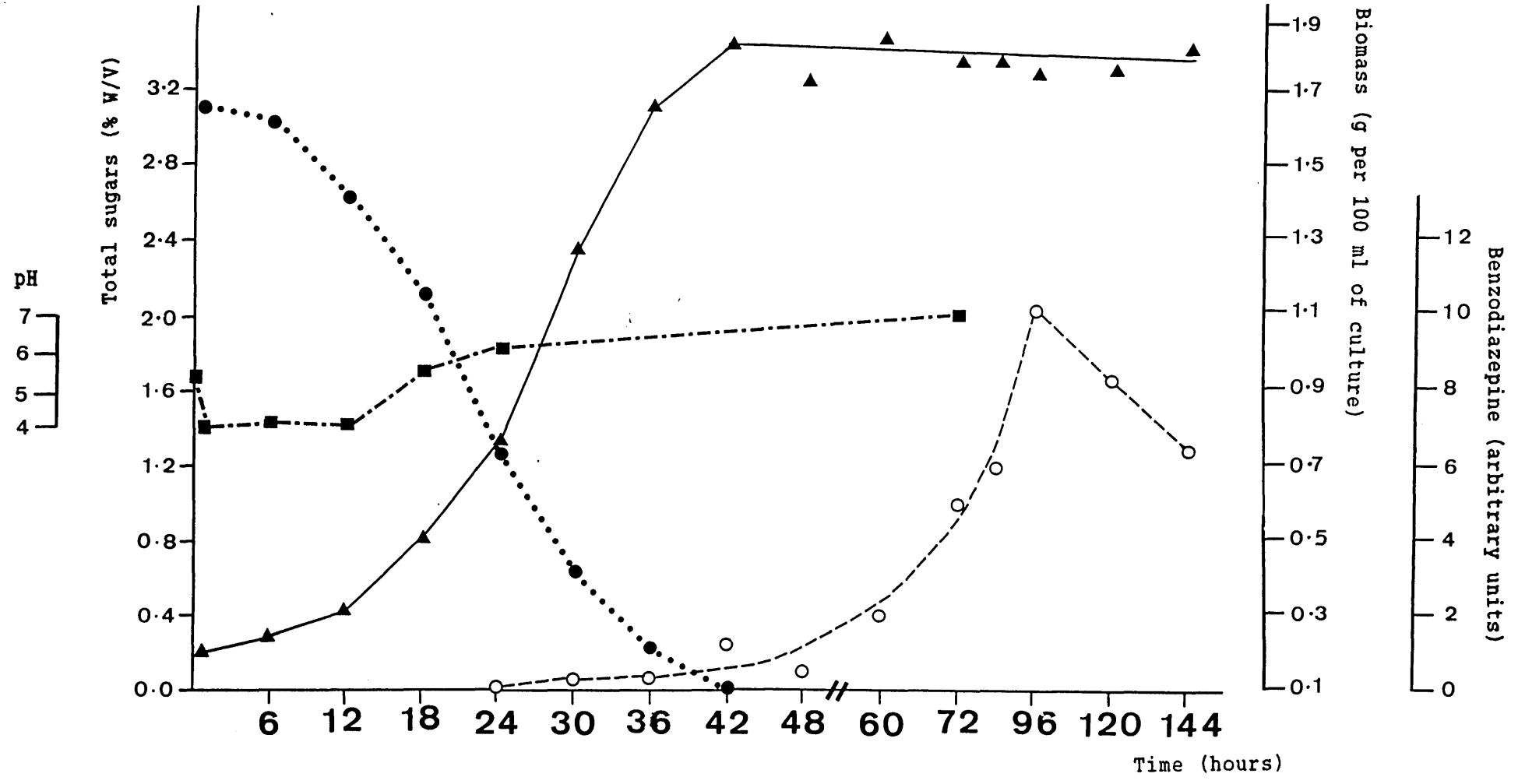


Fig. 20 Progress of a stirred 60 litre fermentation of *P. aurantiogriseum* in calcium chloride-supplemented medium.

▲——▲ , Biomass; ●···● , Total sugars; ■-·-■ , pH;
○– – –○ , Benzodiazepine production.

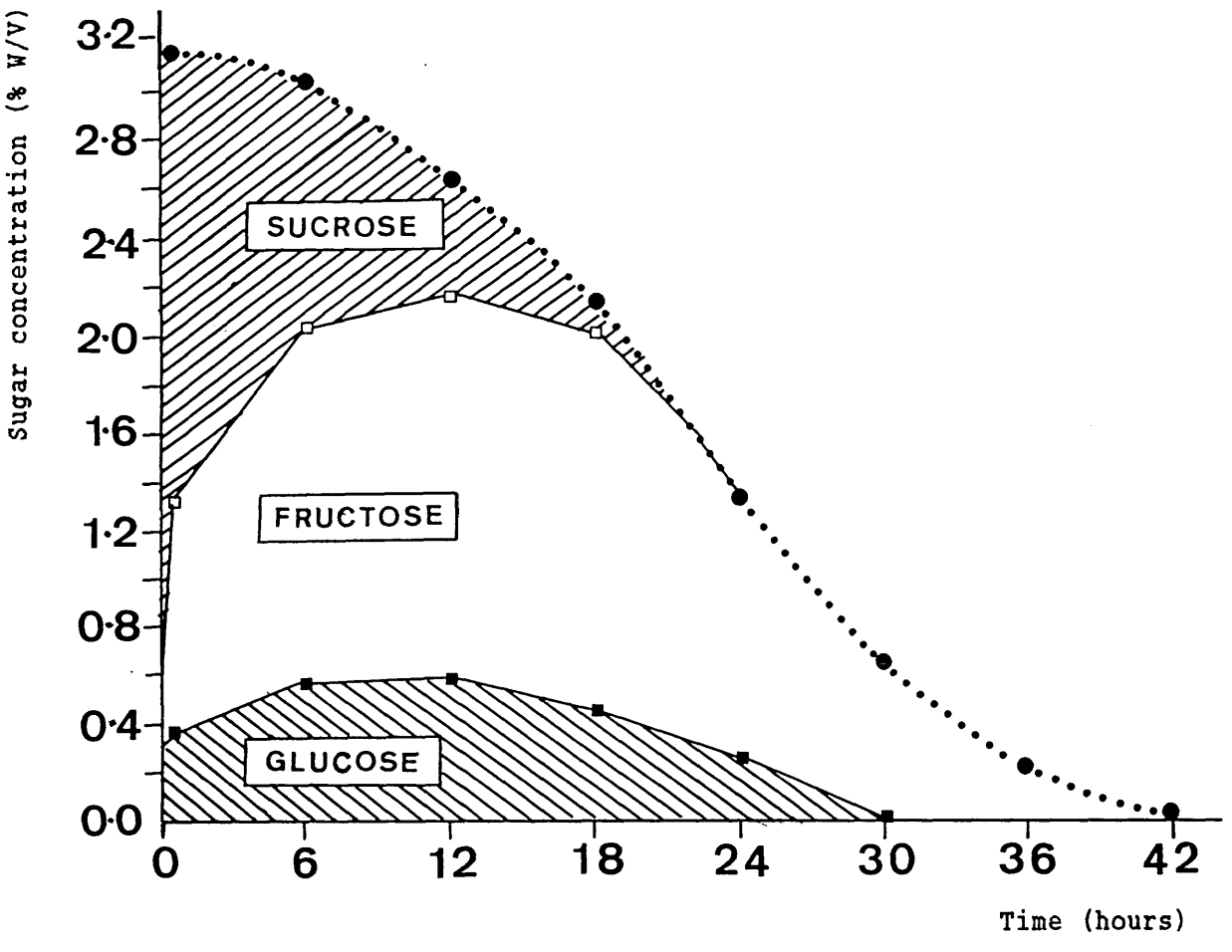


Fig. 21 Distribution of sucrose and constituent monosaccharides during the sugar utilisation phase of a stirred 60 litre fermentation of *P. aurantiogriseum.*
●···●, Total sugars; □—□, reducing sugars before hydrolysis; ■—■, Glucose.

3.3.3 Sugar Analysis.

Approximately 20% of the batched sucrose had been hydrolysed to glucose and fructose during sterilisation (Fig. 21). Each monosaccharide therefore had an initial concentration of about 0.3% W/V. This was lower than that obtained in a large-scale fermentation of *Penicillium paxilli* (Ibba *et al.*, 1987) where 50% sucrose hydrolysis had occurred during sterilisation. The medium used by Ibba *et al.* (1987), however did not contain a calcium chloride supplement.

The dynamics of subsequent enzymic inversion of sucrose and the apparent partially diauxic utilisation of glucose and fructose are set in the context of complete exhaustion of sugars from the broth within 42 hours (Fig. 21). There was clearly considerable invertase activity during the early part of the first day. Throughout most of the first day the amount of fructose was 3-4 times greater than that of glucose implying preferential uptake of glucose.

3.3.4 Culture Morphology and Sporulation.

The primary culture comprised a finely divided hyphal suspension within 24 hours and this growth form persisted in the secondary stage so that the mycelial sample transferred to the fermenter comprised long, infrequently branched hyphae. The mycelial cream-beige colour initially observed in the fermenter did not change for at least the first 18 hours. The green pigmentation first became evident by 24 hours when the mycelium acquired an eau-de-nil colour which became progressively darker until nearly black by 36 hours (Fig. 22). The intensity of this blackness later diminished to a brown colour in the final stages of the fermentation (Fig. 22).

The culture filtrate had changed colour by 48 hours from pale-yellow to amber-brown which intensified gradually throughout the rest of the fermentation.

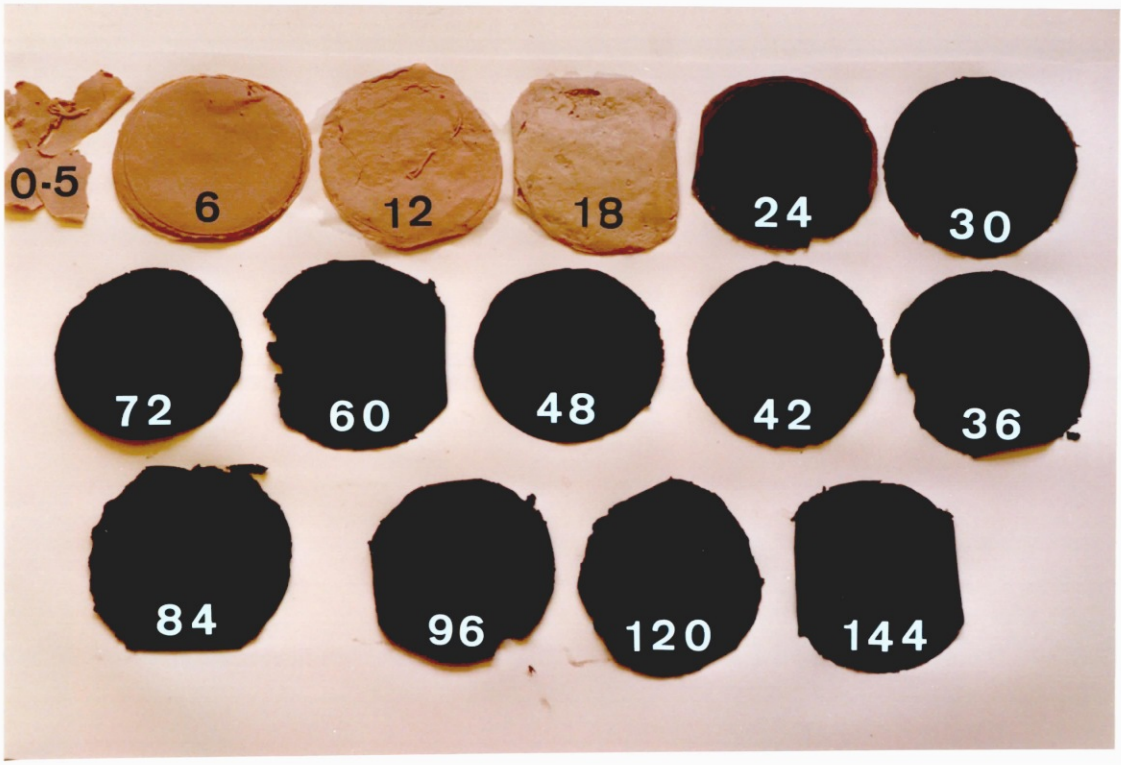


Fig. 22 Mycelial colours of *P. aurantiogriseum* observed during progress of a 60 litre fermentation.

Numbers refer to logged hours from the start of the fermentation.

The calcium-supplemented medium in the stirred fermenters encouraged the filamentous hyphae to proliferate radially during the first few hours to form mycelial aggregates. After 6 hours the hyphae were more frequently branched and hyphal tips had become swollen. Hyphal branching was already suggestive of metulae and phialides by 12 hours, and by 18 hours conidia were borne by fully differentiated penicilli.

Detachment of spores from the sporophores was first apparent by 24 hours but the free spores did not appear to germinate throughout the remainder of the fermentation. By 36 hours the organism had spored profusely, at which time vegetative proliferation had ceased due to the peripherally sporulating nature of the pellets of mycelium.

Pellet formation was initially thought to be advantageous in facilitating filtration to separate biomass from growth stage samples and at the end of the fermentation. However, the large number of spores produced caused blockage of the filters and made filtration more difficult than expected.

3.3.5 Benzodiazepine Production.

Initial evidence of benzodiazepine production (Fig. 20) occurred when free spores were first observed in the broth which coincided with the appearance of a green pigment in the biomass (Fig. 22). Since glucose had been completely exhausted by the time production was escalating, a carbon-catabolite regulation via glucose might be indicated. Accelerated production of the benzodiazepine occurred in a distinct idiophase after the fungus had completed sporulation. After 42 hours, when sugars were no longer detectable in the broth, continued formation of the benzodiazepine must, therefore, have been supported by assimilated carbon sources.

3.4 SELECTION OF A SUITABLE CALCIUM CHLORIDE CONCENTRATION FOR SUBMERGED FERMENTATION OF *PENICILLIUM AURANTIOGRISEUM* PRODUCING METABOLITES OF INTEREST.

The effect of varying calcium chloride concentrations on the culture morphology of *P. aurantiogriseum* was observed. Cultivation of the organism in medium containing a calcium chloride concentration of 0.01% (W/V) resulted in a growth form similar to that of the control culture to which no calcium chloride was added. These cultures were cream in colour and comprised long, thin, unbranched hyphae. These cultures also had an apparent high viscosity. The organism was sensitive to calcium chloride concentrations of 0.1, 0.5 and 2% and responded by differentiating into a sporulating growth form. By the third day the culture with a calcium supplement of 2% had produced 2.4×10^8 spores per ml.

A calcium chloride concentration of 2% W/V was chosen for large-scale fermentation of *P. aurantiogriseum* as this concentration consistently resulted in an extremely fluid culture on account of the highly sporing and fragmented nature of the organism produced by this treatment.

3.5 EFFECT OF CALCIUM CHLORIDE ADDITION ON DIKETOPIPERAZINE PRODUCTION IN A SMALL-SCALE FERMENTATION OF *P. AURANTIOGRISEUM*.

*(Pitt and Poole, 1981)

3.5.1 Biomass.

The accumulation of biomass was initially linear in both the treated and untreated flasks (Fig. 23). However a higher maximum value was achieved in the calcium-supplemented shake-flask due, presumably, to absorption of some of the calcium salts*. The accumulation of biomass occurred to the same extent in the calcium-supplemented shake-flask as in the 60 l fermenter, but maximum accumulation was prolonged by one day in the smaller vessel.

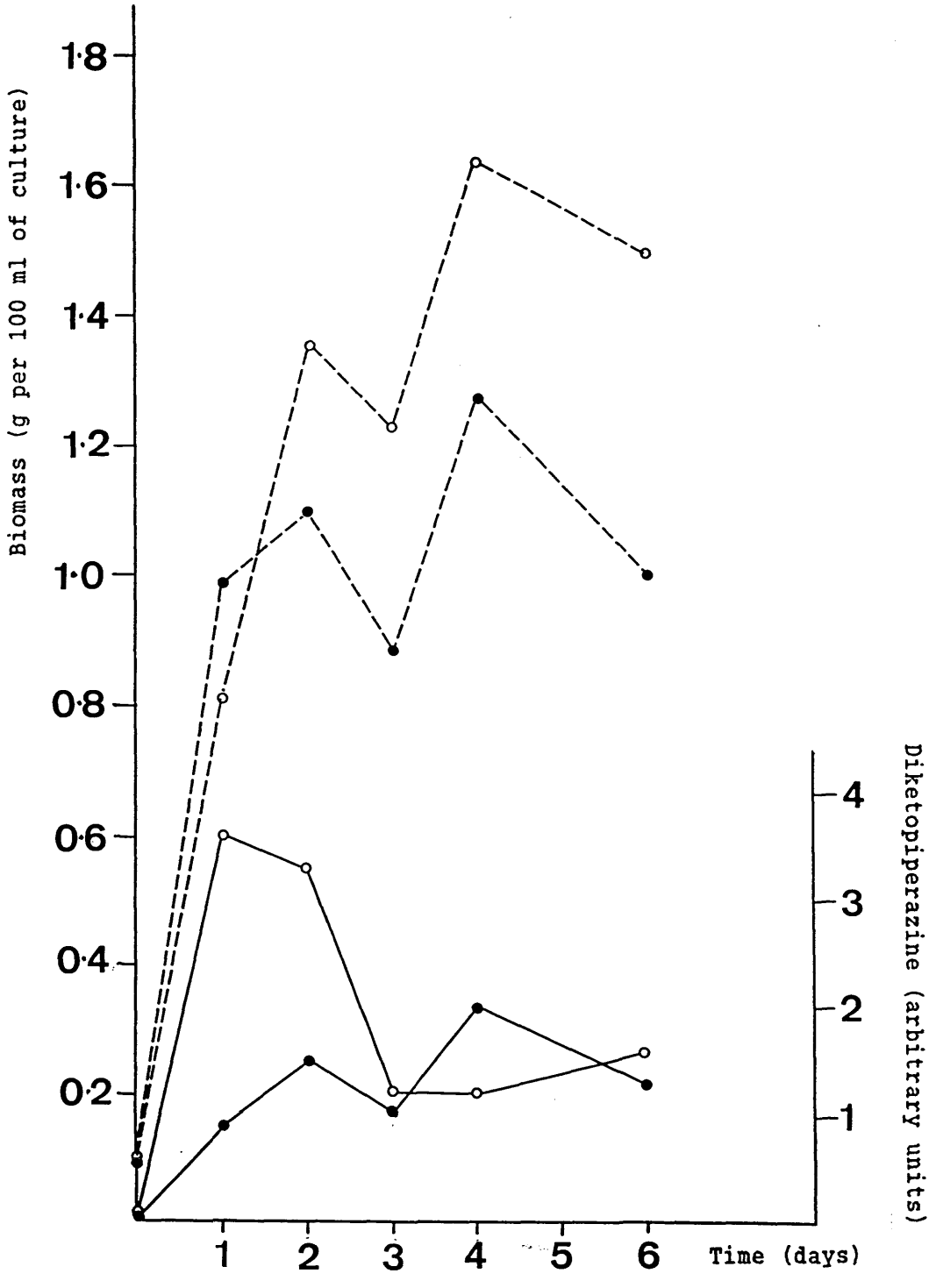


Fig. 23 Biomass accumulation in cultures of *P. aurantiogriseum* and dynamics of diketopiperazine (DKP) production in calcium-supplemented and unsupplemented medium.
●---●, Biomass (CDYE); O----O, Biomass (CDYE+2% $CaCl_2$);
●——●, Total DKP (CDYE); O——O, Total DKP (CDYE+2% $CaCl_2$)

Oxygen-sparging probably provided adequate aeration in the large scale fermentation. However, in the shake-flask, oxygen might have been growth-limiting during the later stages of the fermentation trophophase when there would have been maximum respiratory demand by the accumulating biomass.

Measured values for biomass, after the second day, deviated considerably from the smooth dynamics expected (Fig. 23). Such deviations were most likely artefacts due to the difficulty in obtaining small representative aliquots from a heterogeneous matrix of spores, hyphal fragments and pellets.

3.5.2 Diketopiperazine Production.

The diketopiperazine metabolite was detected in both calcium chloride-supplemented and control media at time 0. These values represented the amount of diketopiperazine carried across from the seed culture. Although Fig. 23 tends to indicate otherwise, production of the diketopiperazine probably proceeded through smooth dynamics in both the calcium-treated and control cultures. The apparent drop in the amount of diketopiperazine on day 3 was consistent with a reduction in the amount of biomass at this time.

At the end of the fermentation very similar quantities of the diketopiperazine were present in both the treated and control flasks. Initial production dynamics, however, were quite different in each flask. There was a significant increase in amount of the diketopiperazine formed in the calcium chloride-treated culture over the first day. Interestingly, the metabolite was most abundant in the culture filtrate by the end of the first day in the treated flask. Production of the diketopiperazine was consistently growth associated but initially occurred more gradually in the control flask. The subsequent apparent gradual disappearance of the diketopiperazine from the treated culture (Fig. 23) might not simply be attributable

to uptake of the metabolite by the cells. It could be that either anabolic or catabolic transformation to one or more compounds had been occurring. If the presumed transformation products differed from the diketopiperazine in chromatographic properties, then the progressive reduction of the HPLC signal in the diketopiperazine assay system, after the first day, could be explained.

The observed decrease in the total amount of the diketopiperazine in the treated culture (Fig. 23) was associated with sporulation of the differentiated organism.

3.5.3 Incorporation of [^{14}C-methylene]-tryptophan into the novel diketopiperazine.

Autoradiography of a TLC-resolved HPLC-fraction containing the diketopiperazine, extracted from mycelium of a culture of *P. aurantiogriseum* fed with ^{14}C-tryptophan, revealed fairly specific incorporation of the radiolabel into the novel diketopiperazine (Fig. 24). Scintillation counting of the HPLC-purified diketopiperazine showed a ^{14}C-activity of 1.32×10^{-2} µCi, representing 0.13% incorporation of the added radiolabel.

3.5.4 Culture Morphology.

Light microscopy of an aliquot of seed-stage culture, removed two hours post inoculation, revealed germ tubes developing from many spores. By 24 hours elongating germ tubes had aggregated to form small pellets visible to the naked eye. The mycelial suspension at this stage was suitable for transfer to production flasks (with and without the addition of calcium chloride). The loosely packed mycelial pellets increased in diameter until the fourth day in calcium chloride-free medium. An increasing pellet size was due to the proliferation of largely unbranched hyphae. This contrasted with the compact nature of pellets in the calcium chloride-treated culture

in which maximum pellet size was attained by the end of the first day. Further increase was precluded as laterally-branching hyphae, with tips fully differentiated into conidiophores, were shedding spores profusely.

The diffuse form of pellets in the vegetatively-growing culture contributed to an apparently greater viscosity in this flask for the first three days. However, by the fourth day a marked change to a highly fluid state was evident probably reflecting breakage of filamentous hyphae. The apparent viscosity of the conidiating culture did not increase excessively as most of the biomass was in the form of small pellets and spores throughout the fermentation.

There was a marked difference in colour of the mycelium when grown with and without calcium addition. The calcium chloride-treated culture showed similar colour changes to those observed in the 60 l fermentation (Fig. 22), progressing from an initial cream-beige through an eau-de-nil to a final dark green colour. However, the untreated culture remained cream-beige in colour throughout the entire culture period. The green colour of the mycelium was shown to be associated with phialide development which became evident during the first day in the treated culture but which did not occur at any stage in the untreated culture.

3.6 STRUCTURE DETERMINATION OF THE NOVEL BENZODIAZEPINE.

3.6.1 Ultra-violet (UV) spectroscopy.

The UV spectrum of the native benzodiazepine revealed an absorption maximum at 226 nm (Fig. 25A) and this wavelength was subsequently used for detection of the benzodiazepine by HPLC.

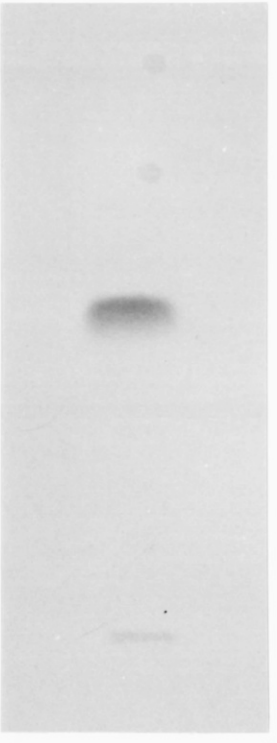
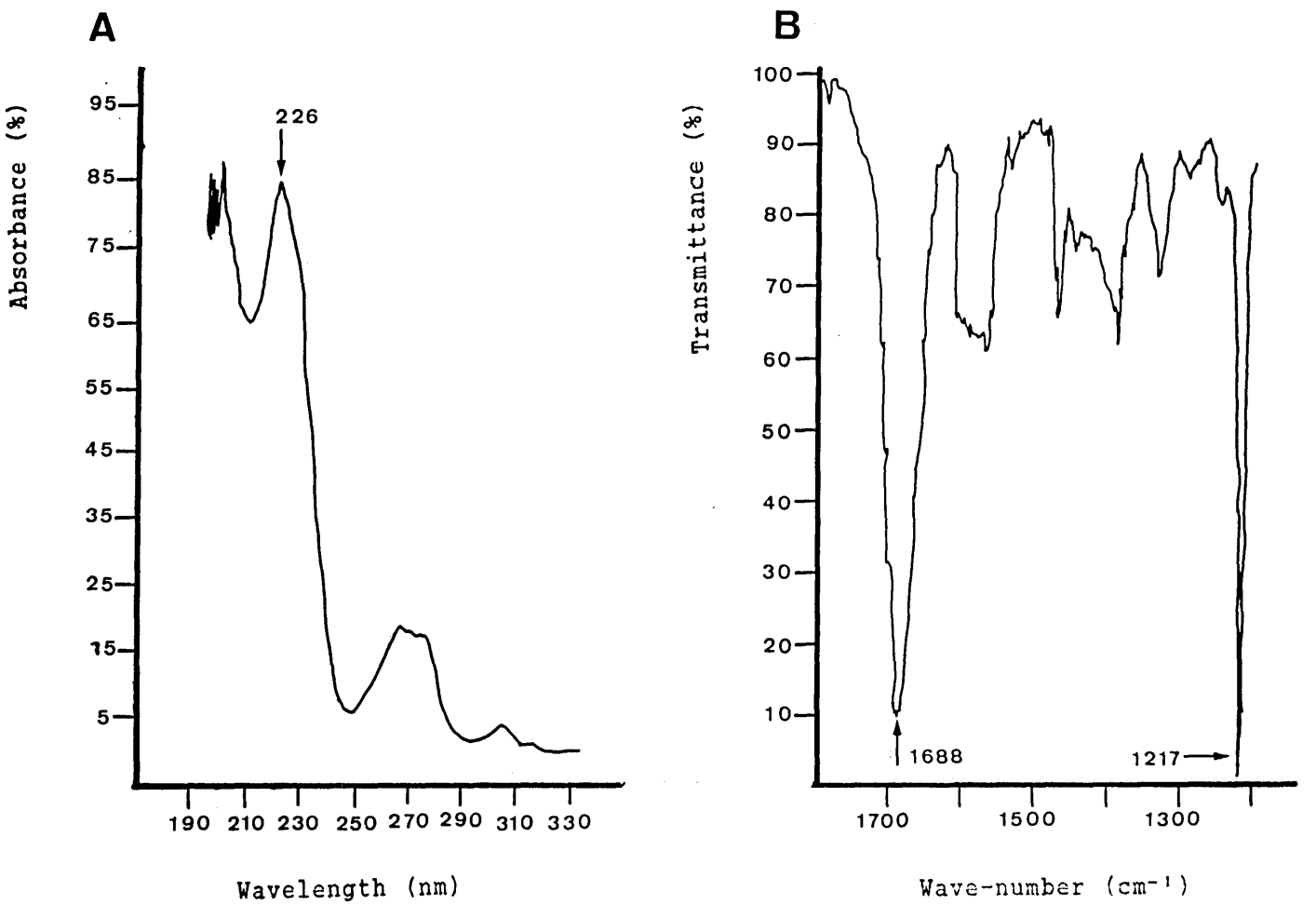


Fig. 24 Autoradiograph of HPLC-purified [^{14}C-methylene]-tryptophan-labelled diketopiperazine.

Solvent system, Chloroform: Acetone (5:1)

Autoradiograph exposure time, 2 weeks

Fig. 25 A) UV spectrum B) IR spectrum of the novel benzodiazepine.



3.6.2 Infra-red spectroscopy.

The infra-red (IR) spectrum of the novel benzodiazepine (BZD) in $CDCl_3$ (Fig. 25B) was obtained against a reference sample of $CDCl_3$. An absorption maximum was observed at 1688 cm^{-1} which probably corresponded to the carbonyl stretching frequency of the glutarimide moiety, possibly hydrogen-bonded to the NH of the amidine. The carbonyl stretching frequency of glutarimide has been reported to be 1705 cm^{-1} and 1710 cm^{-1} by Frank and McPherson (1949) and Horák and Gut (1960), respectively. However, hydrogen-bonding to solvent or intra-molecular H-bonding in cyclic imides may cause a decrease in the carbonyl stretching frequency (Dolphin and Wick, 1977). Thus, the carbonyl IR frequency of the glutarimide actidione (cyclo-heximide) occurred at 1695 cm^{-1} (Kornfeld *et al.*, 1949). The anthranilamide carbonyl stretching frequency of the novel BZD probably occurred in the range 1650-1600 cm^{-1}, which is consistent with values for anthramycin-type compounds (Leimgruber *et al.*, 1965b).

3.6.3 Mass spectrometry.

3.6.3.1 Fast atom bombardment (FAB).

The strong ion observed at m/z 343 was deduced as $(M+H)^+$ since the higher mass ion m/z 365 could be interpreted as $(M+Na)^+$ (Fig. 26). Associated positive ions were evident at m/z 326 and 298 which could correspond respectively to $(M+H-NH_3)^+$ and $(M+H-NH_3-CO)^+$. At higher mass, peaks corresponding to the dimers $(2M+H)^+$ and $(2M+Na)^+$ were present at m/z 685 and 707. A molecular weight of 342 was indicated consistently for both the native and R,S-BZD isomer (Figs. 26, 27).

3.6.3.2 Electron impact (EI).

EI spectra of the two diastereoisomers of the benzodiazepine share several characteristic peaks in common (Figs. 28, 29). Some of the more prominent ions were accurately mass measured. These results are presented in Table 8 which also assigns some fragment losses.

Fig. 26 Positive fast-atom bombardment (FAB) spectrum of the native benzodiazepine.

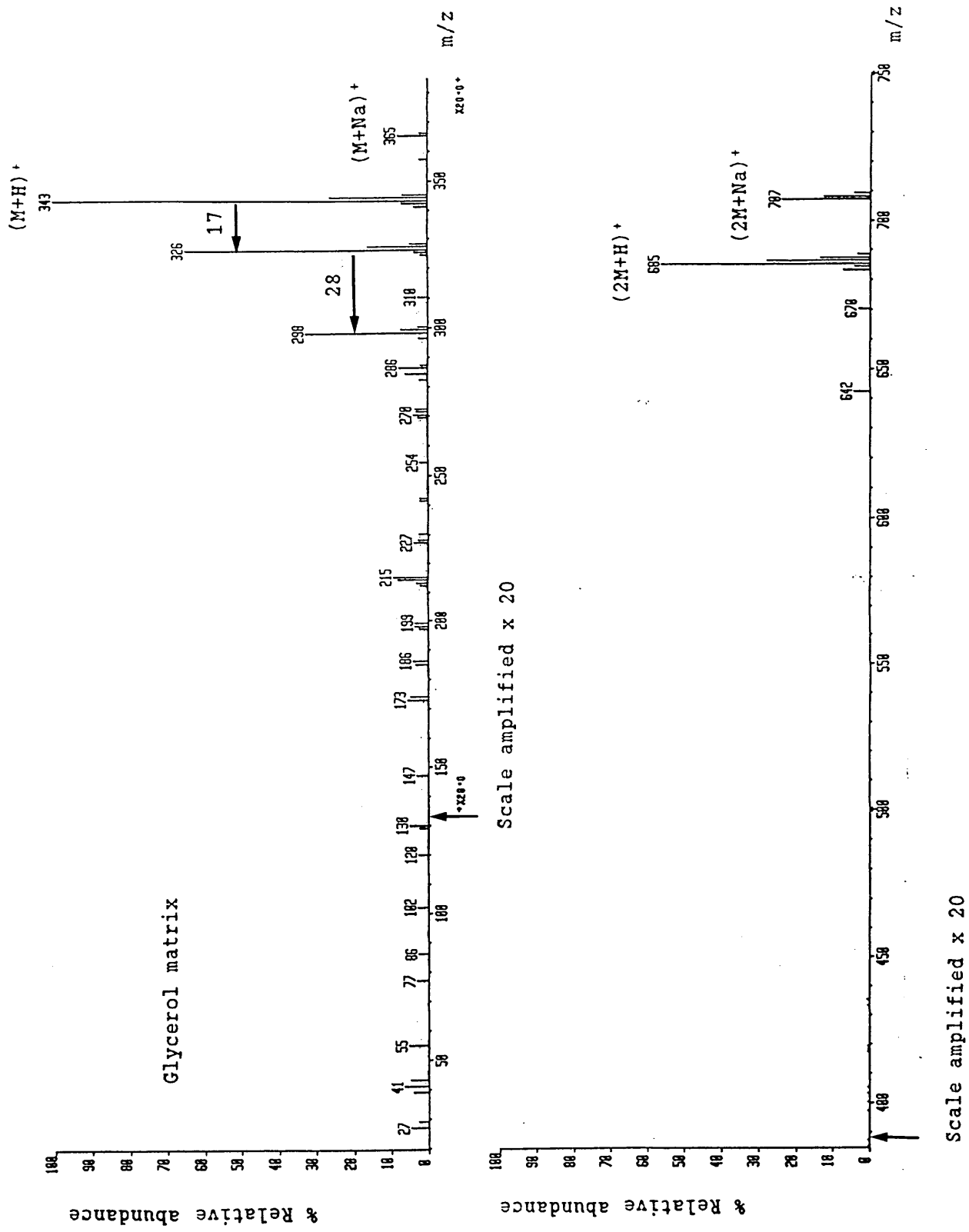


Fig. 27 Positive fast-atom bombardment (FAB) spectrum of the diastereoisomeric benzodiazepine.

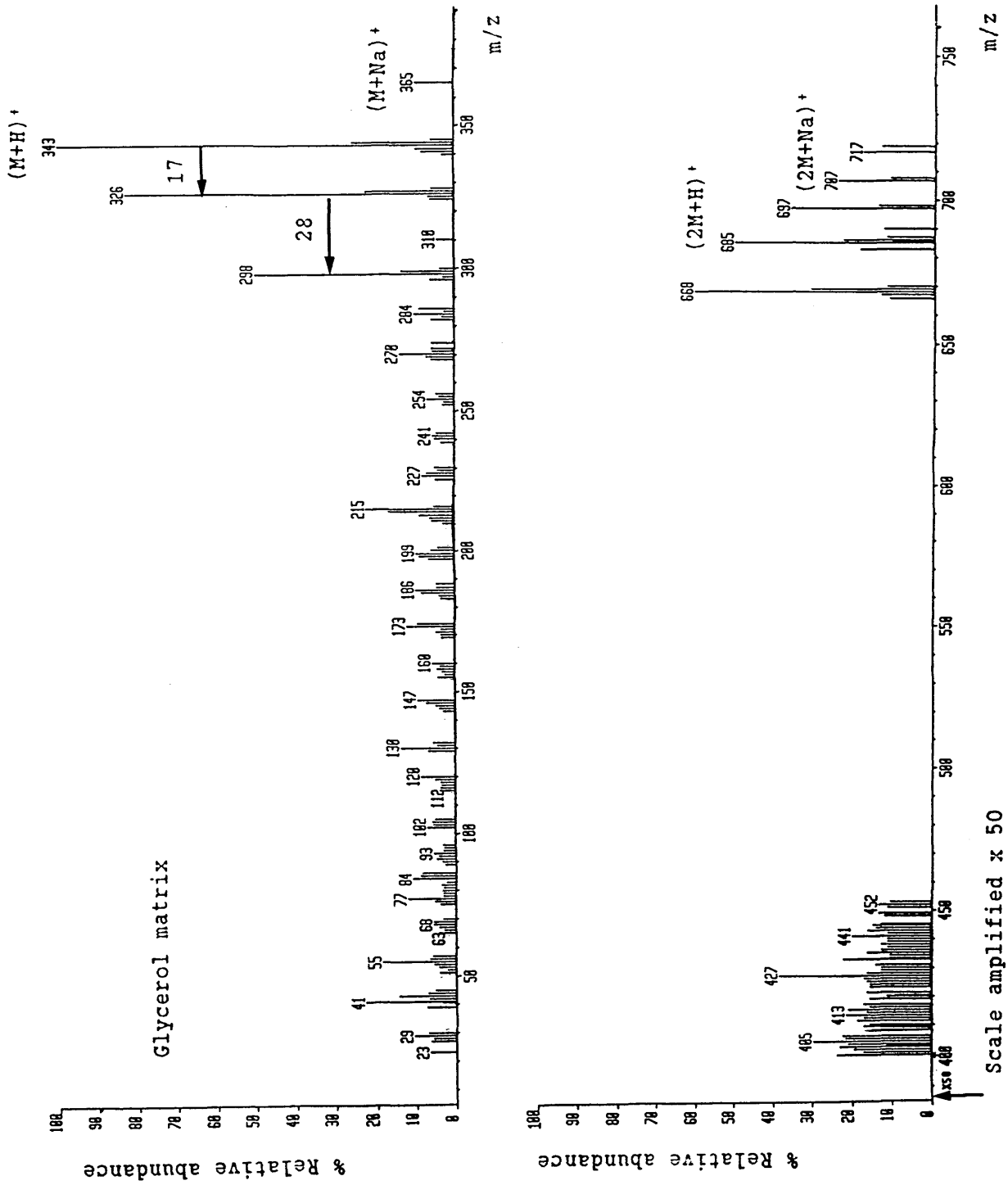


Fig. 28 EI mass spectrum of the native benzodiazepine.
(70 eV; source temperature, 160°C).

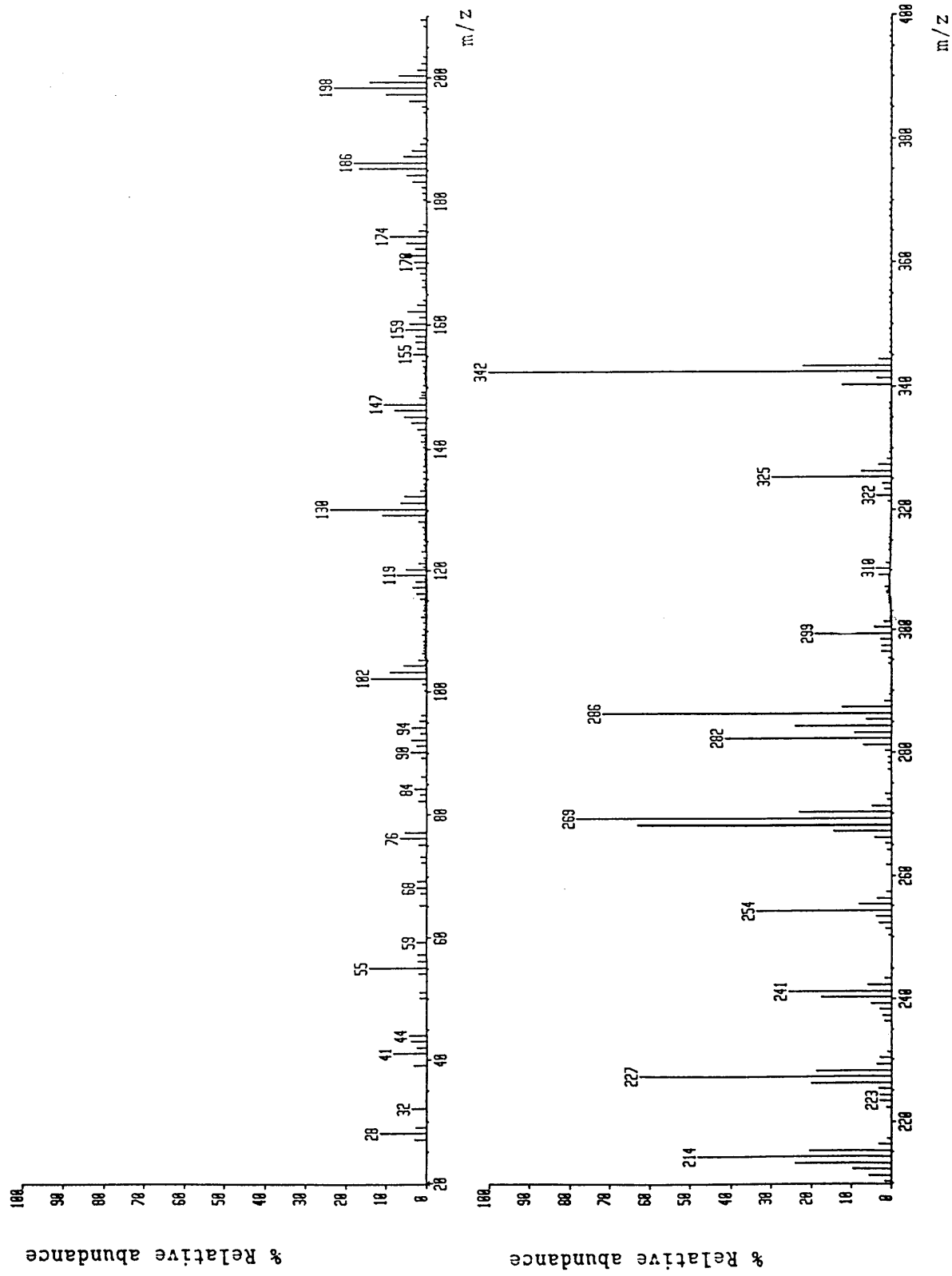


Fig. 29 EI mass spectrum of the diastereoisomeric benzodiazepine. (70 eV; source temperature, 160°C).

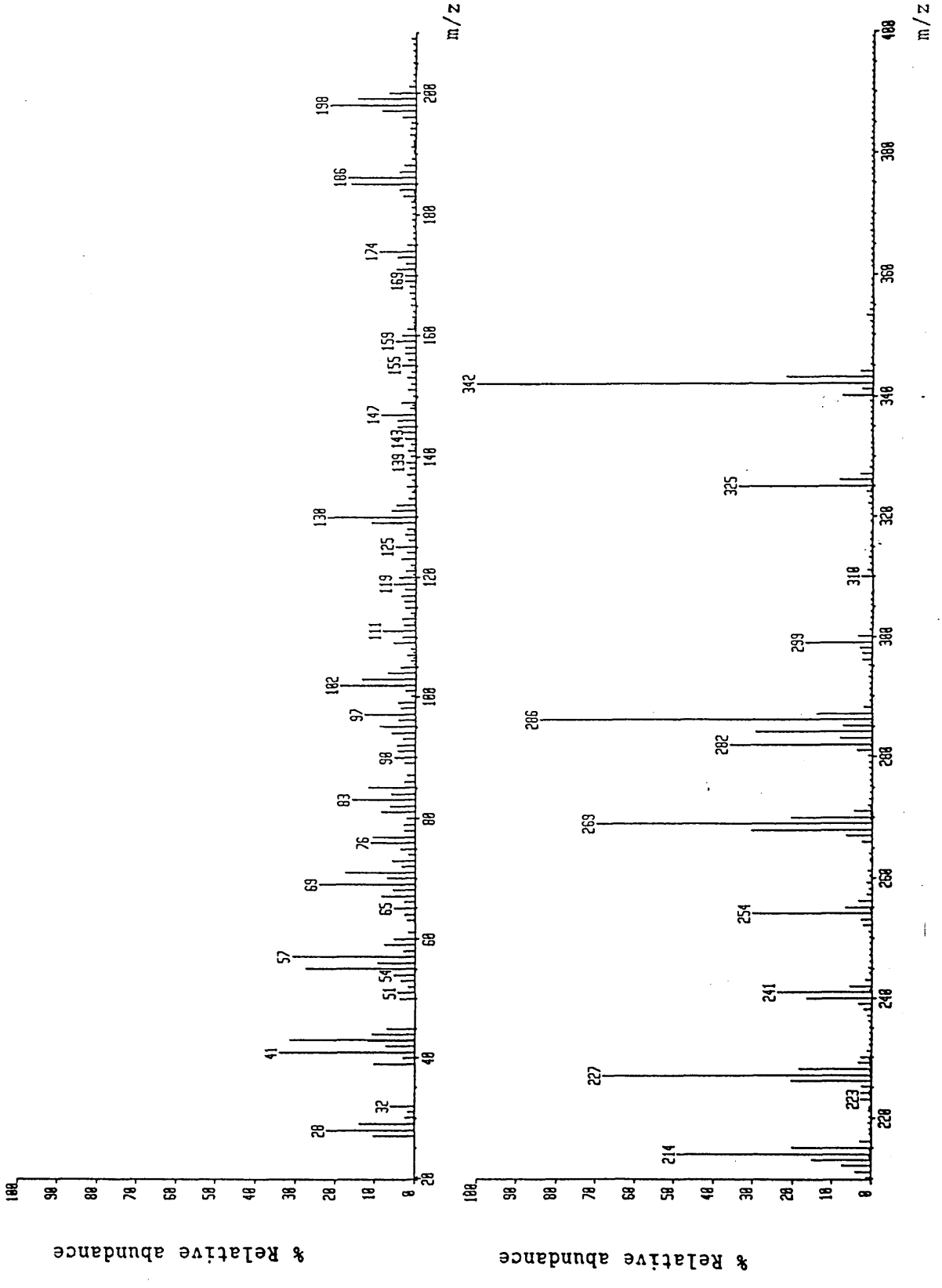


Table 8 Mass measurements of the native benzodiazepine.

Fragment Ion (m/z)	Accurate Mass	Calculated Mass	Molecular Formula
342 M^+	342.1683	342.1692	$C_{18}H_{22}N_4O_3$
325 $(M-NH_3)$	325.1419	325.1426	$C_{18}H_{19}N_3O_3$
299 $(M-C_3H_7)$	299.1145	299.1144	$C_{15}H_{15}N_4O_3$
286 $(M-C_4H_8)$	286.1067	286.1066	$C_{14}H_{14}N_4O_3$
227	227.0691	227.0695	$C_{12}H_9N_3O_2$

3.6.4 ^{1}H-nuclear magnetic resonance (NMR) spectroscopy of the novel benzodiazepine.

The benzodiazepine was initially dissolved in deutero-chloroform but this was an unsatisfactory solvent because, although the resolution was good, there was insufficient dispersion of the proton resonances. The resulting signals were broad with inadequate fine-splitting, particularly at high field (Figs. 30A,B). Deuterated DMSO was subsequently used as a solvent which, although it gave somewhat reduced resolution of individual signals at high field, resulted in a greater dispersion between proton resonances and enabled more J-coupling information to be obtained (Figs. 31A,B).

The benzodiazepine obtained from large-scale fermentation (22.5 mg) was dissolved in D_6-DMSO. This sample had been dried under high vacuum in a pistol apparatus and had been exposed to 180°C because of a fault in the temperature controller. The ^{1}H-NMR spectrum of the inadvertently heated sample (Fig. 32) was more complex than that initially obtained (Fig. 31A) and revealed the formation of a 1:1 mixture of diastereoisomers. The HPLC profile of the pyrolysed benzodiazepine sample corroborated formation of a 1:1 mixture of compounds as an additional peak was observed eluting after 13 minutes (Fig. 33). HPLC of the benzodiazepine sample before pyrolysis had resulted in a single peak eluting after 7 minutes (*c.f.* Fig. 18).

The ^{1}H-spectrum of the 1:1 diastereoisomeric mixture (Fig. 32), when compared with the ^{1}H-spectrum of the native BZD (Fig. 31A), showed two new α-CH resonances at 4.72 and 5.09 ppm. Both signals were shifted downfield with respect to the corresponding resonances in the native isomer. The additional singlet resonance at 8.53 ppm in the 1:1 diastereoisomeric mixture was tentatively assigned as the N-H of leucine of the diastereoisomer (Fig. 17, S.111). The corresponding N-H signal in the native sample (Fig. 17, S.112) appeared as a doublet at 8.87 ppm (Fig. 31A).

The N-H resonances of the anthranilate and glutarimide moieties, appearing at 7.31 and 6.74 ppm in the spectrum of the native benzodiazepine (Fig. 31A), were shifted slightly upfield to 7.25 and 6.72 ppm in the spectrum of the diastereoisomer (Fig. 32). The leucine N-H resonances in both isomers were sharper than the other N-H resonances. Comparing the two isomers, the greatest difference in chemical shift was exhibited by the leucine N-H signals. The N-H resonances of the anthranilate and glutarimide moieties of both isomers could not be assigned definitively as the signals were singlets and J-coupling information therefore could not be obtained.

^1H-^1H COSY NMR spectroscopy of the 1:1 diastereoisomeric mixture was initially performed in $CDCl_3$ (Fig. 34A). The ^1H-^1H COSY spectrum of the 1:1 diastereoisomeric mixture in D_6-DMSO (Fig. 34B) revealed coupling between the N-H at 8.87 ppm of leucine and the α-CH of leucine at 4.39 ppm of the native BZD. A corresponding coupling between the α-proton of leucine at 4.72 ppm and the leucine N-H singlet at 8.5 ppm in the diastereoisomer, however, was not present. The loss of coupling was implicit in the narrowing of the signal at 4.72 ppm in comparison with that at 4.39 ppm (Fig. 32). The apparent loss of coupling in the diastereoisomer may be explained by a change in the geometry at the leucine α-centre of the molecule. In addition, the ^1H-^1H COSY spectrum (Fig. 34B) revealed a greater non-equivalence of the β-CH_2 protons of the leucine in the diastereoisomer than in the native isomer which implied epimerisation at the leucine α-centre. Proton assignments for the native benzodiazepine and the diastereoisomer in D_6-DMSO are presented in Tables 9 and 10 respectively.

Fig. 30A ^{1}H-NMR spectrum of the native benzodiazepine in $CDCl_3$. (*c.f.* COSY spectrum, Fig. 34A).

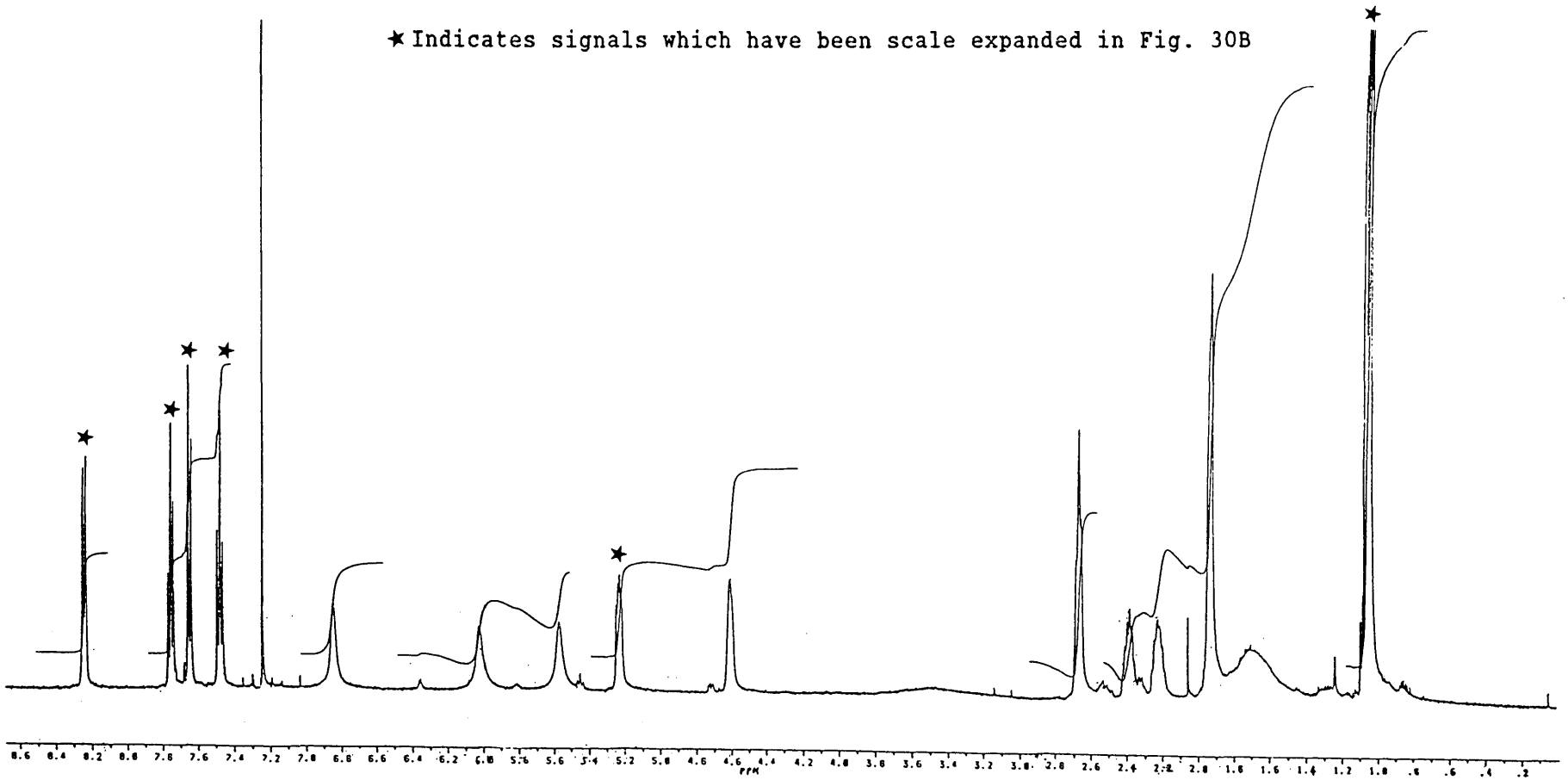
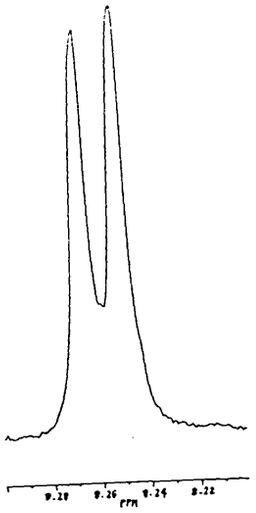
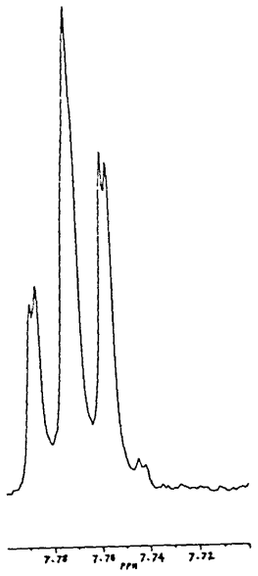


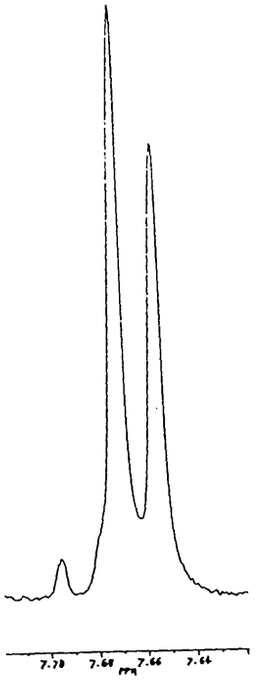
Fig. 30B Scale expanded ^{1}H-NMR resonances of the native benzodiazepine in $CDCl_3$ (*c.f.* Fig. 30A).



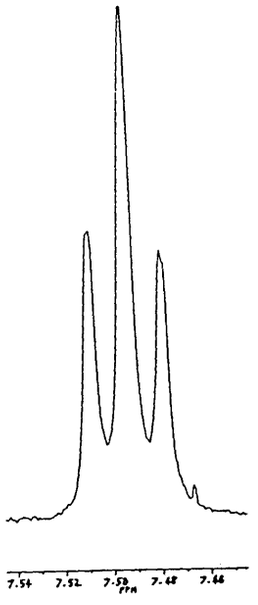
C(7)-H



C(9)-H



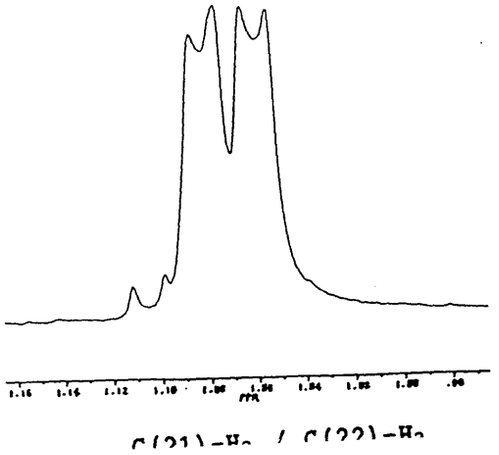
C(10)-H



C(8)-H



C(13)-H



C(21)-H$_a$ / C(22)-H$_a$

Fig. 31A ^{1}H-NMR spectrum of the native benzodiazepine in D_6-DMSO.

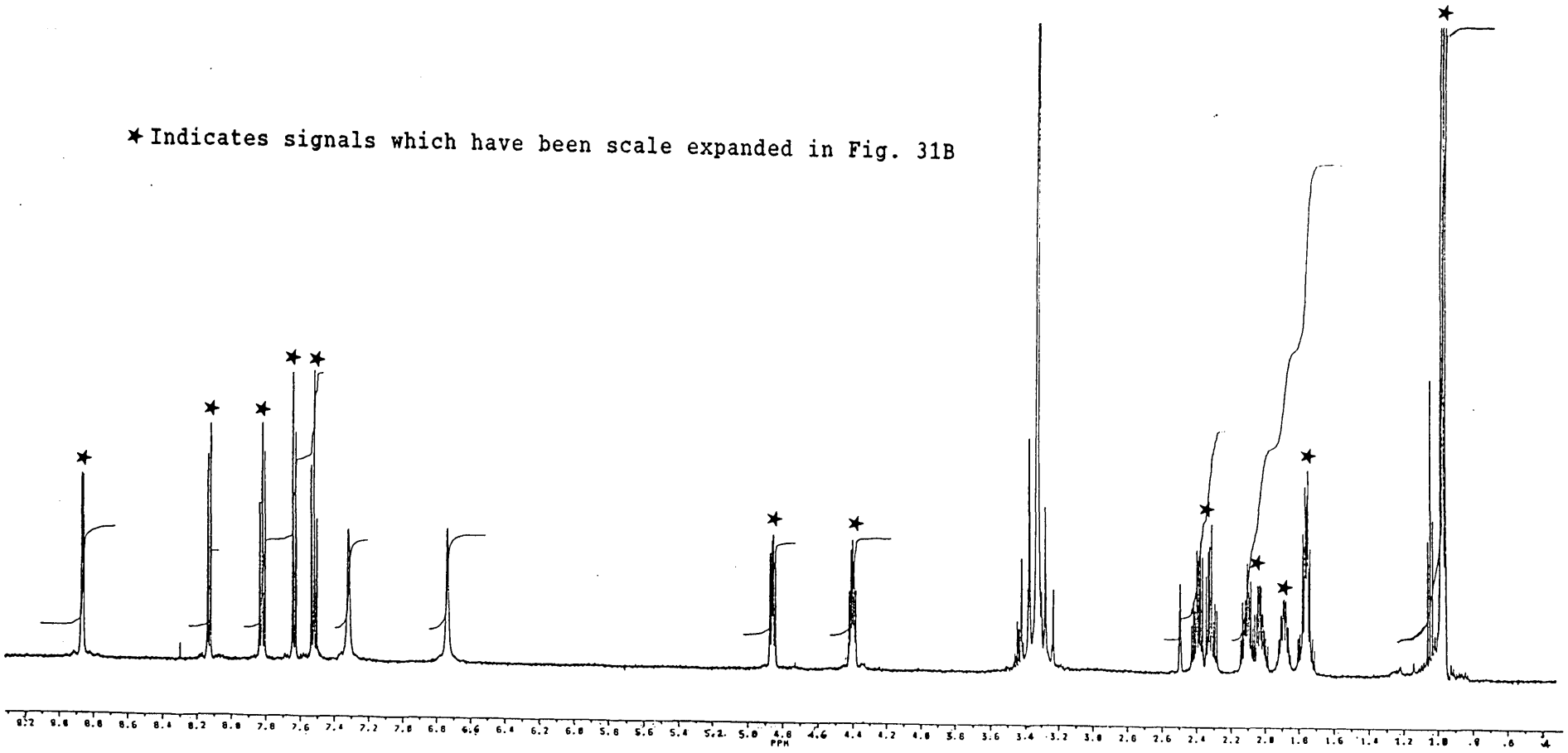


Fig. 31B Scale expanded ^{1}H-NMR resonances of the native benzodiazepine in D_6-DMSO (*c.f.* Fig. 31A).

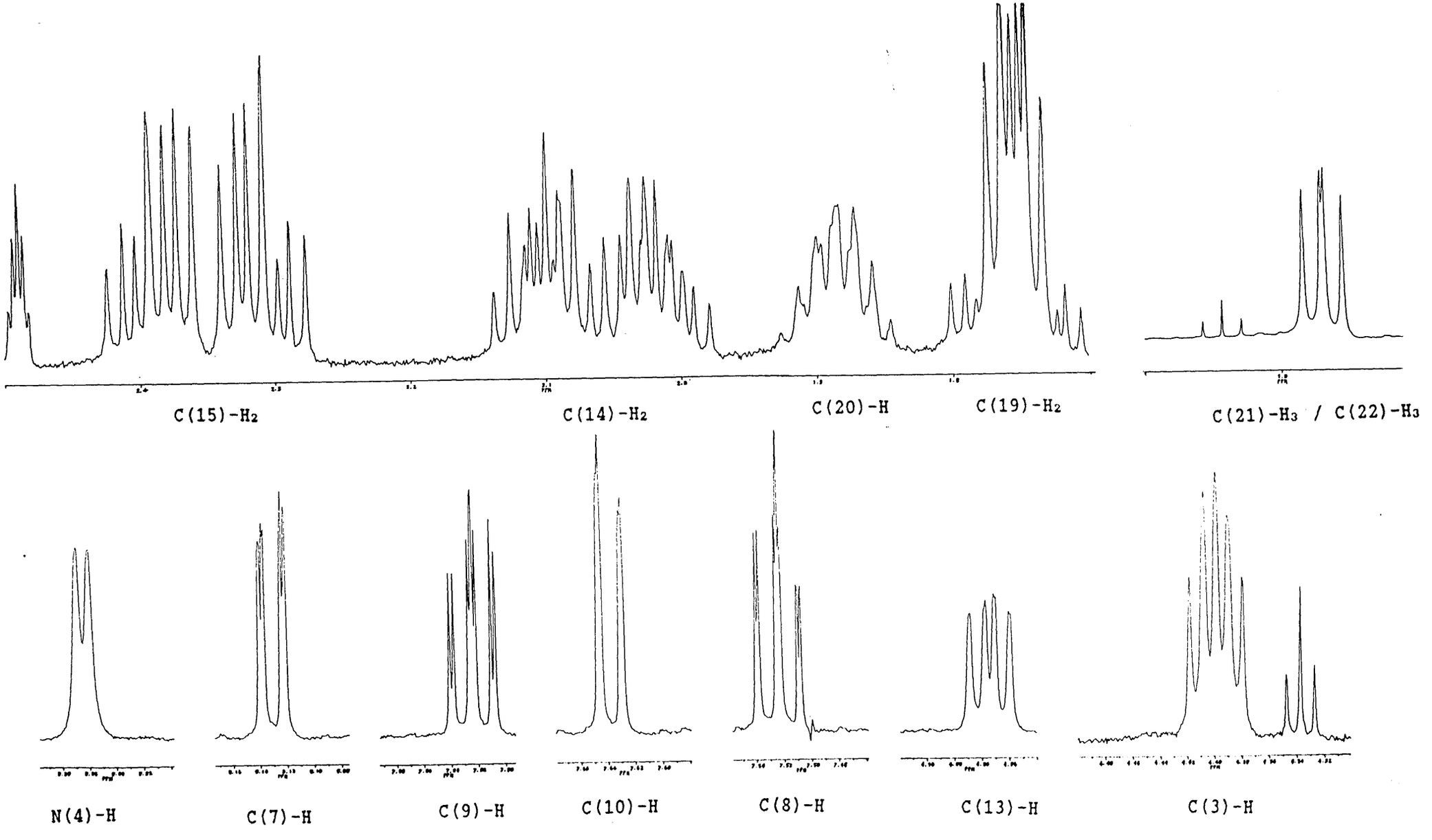
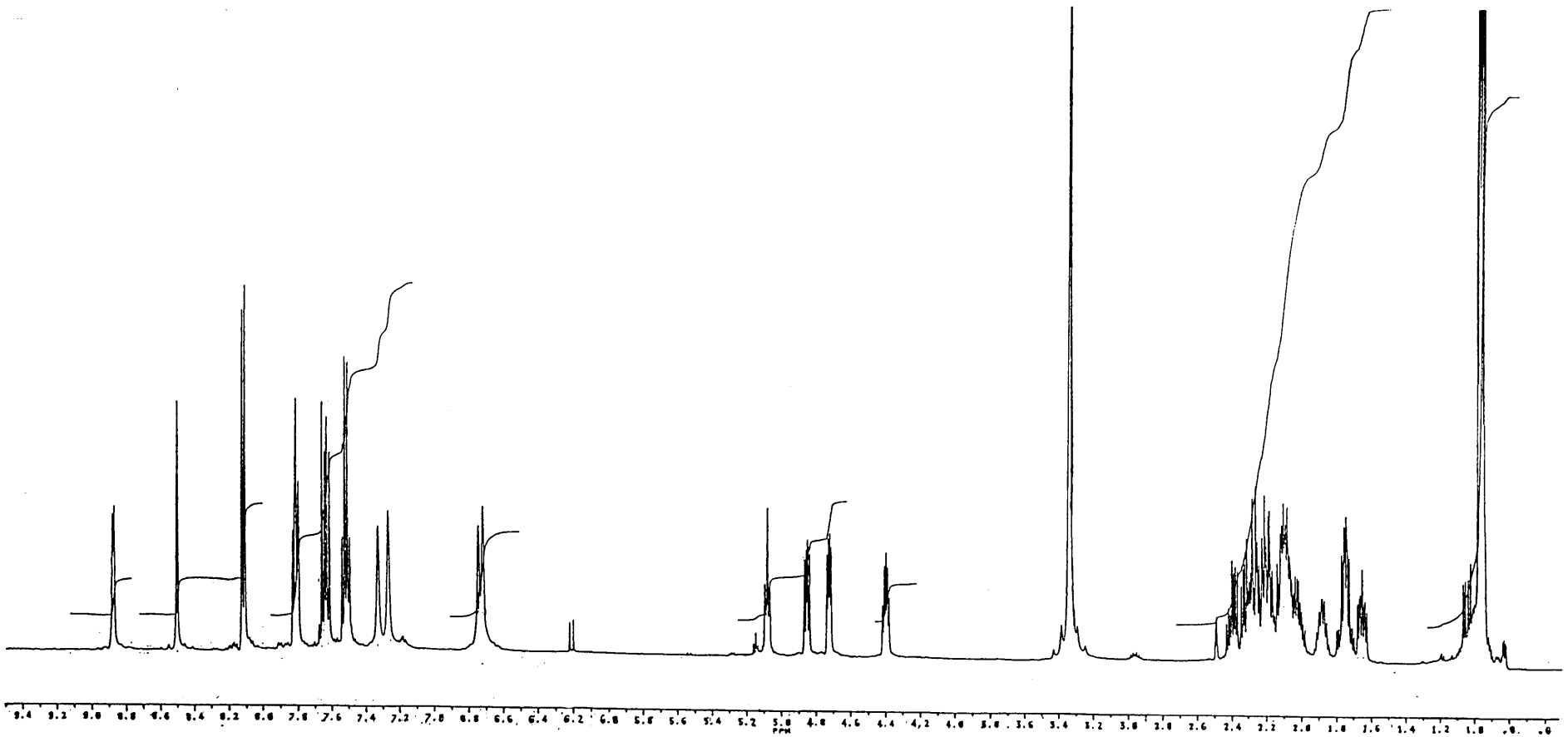


Fig. 32 ^{1}H-NMR spectrum of a 1:1 diastereoisomeric mixture of the novel benzodiazepine in D_6-DMSO.



Chemical shift (ppm)

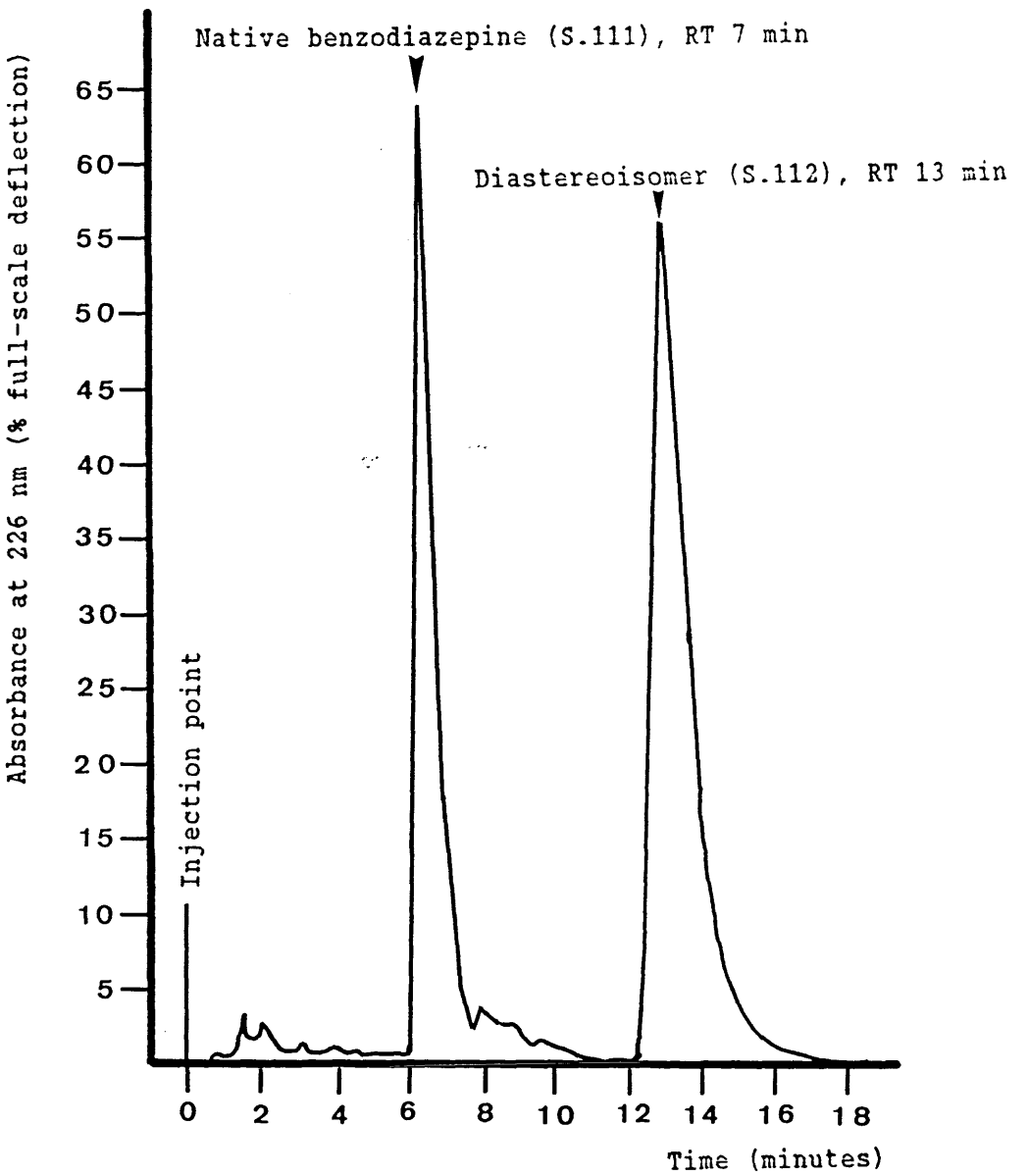
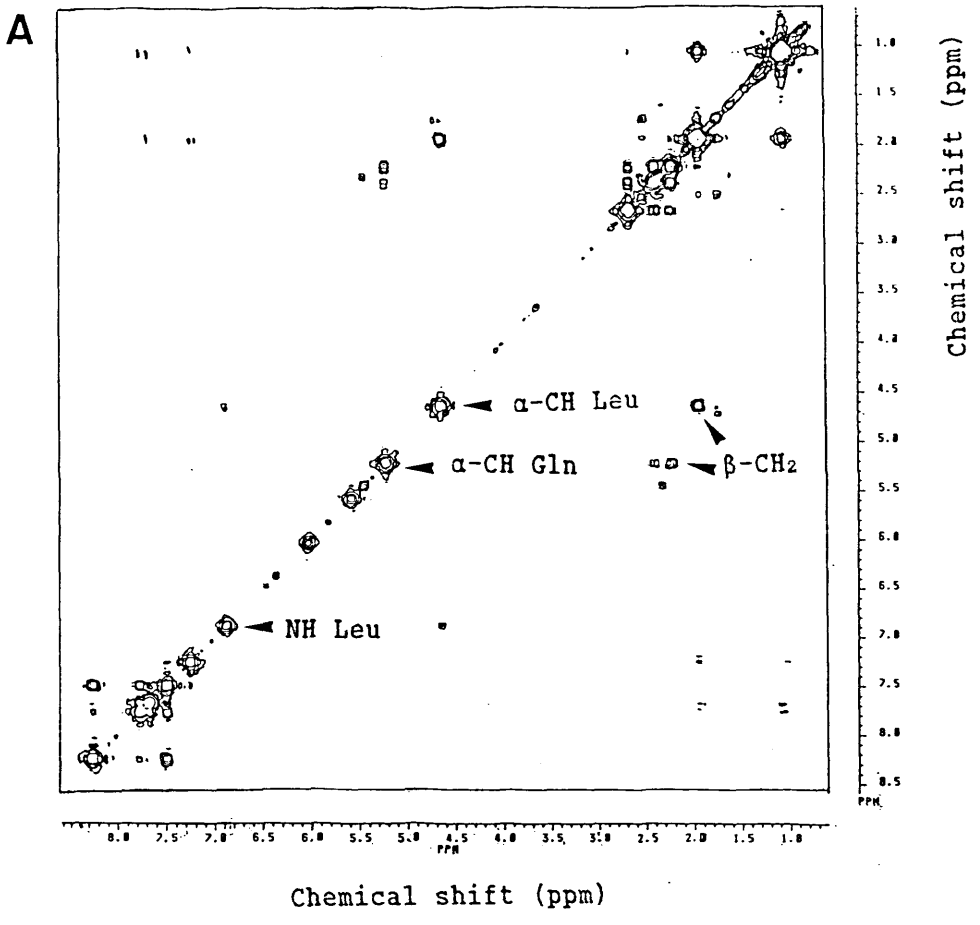


Fig. 33 HPLC profile of a pyrolysed sample of the novel benzodiazepine (1:1 mixture of diastereoisomers).

Fig. 34 ^{1}H-^{1}H COSY NMR spectrum of the novel benzodiazepine (1:1 mixture of diastereoisomers) in (A) $CDCl_3$, (B) D_6-DMSO.



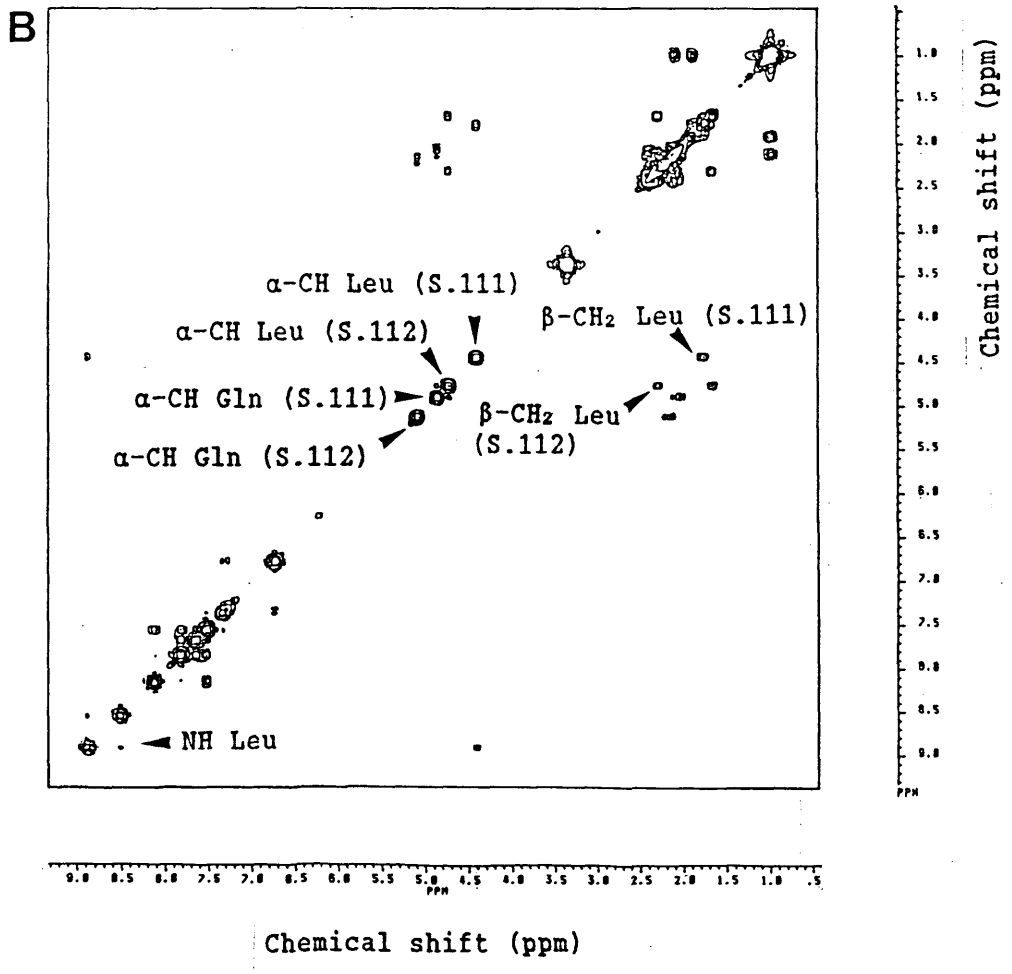


Table 9 ^{1}H-NMR spectroscopic assignments of the native benzodiazepine (S.111) in D_6-DMSO.

Chemical shift (ppm)	Integration and assignment		Position in proposed structure (Fig. 17, S.111)
0.97 Two dds (olp)	6H	2 CH_3	21, 22
1.69 - 1.79 m	2H	CH_2	19
1.84 - 1.93 bsep	1H	CH	20
1.96 - 2.14 m	2H	CH_2	14
2.25 - 2.40 m	2H	[a] CH_2	15
4.39 m	1H	[b] CH	3
4.86 m	1H	CH	13
6.74 bs	1H	[c] NH]	1, 17
7.31 bs	1H	[c] NH]	
7.52 dt	1H	CH	8
7.63 dd	1H	[d] CH	10
7.81 dt	1H	CH	9
8.13 dd	1H	[d] CH	7
8.87 bd	1H	[e] NH	4

Abbreviations used for multiplicities: dd(s), double doublet(s); olp, overlapping; m, multiplet; bsep, broad septuplet; bs, broad singlet; dt, double triplet; bd, broad doublet.

[a] C-15 is adjacent to a carbonyl group which has a deshielding effect on neighbouring protons. A downfield shift of the protons attached to C-15 with respect to the protons on C-14 was therefore observed.

[b] The ^{1}H-^{1}H NMR COSY experiment (Fig. 34B) defined this assignment.

[c] The anthranilyl and glutaminyl N-H resonances could not be differentiated for lack of coupling.

[d] H-7 was characteristically shifted downfield with respect to H-10 since it was subject to the deshielding effect of the proximate carbonyl group at C-5.

[e] This assignment was supported by evidence from the ^{1}H-^{1}H COSY experiment (Fig. 34B) and by comparison with the signals obtained from ^{1}H-NMR spectroscopy of cyclo-anthranilyl-leucine dipeptide (Fig. 36).

Table 10 ^{1}H-NMR spectroscopic assignments of the diastereoisomer (S.112) in D_6-DMSO.

Chemical shift (ppm)	Integration and assignment		Position in proposed structure (Fig. 17, S.112)
0.97 m	6H	2 CH_3	21, 22
1.65 m	1H	CH	19
2.10 m	1H	CH	20
2.20 m	2H	CH_2	14
2.00-2.30 m	2H	CH_2	15
2.28 m	1H	CH	19
4.72 dd	1H	CH	3
5.09 bt	1H	CH	13
6.72 bs	1H	NH]	1, 17
7.25 bs	1H	NH]	
7.51 dt	1H	CH	8
7.60 dd	1H	CH	10
7.81 dt	1H	CH	9
8.13 d	1H	CH	7
8.53 d	1H	NH	4

Abbreviations used for multiplicities: see footnote in Table 9.

3.6.5 ^{1}H-NMR spectroscopy of cyclo-anthranilyl-leucine dipeptide.

A benzodiazepine cyclic dipeptide (Fig. 35A, S.116), comprising anthranilate and leucine, which had been chemically synthesised was dissolved in D_6-DMSO for analysis by ^{1}H-NMR spectroscopy. The resultant spectrum (Fig. 36) aided interpretation of the ^{1}H-NMR spectrum of the novel benzodiazepine. Proton assignments and J-coupling values of the dipeptide (S.116) are presented in Table 11.

The anthranilyl-leucine (anth-leu) dipeptide eluted slightly before the native benzodiazepine (S.111) from a reverse phase (C_{18}) HPLC column, having a retention time (RT) of 5-6 min (Fig. 37). In order to ascertain that this was the true RT and not the result of a slight variation in operating conditions, HPLC was performed on a mixed sample of the native benzodiazepine and the anth-leu dipeptide which indicated the presence of two compounds eluting in succession since distinct peaks were observed.

3.6.6 ^{1}H-NMR spectroscopy of L-pyroglutamide.

Initially the glutarimide moiety of the benzodiazepine was considered to have rearranged by pyrolysis to a 5-membered pyroglutamine structure, a well known chemical transformation (Bodanszky and Martinez, 1981), as shown in Fig. 35B. This idea was originally formulated on account of considerable change in signal-envelope at the high field region of the spectrum of a pyrolysed sample of the benzodiazepine (Fig. 32). However, this proposal was clearly incorrect since three discrete N-H signals were observed and a primary amide resonance was not apparent (Fig. 32). A reference ^{1}H-NMR spectrum of L-pyroglutamide (S.117) in D_6-DMSO (Fig. 38) revealed a pattern for N-H resonances which was sufficiently different from that obtained for the diastereoisomer (Fig. 32). The secondary amide N-H proton of L-pyroglutamide appeared as a singlet at 7.74 ppm (Fig. 38) and the signal was sharper than those of the

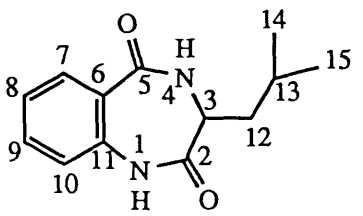
two primary amide protons. These signals were base-line separated (indicating restricted rotation about the primary amide bond) and appeared as slightly broadened singlets at 7.38 and 7.05 ppm. The difference in chemical shift values for the primary amide resonances of L-pyroglutamide was 0.33 ppm and for the N-H signals of the diastereoisomer was 0.53 ppm. The chemical shift difference in the N-H signals of the diastereoisomer was sufficiently great to dismiss the idea of these being primary amide protons.

3.6.7 ^{1}H-NMR spectroscopy of the benzodiazepine after prolonged exposure to $CDCl_3$.

A sample of native benzodiazepine which had been in contact with $CDCl_3$ for three days and consequently had become brown in colour, was used for ^{1}H-NMR spectroscopy on one occasion. New α-CH resonances were observed for the leucine and glutarimide moieties at 4.74 and 5.48 ppm respectively (Fig. 39), shifted slightly downfield with respect to the corresponding signals obtained for a freshly prepared solution of the benzodiazepine in $CDCl_3$ (Figs. 30A,B). Three additional signals were observed at 5.82, 5.90 and 6.39 ppm in the chloroform-treated sample, corresponding to N-H resonances which were more tightly grouped than those of the native benzodiazepine (Fig. 39). The appearance of new signals was attributable to a tautomeric form of the benzodiazepine (Fig. 17, S.113) in which the former exo-cyclic double bond had migrated to be in conjugation with the aromatic ring. The brown colour of the tautomeric compound (S.113) was consistent with an extended chromophore resulting from conjugation. The presence of a new proton on N(12) connecting the benzodiazepine and glutarimide rings in the tautomer was evident by the increased width of the new α-CH resonance of the glutarimide moiety at 5.48 ppm with respect to that at 5.24 ppm (Fig. 39) which indicated the resultant additional coupling.

Fig. 35A Structures of compounds used as an aid in spectral interpretation of the novel benzodiazepine.

S.116 cyclo-anthranilyl-leucine dipeptide



S.117 L-pyroglutamide

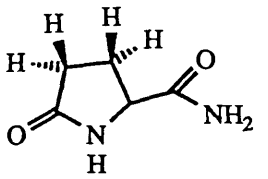
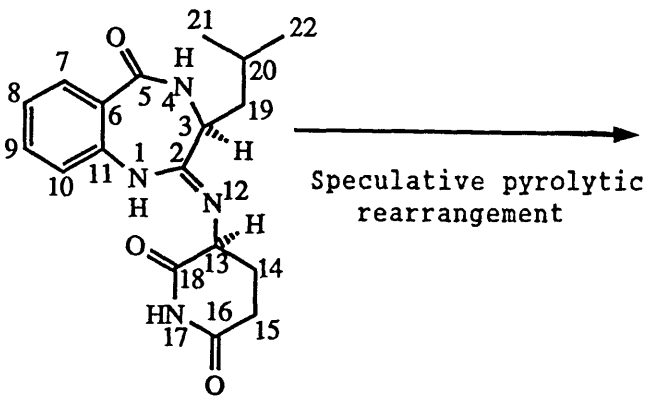


Fig. 35B Formation of the speculative pyroglutaminyl derivative of the benzodiazepine.

S.111 Native benzodiazepine



Speculative pyrolytic rearrangement

S.118 Pyroglutaminyl derivative

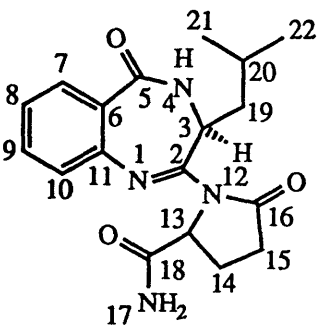
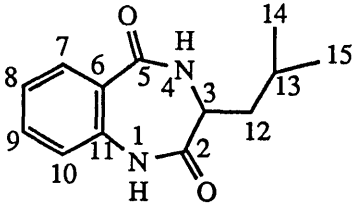


Fig. 36 ^{1}H-NMR spectrum of cyclo-anthranilyl-leucine dipeptide in D_6-DMSO.

S.116 cyclo-anthranilyl-leucine dipeptide



Chemical shift (ppm)

Table 11 ^{1}H-NMR spectroscopic assignments of cyclo-anthranilyl-leucine dipeptide ($C_{13}H_{16}N_2O_2$) in D_6-DMSO.

Chemical shift (ppm)	Integration and assignment		Position in structure (Fig. 35A)	Coupling constant (H_z)
0.77	3H	CH_3]	14, 15	J = 6.60
0.85	3H	CH_3]		J = 6.60
1.55	2H	CH_2	12	J = 7.20
1.70	1H	CH	13	J = 6.80
3.60	1H	CH	3	J = 3.40
7.09	1H	CH	10	J_{10-9} = 8.10
				J_{10-8} = 1.05
7.21	1H	CH	8	J_{8-7} = 7.80
				J_{8-9} = 7.30
				J_{8-10} = 1.05
7.51	1H	CH	9	J_{9-8} = 7.30
				J_{9-10} = 8.10
				J_{9-7} = 1.50
7.75	1H	CH	7	J_{7-8} = 7.80
				J_{7-9} = 1.50
8.43 d	1H	NH	4	J(N^4H-C^3H) = 5.80
10.36 s	1H	NH	1	

Abbreviations: J, spin-spin coupling constant;
d, doublet; s, singlet.

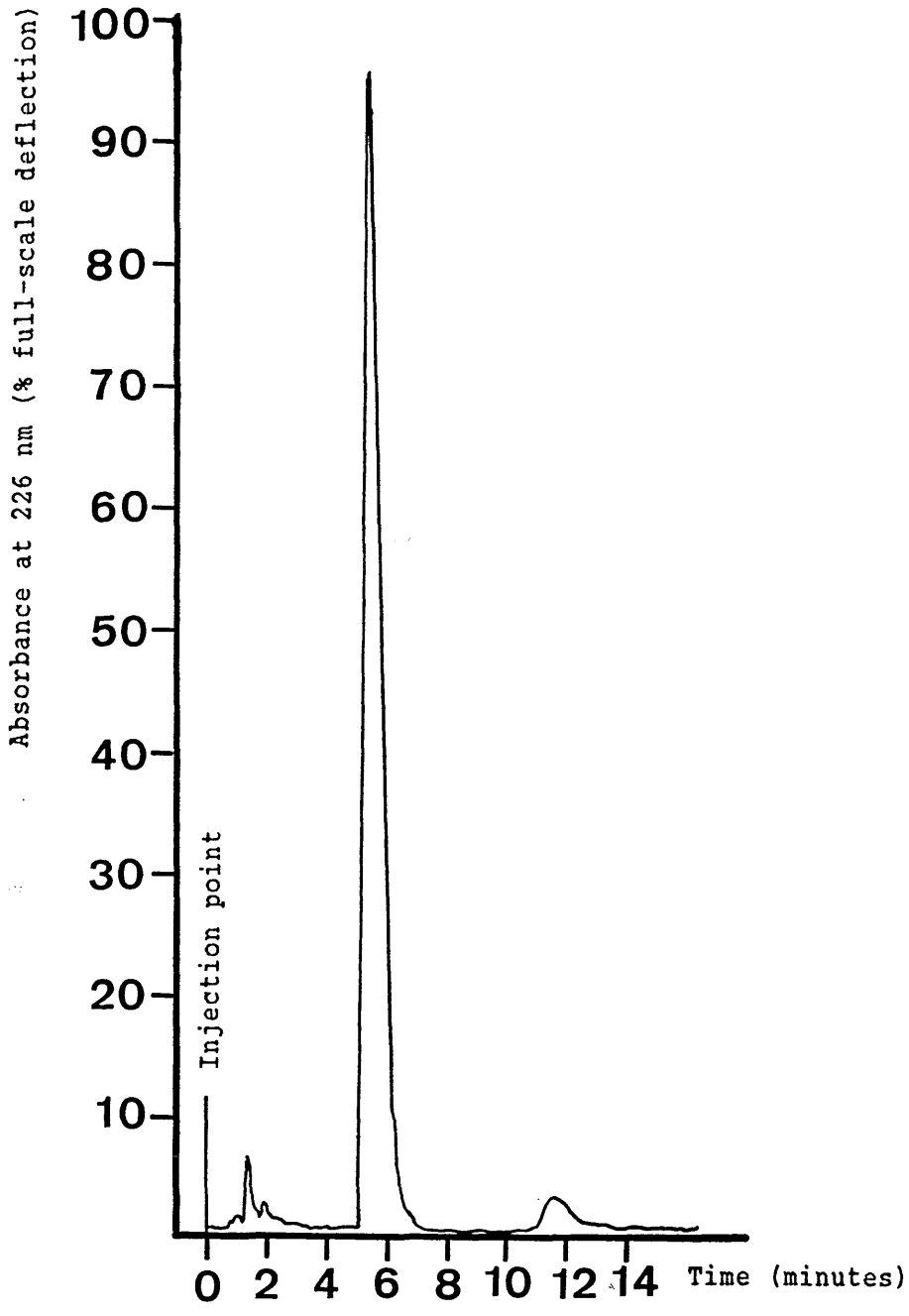


Fig. 37 HPLC profile of cyclo-anthranilyl-leucine dipeptide.

Fig. 38 ^{1}H-NMR spectrum of L-pyroglutamide.

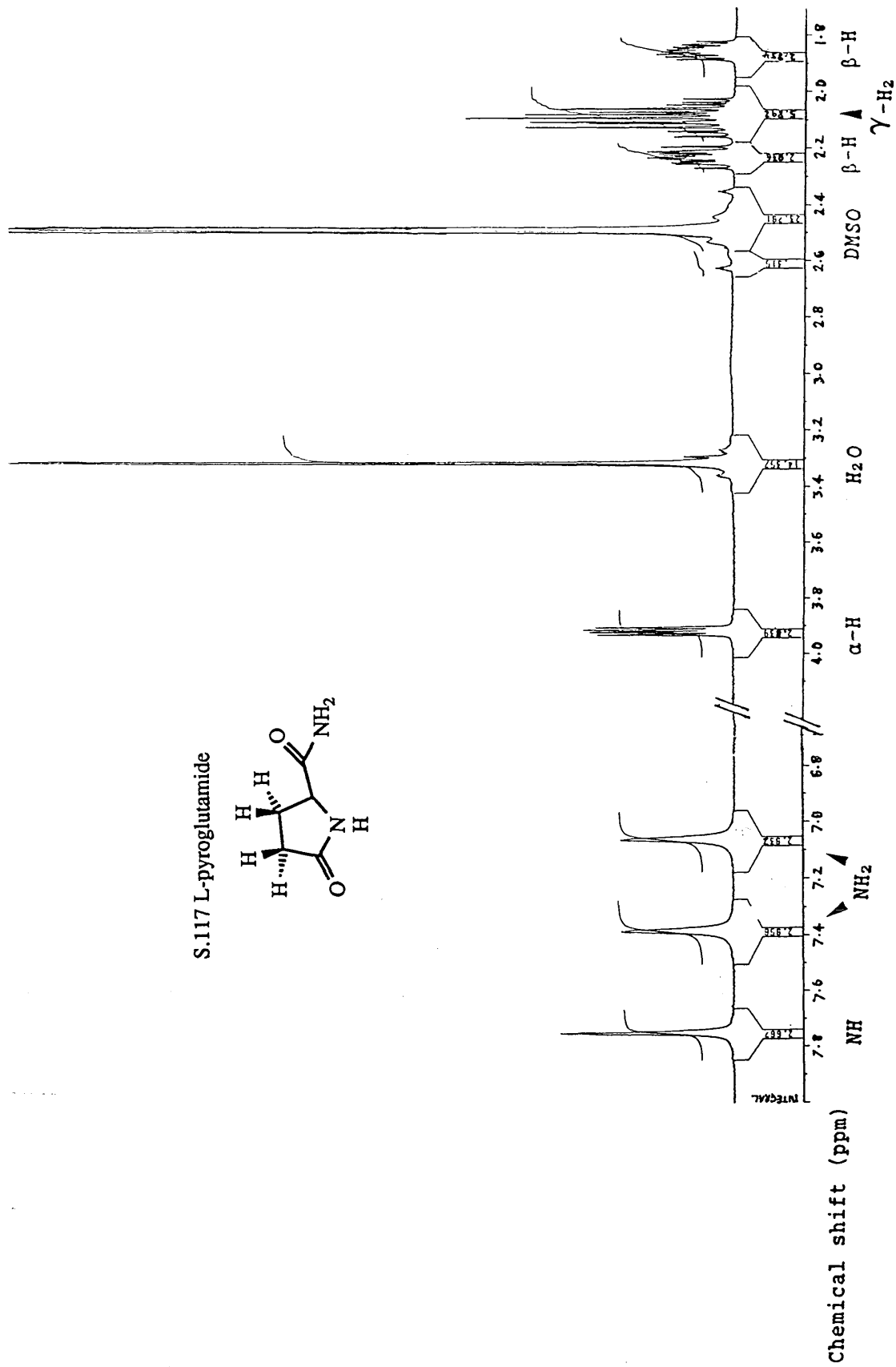
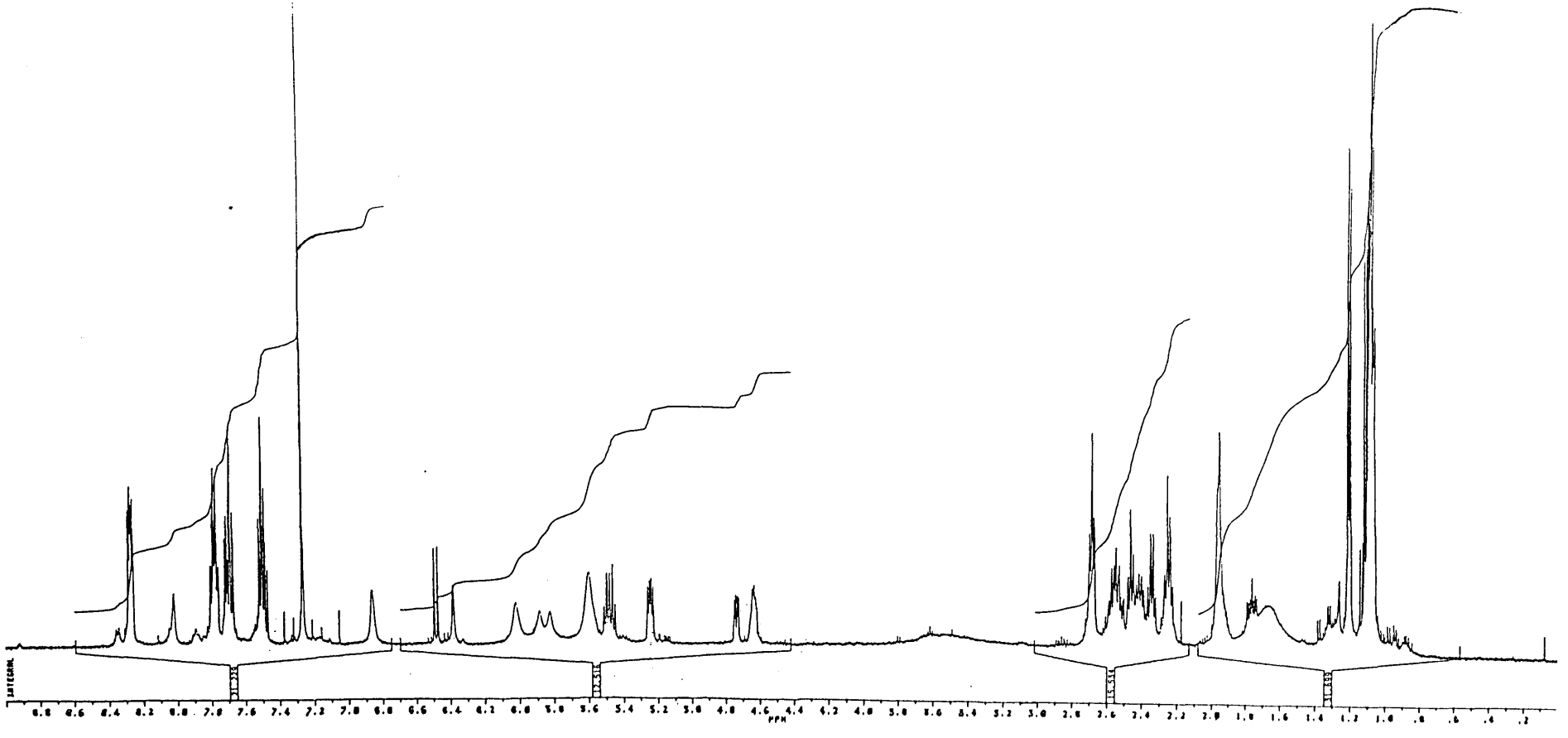


Fig. 39 ^{1}H-NMR spectrum of a sample of the native benzodiazepine which had been exposed to chloroform for three days.



Chemical shift (ppm)

3.6.8 ^{13}C-NMR spectroscopy of the novel benzodiazepine.

^{13}C-NMR spectroscopy of the native benzodiazepine was performed in D_6-DMSO (Fig. 40) although initially $CDCl_3$ had been used (Fig. 41). Specified resonances are presented on an expanded scale (Fig. 42A,B).

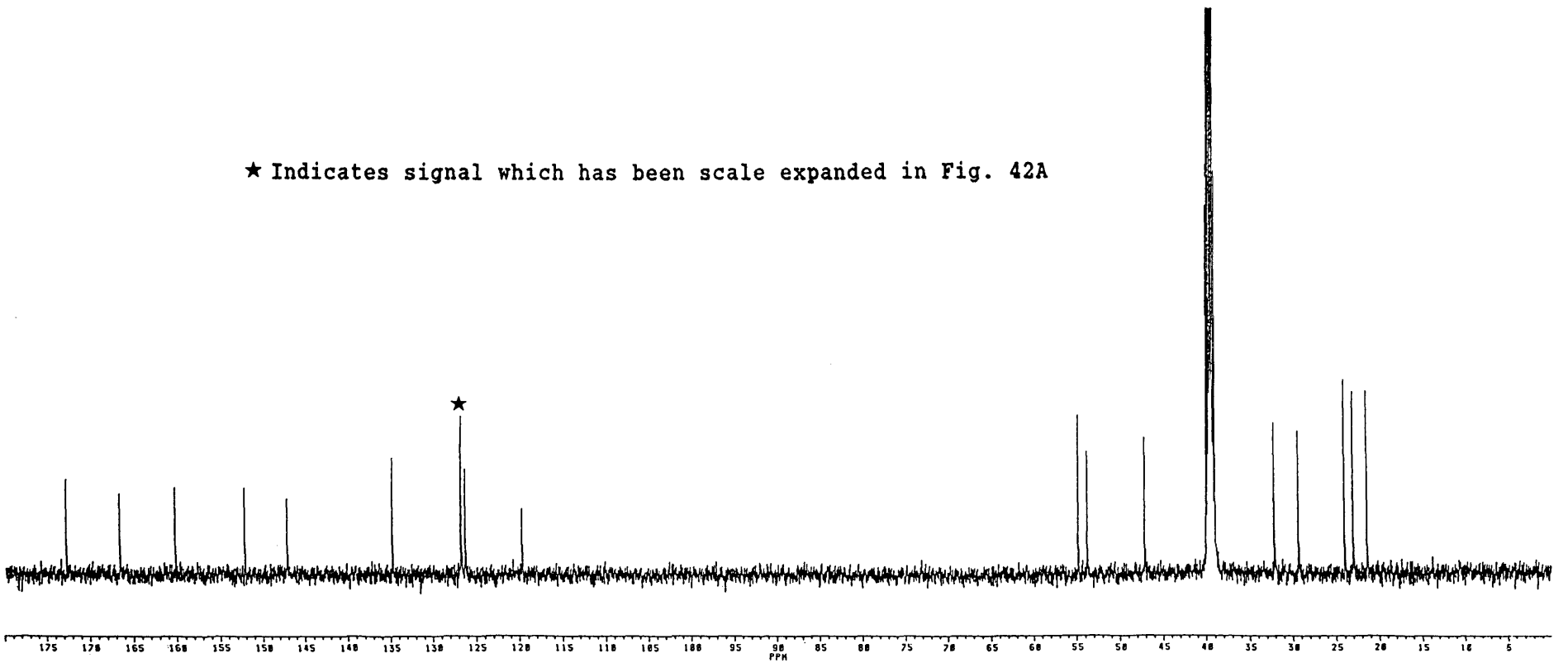
The ^{13}C-NMR spectrum of a 1:1 diastereoisomeric mixture (formed by pyrolysis) showed a doubling up of all resonances (Fig. 43). Enhanced resolution of certain resonances is shown in Fig. 44.

^{13}C-NMR spectroscopic assignments of the native benzodiazepine in D_6-DMSO are presented in Table 12.

Table 13 shows chemical shift values for ^{13}C-resonances of the native benzodiazepine in $CDCl_3$ and D_6-DMSO and chemical shift differences between the two diastereoisomers in D_6-DMSO. Examination of chemical shift differences between relevant carbons in the diastereoisomers (Table 13) revealed the greatest discrepancy at the leucine β-carbons (C-19 in Fig. 17, S.111, S.112). This finding corroborated ^{1}H-NMR spectroscopic evidence of increased non-equivalence of the leucine β-protons of the diastereoisomer (S.112) with respect to the corresponding protons in the native isomer (S.111). Other significant differences in chemical shift (Table 13) presumably reflect steric interaction of the two substituents on the benzodiazepine ring.

Fig. 40 ^{13}C-NMR spectrum of the native benzodiazepine in D_6-DMSO.

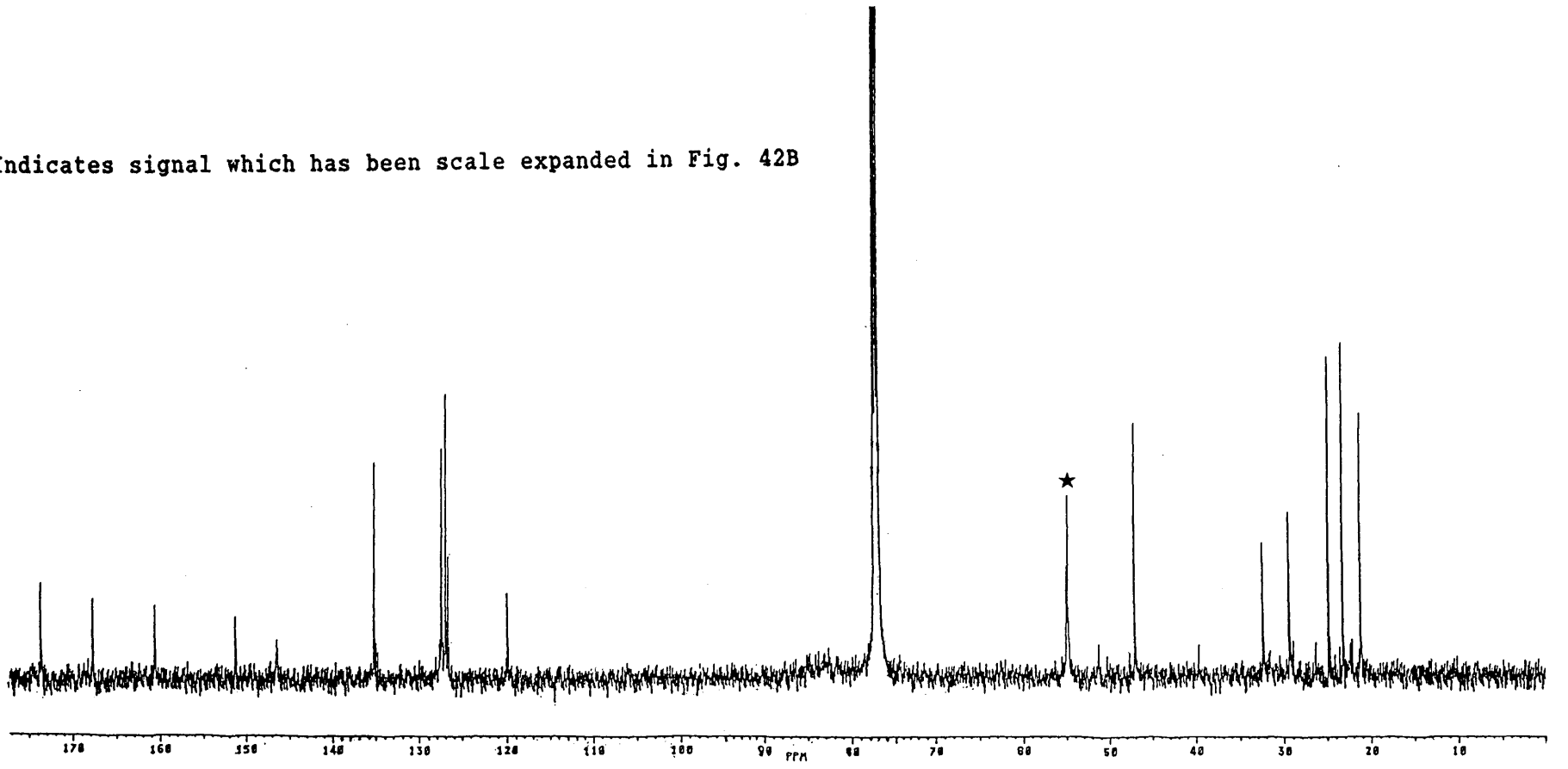
★ Indicates signal which has been scale expanded in Fig. 42A



Chemical shift (ppm)

Fig. 41 ^{13}C-NMR spectrum of the native benzodiazepine in $CDCl_3$.

★ Indicates signal which has been scale expanded in Fig. 42B



Chemical shift (ppm)

Fig. 42A Scale expanded ^{13}C-NMR signal at 126.7 ppm (*c.f.* Fig. 40).

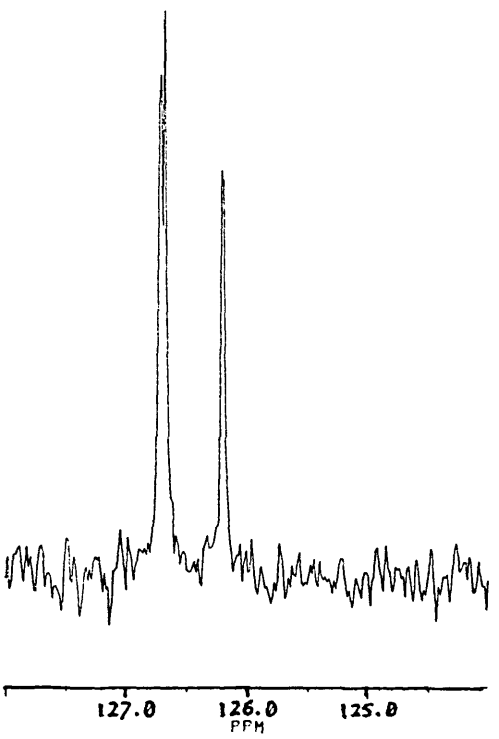


Fig. 42B Scale expanded ^{13}C-NMR signal at 54.9 ppm (*c.f.* Fig. 41).

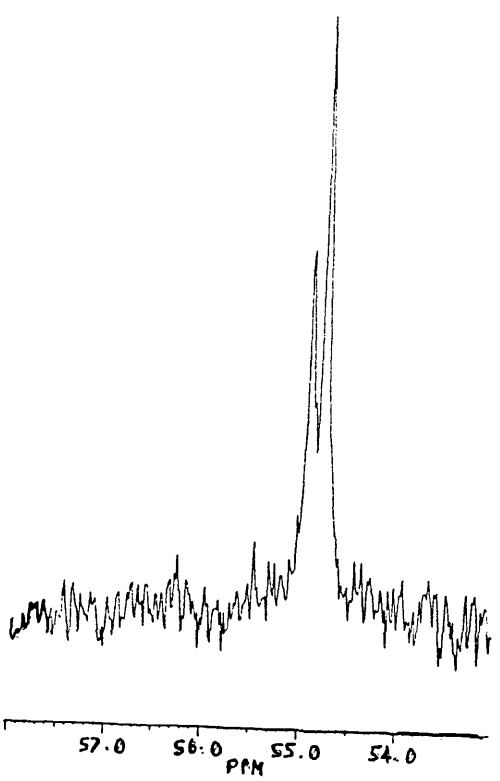


Fig. 43 ^{13}C-NMR spectrum of a 1:1 diastereoisomeric mixture of the novel benzodiazepine in D_6-DMSO.

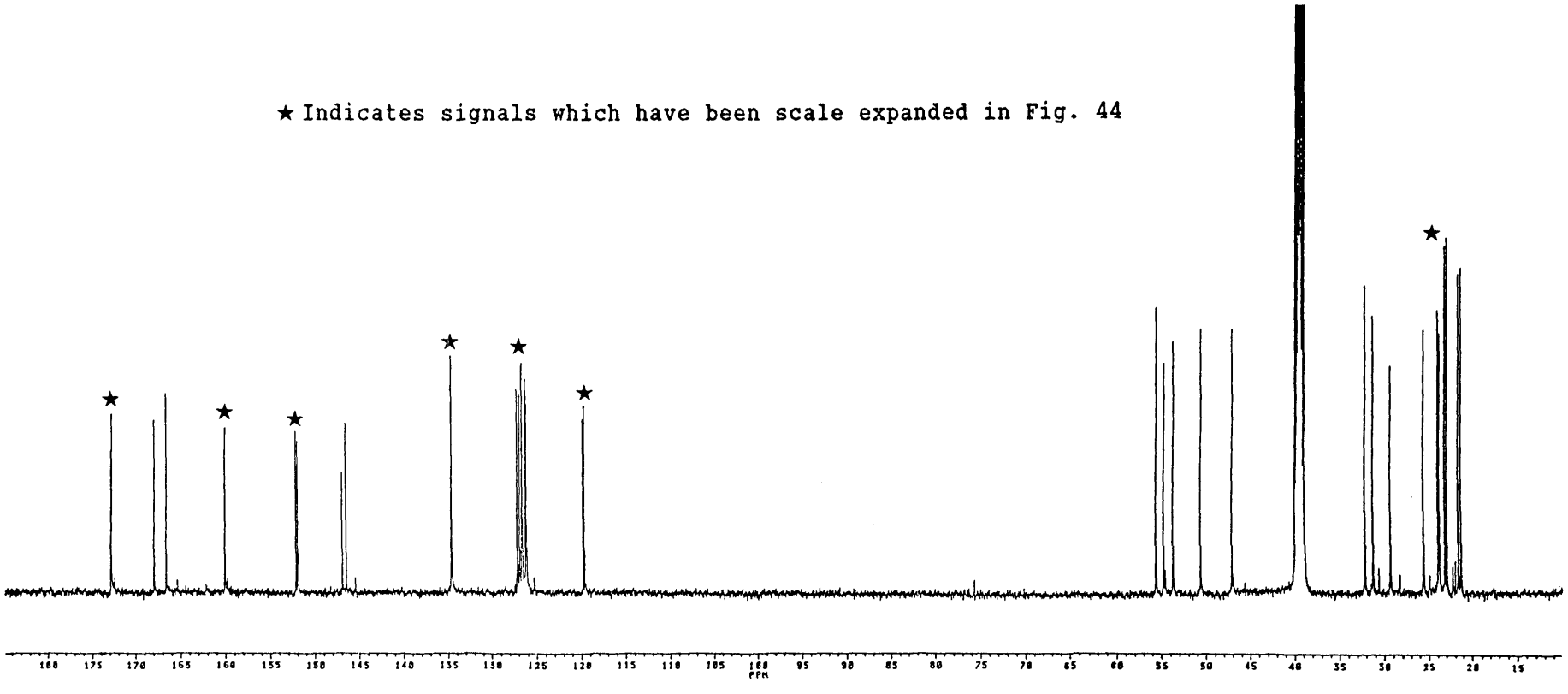


Fig. 44 Scale expanded ^{13}C-NMR signals (1:1 diastereoisomeric mixture of the novel benzodiazepine) in D_6-DMSO (*c.f.* Fig. 43).

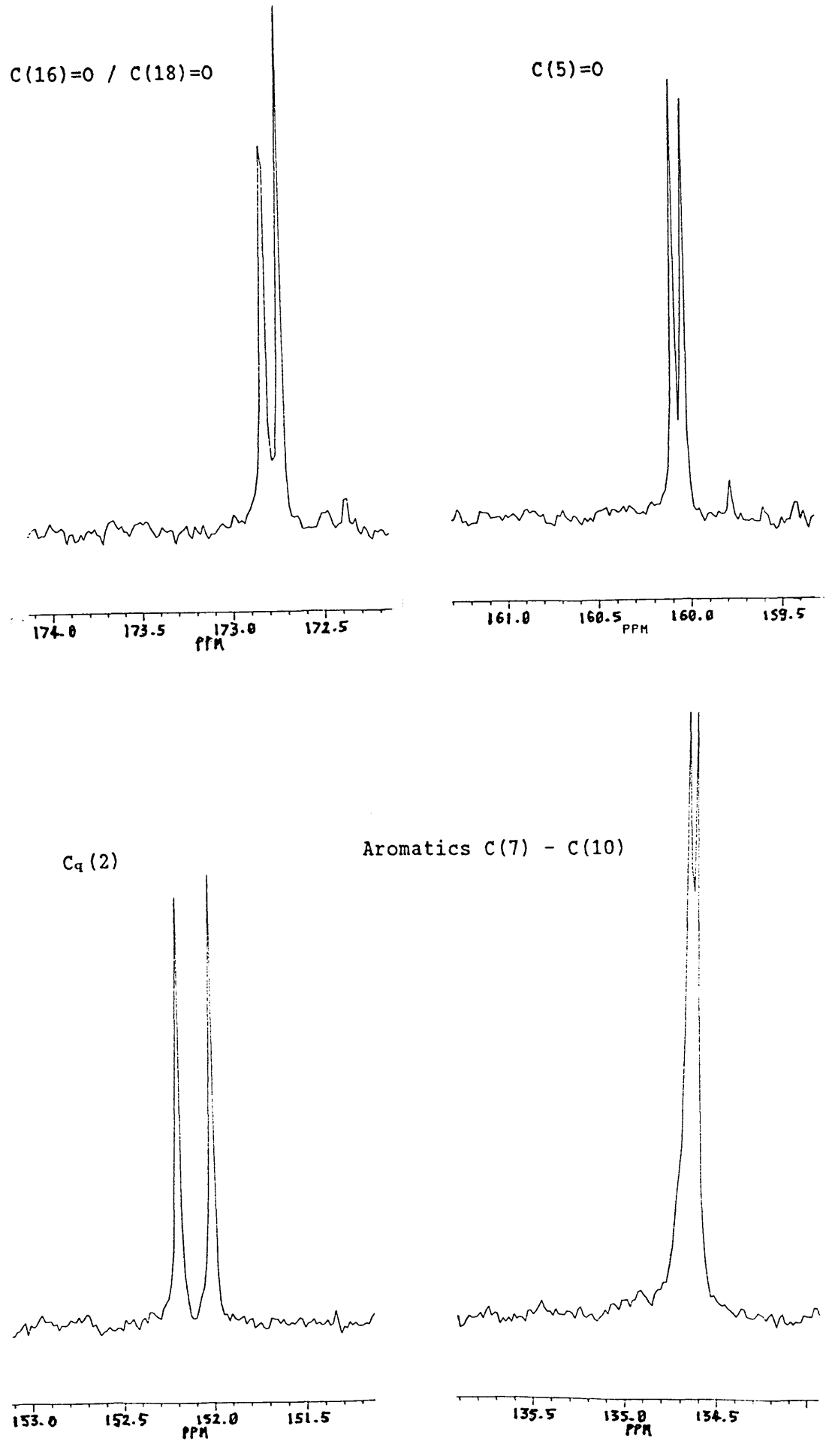


Fig. 44 cont.

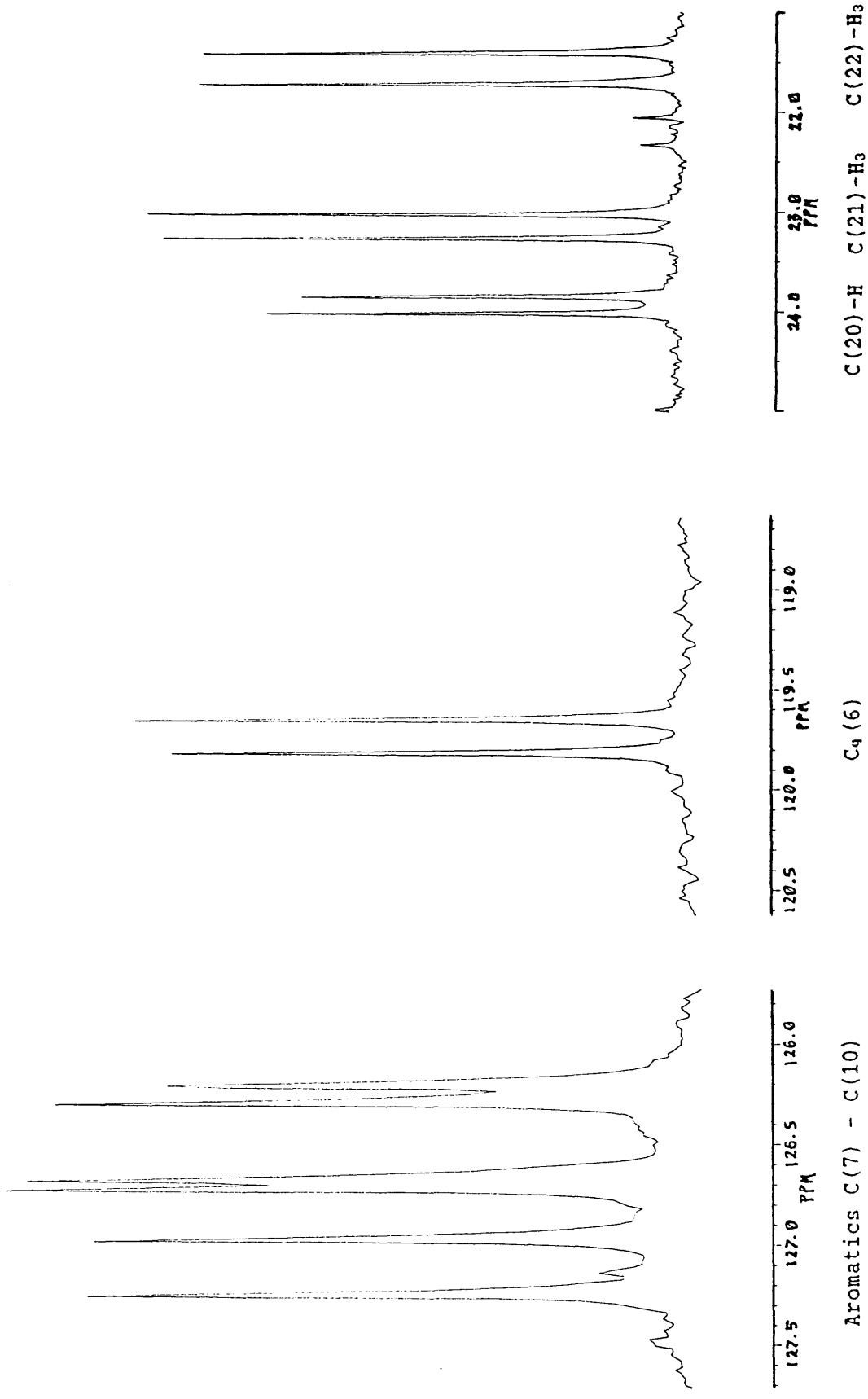

24.0
23.0
PPM
22.0
C(20)-H C(21)-H3 C(22)-H3
120.5
120.0
119.5
PPM
119.0
Cq (6)
127.5
127.0
126.5
PPM
126.0
Aromatics C(7) - C(10)

Table 12 ^{13}C-NMR spectroscopic assignments of the native benzodiazepine in D_6-DMSO.

Chemical shift (ppm)	Assignment	Position in proposed structure (Fig. 17, S.111)
21.41	CH_3	21, 22
23.02	CH_3	
24.01	CH	20
29.34	CH_2	14
32.16	CH_2	15
47.18	CH_2	9
53.80	CH	3, 13
54.85	CH	
119.66	C_q	6
126.20	CH	
126.68	CH	7-10
126.71	CH	
134.70	CH	
147.00	C_q	11
152.04	C_q	2
160.11	* C=O	5
166.59	C=O	16, 18
172.80	C=O	

* In the absence of a fully ^1H-coupled ^{13}C spectrum, carbonyl assignments were made by analogy with auranthine (Yeulet *et al.*, 1986).

Assignments were otherwise made by analogy with asperlicin (Leisch *et al.*, 1985).

Table 13 ^{13}C-NMR spectroscopy of the benzodiazepine (BZD): chemical shift values of the native BZD (S.111) in $CDCl_3$ and D_6-DMSO and shift differences between the two diastereoisomers (S.111 and S.112) in D_6-DMSO.

Chemical shift of native BZD (ppm) in:		Chemical shift of 1:1 mixture of diastereoisomers (ppm) in D_6-DMSO		Difference in chemical shift (ppm)	Position in proposed structure
$CDCl_3$	D_6-DMSO	A (S.111)	B (S.112)	(A-B)	(Fig. 17, S.111)
21.16	21.41	21.40,	21.71	-0.31	21, 22
23.18	23.02	23.02,	23.26	-0.24	
24.79	24.01	24.01,	23.85	0.16	20
29.33	29.34	29.35,	25.65	3.70	14
32.27	32.16	32.16,	31.28	0.88	15
46.99	47.18	47.18,	39.33*	7.85$	19
54.77	53.80	53.80,	50.71	3.09	3, 13
54.92	54.85	54.68,	55.84	-1.16	
119.81	119.66	119.64,	119.81	-0.17	6
126.62	126.20	126.18,	126.27	-0.09	
126.90	126.68	126.69,	126.65	0.04	7-10
127.38	126.71	126.95,	127.23	-0.28	
135.07	134.70	134.67,	134.63	0.04	
146.46	147.00	146.98,	146.53	0.45	11
151.29	152.04	152.00,	152.19	-0.19	2
160.63	160.11	160.09,	160.03	0.06	5
167.74	166.59	166.92,	167.60	-0.68	16, 18
173.64	172.80	172.82,	172.74	-0.72	

* This signal was obscured by DMSO resonances.

$ Denotes the greatest difference in chemical shift.

3.7 STRUCTURE ELUCIDATION OF THE DIKETOPIPERAZINE.

Purity of the diketopiperazine (Fig. 17, S.114) was indicated in HPLC analysis since a single peak was obtained under two different solvent conditions. A single spot was apparent in thin-layer chromatography, consistent with chemical purity of the compound.

3.7.1 UV spectroscopy.

A wavelength of 250 nm was used routinely in HPLC analysis to detect the diketopiperazine. The UV spectrum of the purified diketopiperazine revealed an absorption maximum at 244 nm (Fig. 45). The extinction coefficient was not determined since an insufficient amount of diketopiperazine was available at this stage for accurate gravimetric measurement.

3.7.2 Mass spectrometry.

The electron-impact (EI) mass spectrum (Fig. 46), obtained at 70 eV, revealed a molecular ion of 443 and several prominent fragment ions. The fragmentation pattern was sufficiently informative to make further analysis by fast-atom bombardment (FAB) mass spectrometry unnecessary. The low voltage (12 eV) mass spectrum revealed prominent peaks at the following m/z: 443, 401, 374 and 332. Accurate mass measurements of some fragment ions, together with molecular formula assignments, are shown in Table 14. Metastable ion analysis was performed using a link-scanning technique. The particular method employed involved maintaining the ratio of magnetic field strength to accelerating voltage (B/E) at a constant value to yield the subset of ions fragmenting from a given parent ion. The relevant daughter ions obtained by link-scanning analysis are presented in Table 15. A summary of key ions in the fragmentation is illustrated (Fig. 47). Fig. 48 indicates some of the molecular cleavages and other deduced fragmentations are described in Table 16.

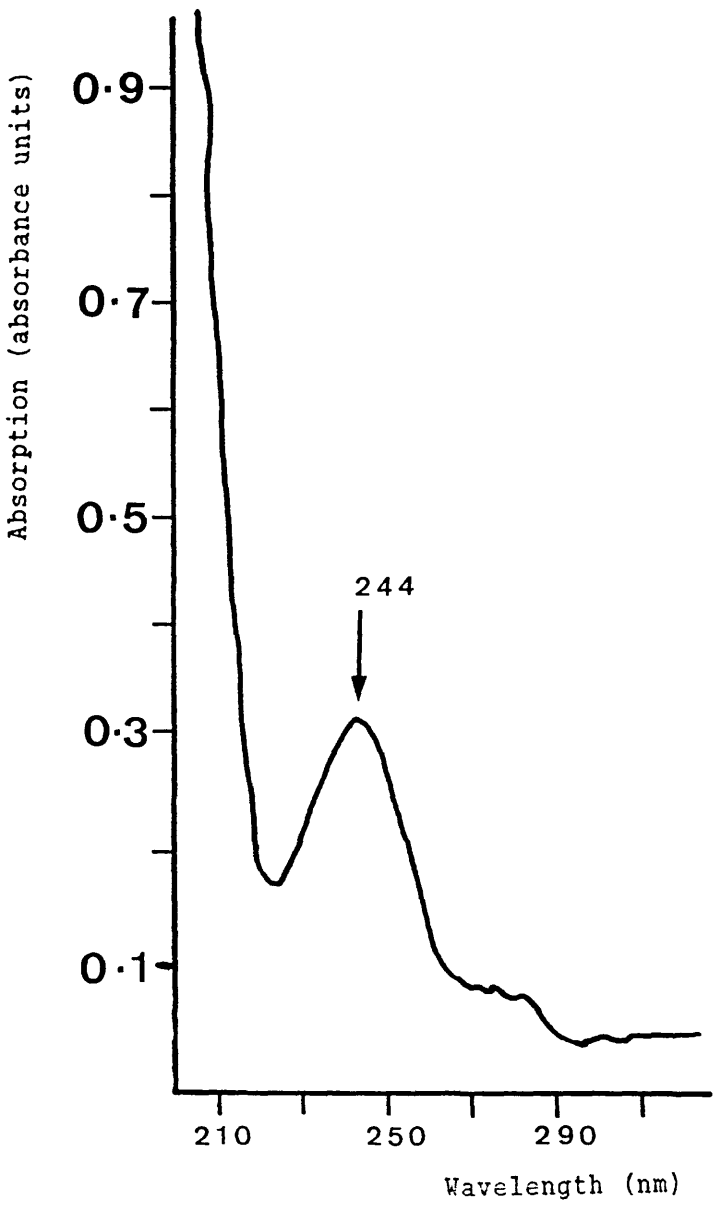


Fig. 45 Ultra-violet spectrum of the novel diketopiperazine in ethanol.

Fig. 46 Electron impact mass spectrum of the novel diketopiperazine (70 eV; source temperature, 190°C).

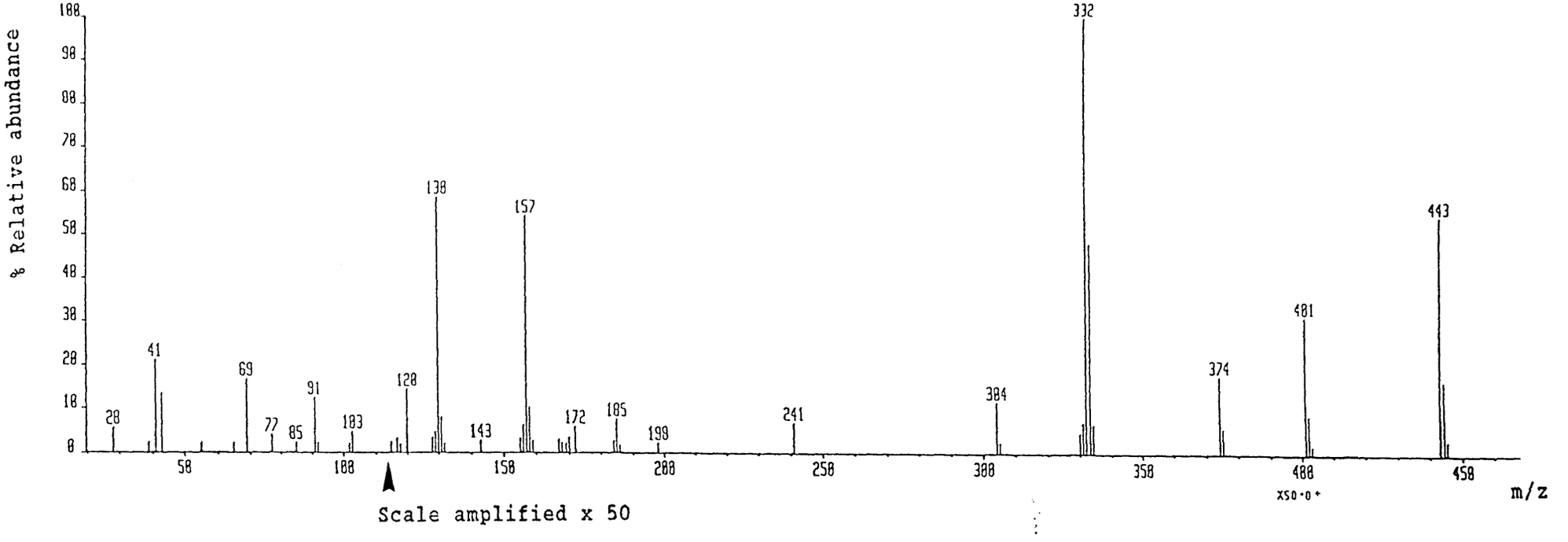


Table 14 Accurate mass measurements of the novel diketopiperazine.

Fragment Ion (m/z)	Accurate Mass	Calculated Mass	Molecular Formula
443 (M^+)	443.2209	443.2209	$C_{27}H_{29}N_3O_3$
332	332.1403	332.1399	$C_{20}H_{18}N_3O_2$
241	241.0849	241.0851	$C_{13}H_{11}N_3O_2$
157	157.0766	157.0766	$C_{10}H_9N_2$
120	120.0814	120.0813	$C_8H_{10}N_1$
91	91.0547	91.0548	C_7H_7

Table 15 Metastable ion analysis of the diketopiperazine.

Parent Ion (m/z)	Daughter Ions (m/z)
443	401 374
401	332 310 198 185 130
374	332 284 219 172
332	304 241 220 212 185 157 130 120
241	224 212 198 186 169 157 130
157	130

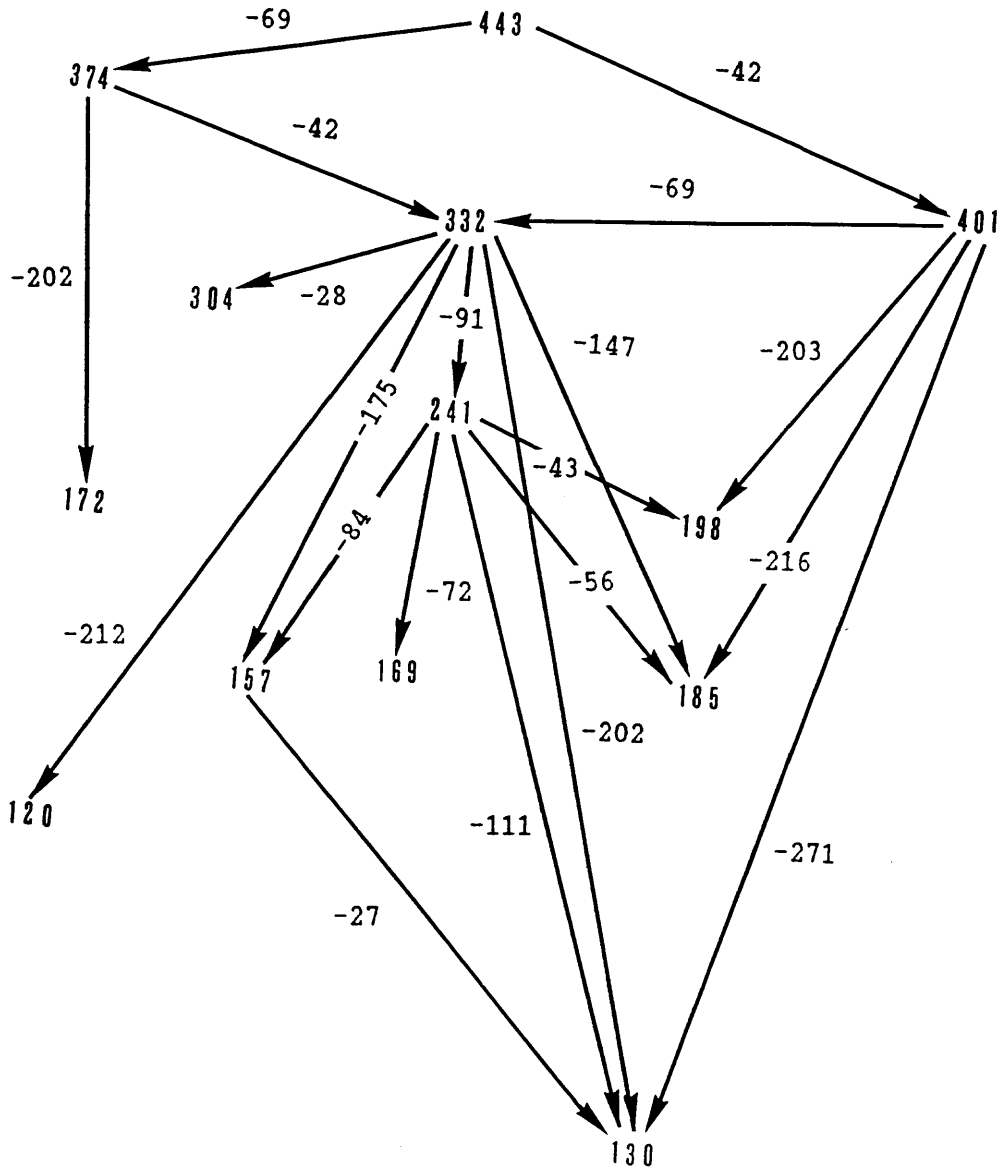
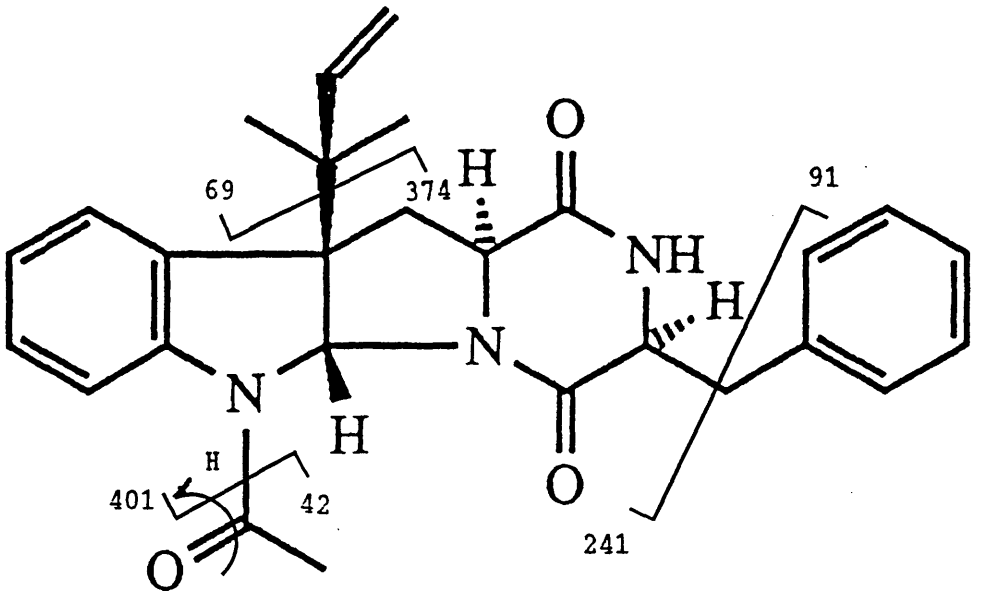


Fig. 47 Summary of key ions in the fragmentation of link-scanned mass spectrometry of the novel diketopiperazine.

Fig. 48 Structure of the novel diketopiperazine showing mass spectrometric cleavages and fragments with charge retention.



443-[69] = 374; 443-[42] = 401; 443-[69+42] = 332;

332-[91] = 241

Structure of the novel diketopiperazine showing adopted numbering system.

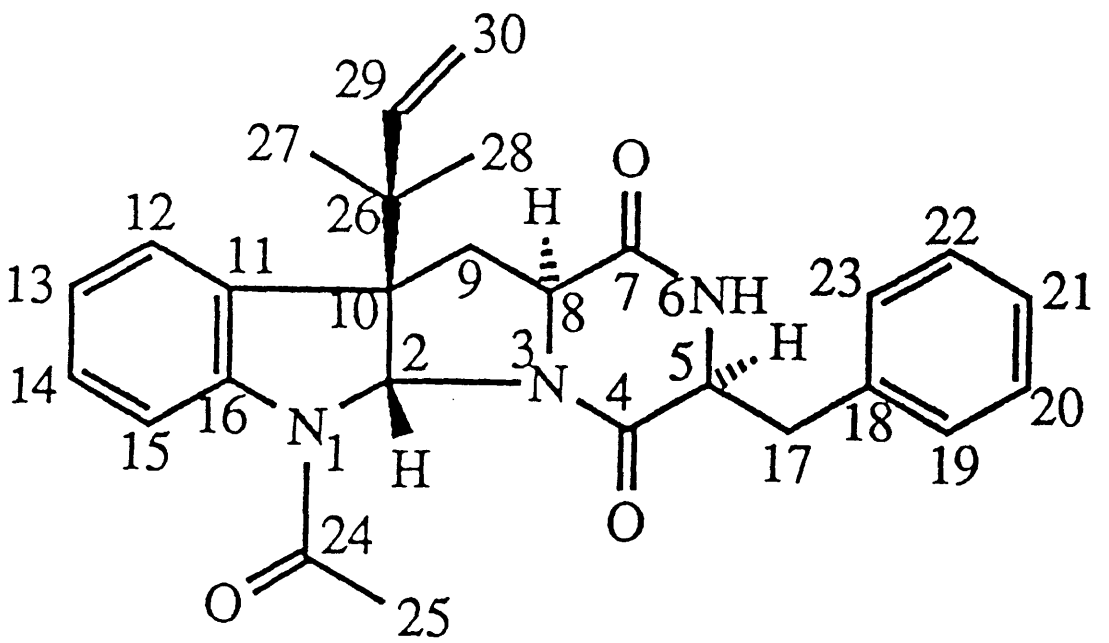
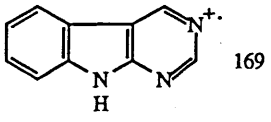
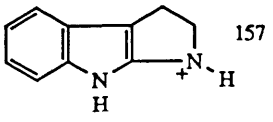
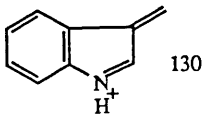
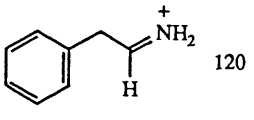


Table 16 Mass spectrometric fragmentations of the diketopiperazine.

Observed ion (m/z)	Structure or possible formula of observed ion * See Fig. 48	Mass difference (parent ion - observed ion)	Molecular assignment of extruded fragment
443#	*	M^+	
401		- 42	C_2H_2O (ketene)
374		- 69	C_5H_9 (prenyl)
401#	*	Parent ion	
332		- 69	C_5H_9 (prenyl)
198	*	- 203	Loss of 69+91+43
185	*	- 216	Loss of 69+91+56
130	*	- 271	Loss of 69+91+84+27
374#	*	Parent ion	
332		- 43	CH_3CO
172		- 202	
332#	*	Parent ion	
304		- 28	
185		- 147	Loss of 91+56
157		- 175	Loss of 91+84
130		- 202	Loss of 91+84+27
120		- 212	
241#	*	Parent ion	
198		- 43	NHCO in ring D*
185		- 56	NHCHCO in ring D*
169	 169	- 72	$C_3H_4O_2$ or C_2H_3O+CHO
157		- 84	CONHCHCO in ring D*
157#	 157	Parent ion	
130	 130	- 27	HCN in ring C*
120	 120		
91	C_7H_7		
77	C_6H_5 (Phe)		
69	C_5H_9		
43	HNCO, CH_3CO or C_3H_7		
42	C_2H_2O		
41	C_3H_5 (of prenyl)		

# Indicates ions which were analysed by link-scanning.

3.7.3 ^{1}H-NMR spectroscopy of the novel diketopiperazine.

The ^{1}H-NMR spectrum of the diketopiperazine in D_6-DMSO is presented in Fig. 49. The resonance at 2.49 ppm was due to DMSO. The aromatic protons of the tryptophan moiety of the diketopiperazine showed a characteristic pattern of a 4-spin system: a doublet resonance was observed at both H-12 and H-15 (termini of the 4-spin system) and triplets for both H-13 and H-14. The signal at 1.60 ppm, due to one of the protons on C-9, was at an unusually high-field position. A long range coupling of 1.3 H_z was observed between the two α-protons of the amino acids and was consistent with a *cis* disposition of the protons with amide to imidate-type tautomerism of the novel diketopiperazine (DKP). A boat conformation of the DKP ring was also indicated.

H-8 and H-9 resonances were compared in other compounds possessing certain structural analogies with the novel DKP namely, verrucofortine, ditryptophenaline, LL-S490β, roquefortine and amauromine (known also as nigrifortine). The structures of these compounds are shown in Fig. 50. Chemical shift values of H-8 and H-9 protons in these compounds and relevant spin-spin coupling constants are presented in Fig. 51. The H-9 resonances were coincident in roquefortine and amauromine (Fig. 51) which was indicative of structural rigidity and therefore these compounds were not good model systems. H-8 and H-9 resonances of ditryptophenaline in $CDCl_3$ were the most closely correlated with those of the novel diketopiperazine and J-coupling values of ditryptophenaline and verrucofortine were similar to those of the novel compound (Fig. 51). Long range coupling between amino acid α-protons was also observed in verrucofortine (Hodge *et al.*, 1988) and ditryptophenaline (Maes *et al.*, 1986) in $CDCl_3$ and the authors attributed this to a boat conformation of the diketopiperazine ring. The long range coupling between H-5 and H-8 in diketopiperazines is indicative of a positive degree of folding of

the DKP ring (Davies and Khaled, 1976). In this conformation the α-hydrogens are pseudo-axial and in ditryptophenaline and the novel diketopiperazine the buckled DKP ring still allows interaction of the phenyl ring with the DKP nucleus but avoids steric interaction of the bulky substituents.

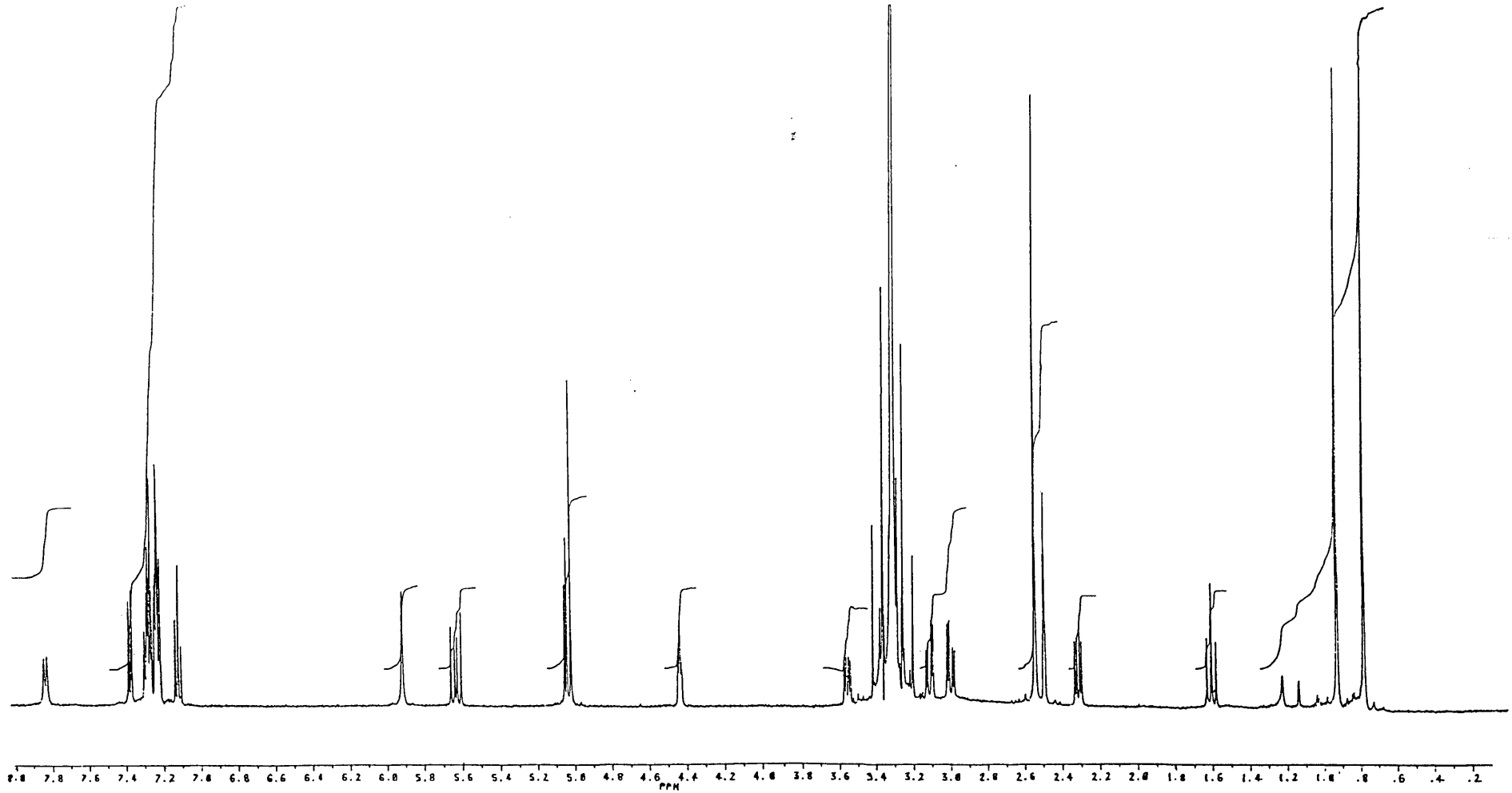
In chloroform, as reported by Maes *et al.* (1986), ditryptophenaline exists in a conformation similar to the solid-state arrangement determined by X-ray crystallography (Springer *et al.*, 1977) which involves a positive degree of folding of the DKP ring with the phenyl group in close proximity.

The similarity of the vicinal couplings, J(α,β), of the phenylalanine residue (*ca.* 4.6 H_z) of the novel DKP is consistent with corresponding couplings observed in ditryptophenaline (*ca.* 4 H_z) which was reported by Maes *et al.* (1986) to indicate that the α- and β-hydogens were *gauche* (Kopple and Ohnishi, 1969) *i.e.* having a dihedral angle of 60°, similar to the solid-state conformation.

Contrary to the results of Maes *et al.* (1986), but consistent with results obtained in the present study, Vicar *et al.* (1973) and Young *et al.* (1976) determined independently that for cyclo-(L-phenylalanine-L-proline) the unfolded rotamer with the aromatic ring extended towards N(1) was preferred in chloroform solution. In polar solvents, however, the folded conformer was shown to predominate and it was suggested (Vicar *et al.*, 1973) that this was due to additional stabilisation resulting from interaction between the aromatic and peptide π systems.

The novel diketopiperazine in D_6-DMSO was therefore concluded to adopt a folded conformation in which the phenylalanine ring could interact with the DKP ring which would also account for the observed upfield shift of the tryptophan β-protons (H-9).

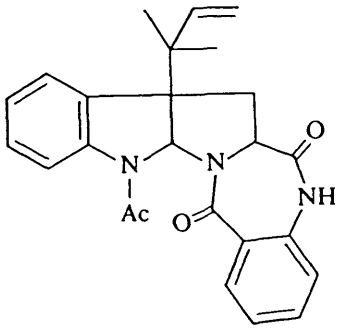
Fig. 49 ^{1}H-NMR spectrum of the novel diketopiperazine in D_6-DMSO.



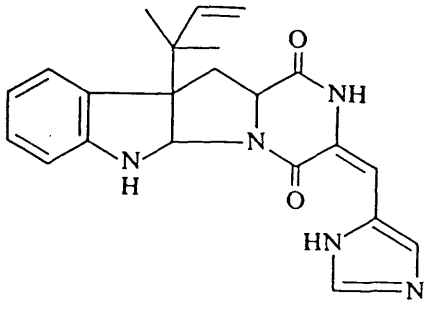
Chemical shift (ppm)

Fig. 50 Structures of compounds having analagous parts to the novel diketopiperazine.

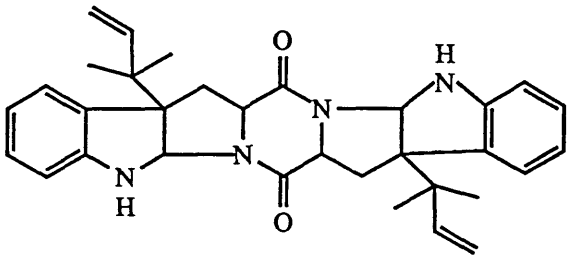
S.20 LL-S490β



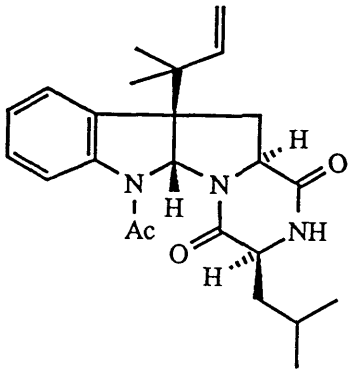
S.24 Roquefortine



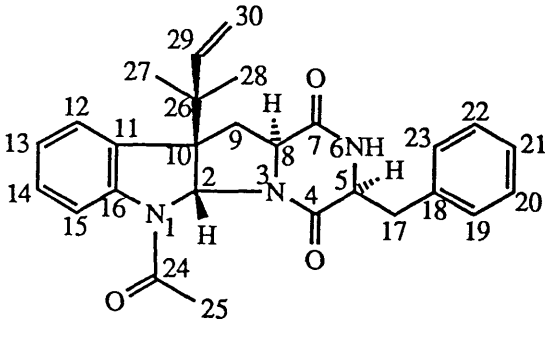
S.36 Amauromine (Nigrifortine)



S.37 Verrucofortine



S.114 Novel diketopiperazine



S.119 Ditryptophenaline

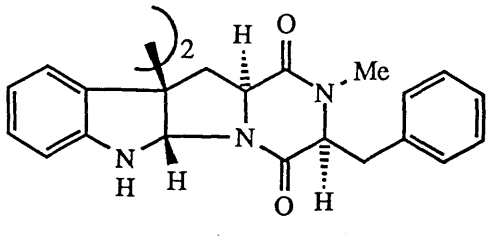
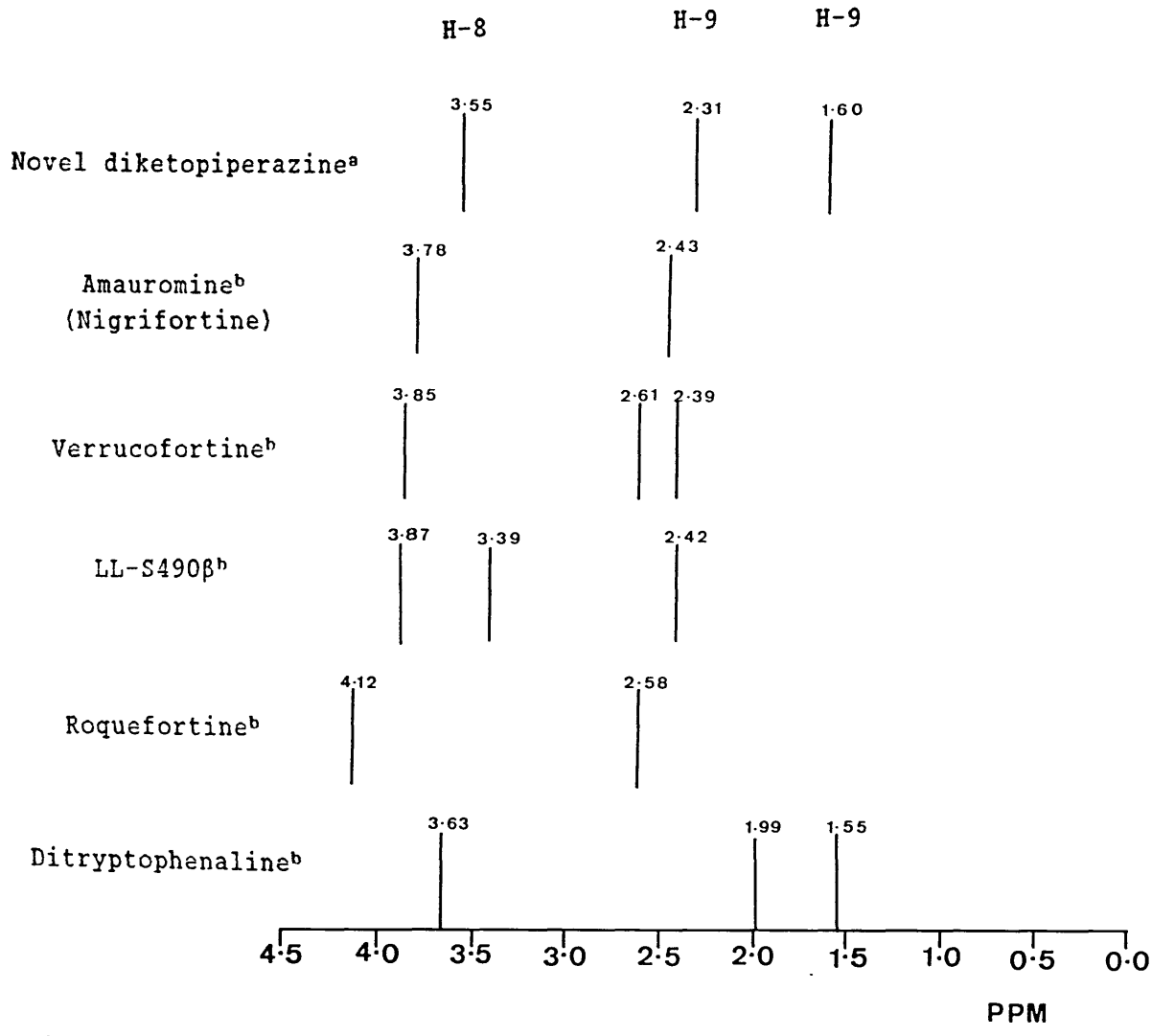


Fig. 51 Comparison of ^{1}H-NMR spectroscopic signals of α- and β-protons of the tryptophan moiety of tryptophan-containing fungal metabolites.



[a] in D_6-DMSO [b] in $CDCl_3$

	Spin-spin coupling constants (H_z)		
	Novel DKP	Verrucofortine	Ditryptophenaline
J H_9 to H_9	12.2	12.5	12.4
J *trans* to H-8	11.8	11.5	12.2
J *cis* to H-8	5.4	5.9	4.9
J H_9 to H_5	1.3	1.0	1.2

3.7.3.1 Structural information obtained by ^1H-^1H COSY spectroscopy.

A two-dimensional proton-NMR COSY spectrum of the diketopiperazine in D_6-DMSO was obtained (Fig. 52). One of the protons on C-9, resonating at 1.60 ppm, showed a coupling to the geminal proton at 2.31 ppm and also to the proton on C-8 at 3.55 ppm. The protons on C-17, resonating at 2.99 ppm and 3.11 ppm, were mutually coupled. The phenylalanine α-proton (on C-5) resonating at 4.43 ppm was coupled to the adjacent β-protons (on C-17). A coupling was observed between the protons resonating at 5.02 ppm (attached to C-30) and that at 5.63 ppm on C-29. In the indolic region of the molecule, H-13 at 7.12 ppm was coupled to H-14 at 7.24 ppm and to H-12 at 7.37 ppm. In addition, H-14 was coupled to H-15 at 7.81 ppm.

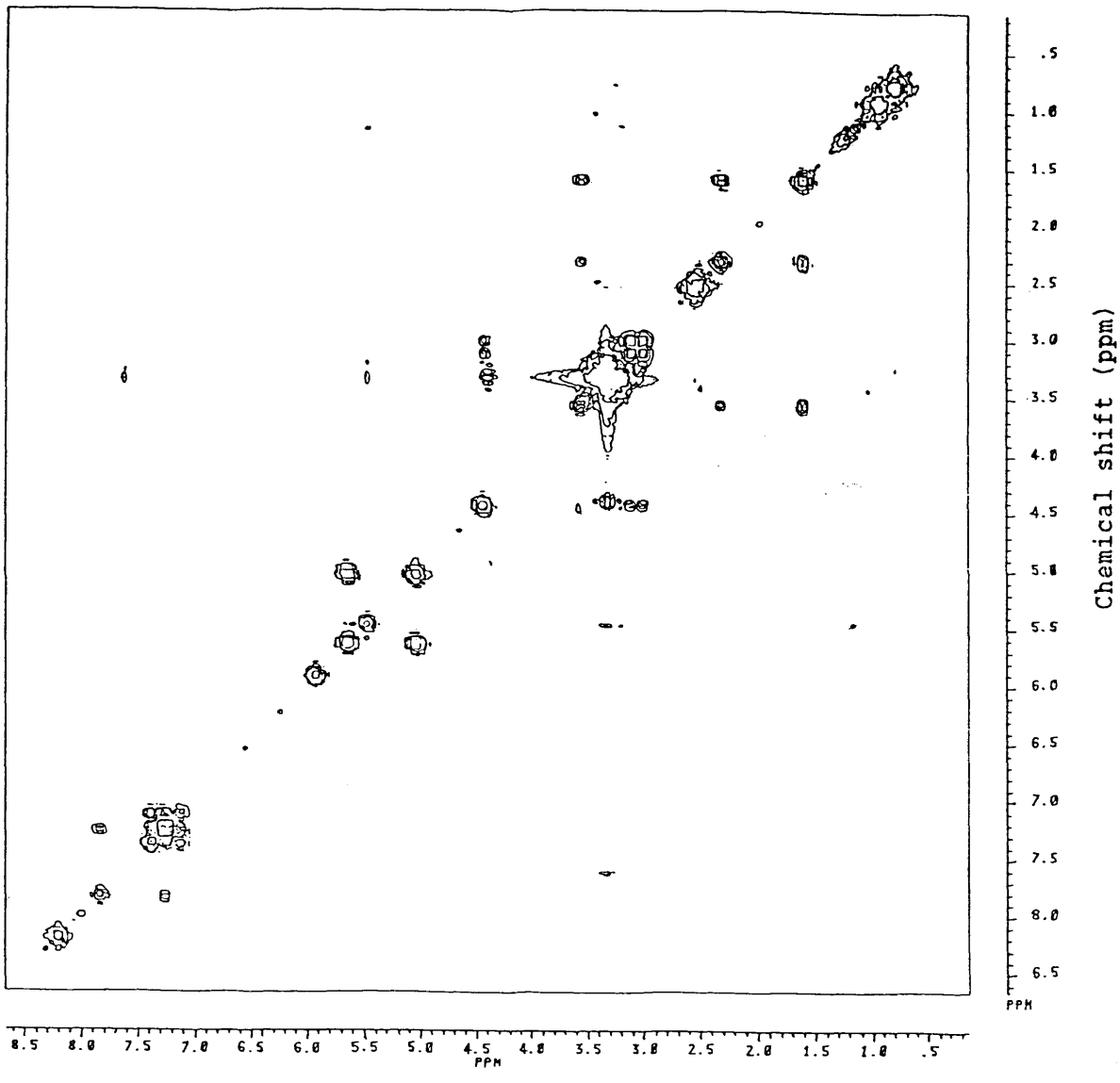
3.7.3.2 Proton assignments of the novel diketopiperazine.

The proton assignments are shown in Table 17, together with relevant J-coupling values. It is noteworthy that the methine proton signal H-2 of the novel diketopiperazine at 5.92 ppm and of verrucofortine at 6.01 ppm (Hodge *et al.*, 1988) occurs at lower field than that in roquefortine (5.70 ppm) and amauromine (5.42 ppm) which are not acetylated at N(1).

3.7.3.3 Nuclear Overhauser Effect (NOE) experiments performed on the novel diketopiperazine.

Table 18 indicates the protons that were irradiated in NOE experiments and the responding protons which were located close in space. The relative stereochemistry between such protons was thereby established. The inverted prenyl group and the proton on C-2 were *cis* to one another. The high field β-proton of tryptophan at 1.60 ppm was *cis* to the prenyl group. The low field (at 2.31 ppm) β-proton on C-9 was *cis* to to the α-proton of tryptophan (on C-8). H-12 was determined to be close in space to the low field β-proton of tryptophan.

Fig. 52 ^{1}H-^{1}H COSY NMR spectrum of the novel diketopiperazine in D_6-DMSO.



Chemical shift (ppm)

Table 17 Assignment of protons of the novel diketopiperazine.

Chemical shift (ppm)	Integration and assignment		Position in structure (Fig. 48)	Coupling constant (Hz)	
0.78 s	3H	CH_3	27, 28		
0.93 s	3H	CH_3			
1.60 t	1H	CH_2	9	J_{gem}	= 12.2
				J *trans* to H-8	= 11.8
2.31 dd	1H	CH_2	9	J_{gem}	= 12.2
				J *cis* to H-8	= 5.4
2.54 s	3H	CH_3	25		
2.99 dd	1H	CH_2	17	J_{gem}	= 14.0
				J to H-5	= 5.0
3.11 dd	1H	CH_2	17	J_{gem}	= 14.0
				J to H-5	= 4.3
3.55 ddd	1H	CH	8	J *trans* to H-9	= 11.8
				J *cis* to H-9	= 5.4
				J to H-5	= 1.3
4.43 bt	1H	CH	5	*J to H-17	= 4.3
				J to H-17	= 5.0
				J to H-8	= 1.3
5.02 m	2H	CH_2	30	J *trans* to H-29	= 17.3
				J *cis* to H-29	= 10.6
5.63 dd	1H	CH	29	$J *trans* to H-30	= 17.3
				J *cis* to H-30	= 10.6
5.92 s	1H	CH	2		
7.12 dt	1H	CH	13	J_{13-12} ortho	= 7.7
				J_{13-14} ortho	= 7.5
				J_{13-15} meta	= 1.1
7.24 dt	1H	CH	14	Coupled to H-13 in COSY	
7.17-7.26	5H	CH	19-23		
7.37 dd	1H	CH	12	J_{12-13} ortho	= 7.7
				J_{12-14} meta	= 0.86
7.81 bd	1H	CH	15	J_{15-14} ortho	= 8.0
8.19 s	1H	NH	6		

* *Cis* and *trans* couplings cannot be differentiated in Phe due to free rotation about the C_α-C_β bond.

$ In decoupling experiments, H-29 (at 5.63 ppm) was irradiated at two different power settings and the signal was transformed to a second-order multiplet centred at 5.02 ppm.

Abbreviations: s, singlet; t, triplet; dd, double doublet; ddd, double double doublet; m, multiplet; dt, double triplet; bd, broad doublet; bt, broad triplet; gem, geminal; Phe, phenylalanine.

Table 18 Nuclear Overhauser Effect (NOE) experiments on the novel diketopiperazine.

Experiment number	Proton(s) irradiated (Fig. 48)	Responding proton(s) (Fig. 48)
1	N(6)-H at 8.19 ppm	a) CH_α Phe (H-5) at 4.43 ppm
2	H-29 at 5.63 ppm	a) H-2 at 5.92 ppm b) *cis* H-30 at 5.02 ppm
3	H-2 at 5.92 ppm	a) H-29 at 5.63 ppm b) C(25)-H_3 at 2.54 ppm
4	H-15 at 7.81 ppm	a) H-14 at 7.24 ppm Confirmed the proton assignments in COSY NMR
5	β-CH_2 of Trp (H-9) at 2.31 ppm	a) β-CH_2 of Trp (H-9) at 1.60 ppm b) α-CH of Trp (H-8) at 3.55 ppm c) H-12 at 7.37 ppm
6	β-CH_2 of Trp (H-9) at 1.60 ppm	a) β-CH_2 of Trp (H-9) at 2.31 ppm b) H-29 at 5.63 ppm (weak response)
7	α-CH of Phe (H-5) at 4.43 ppm	a) β-CH_2 of Phe (H-17) at 2.99 and 3.11 ppm b) Phe ortho aromatic CH's (H-19,23) at 7.17 ppm c) N(6)-H at 8.19 ppm
8	CH_3 of prenyl at 0.78 ppm	a) C(25)-H_3 at 2.54 ppm (weak response) b) *cis* H-30 at 5.02 ppm c) H-29 at 5.63 ppm d) H-2 at 5.92 ppm e) H-12 at 7.37 ppm
9	CH_3 of prenyl at 0.93 ppm	a) β-CH_2 of Trp (H-9) at 1.60 ppm b) *cis* H-30 at 5.02 ppm c) *trans* H-30 at 5.63 ppm d) H-2 at 5.92 ppm

3.7.4 ^{13}C-NMR spectroscopy of the novel diketopiperazine.

^{13}C-spectroscopy of the novel diketopiperazine was performed in D_6-DMSO which resonated at 39.5 ppm. The ^{13}C-spectrum indicated 25 carbon lines (Fig. 53) two of which, at 129.97 and 128.06 ppm, represented coincident transitions of the phenylalanine ortho/meta-carbons. One quaternary carbon signal at 39.94 ppm was obscured by the D_6-DMSO resonances.

The DEPT experiment factorised the carbon spectrum so that CH_3 and CH resonances gave a positive response whereas CH_2 resonances gave a negative response (Fig. 54). Quarternary and carbonyl carbons do not produce a signal in this spectroscopic technique and therefore resonances in Fig. 53 that did not feature in Fig. 54 could be assigned as quaternary and carbonyl carbon atoms.

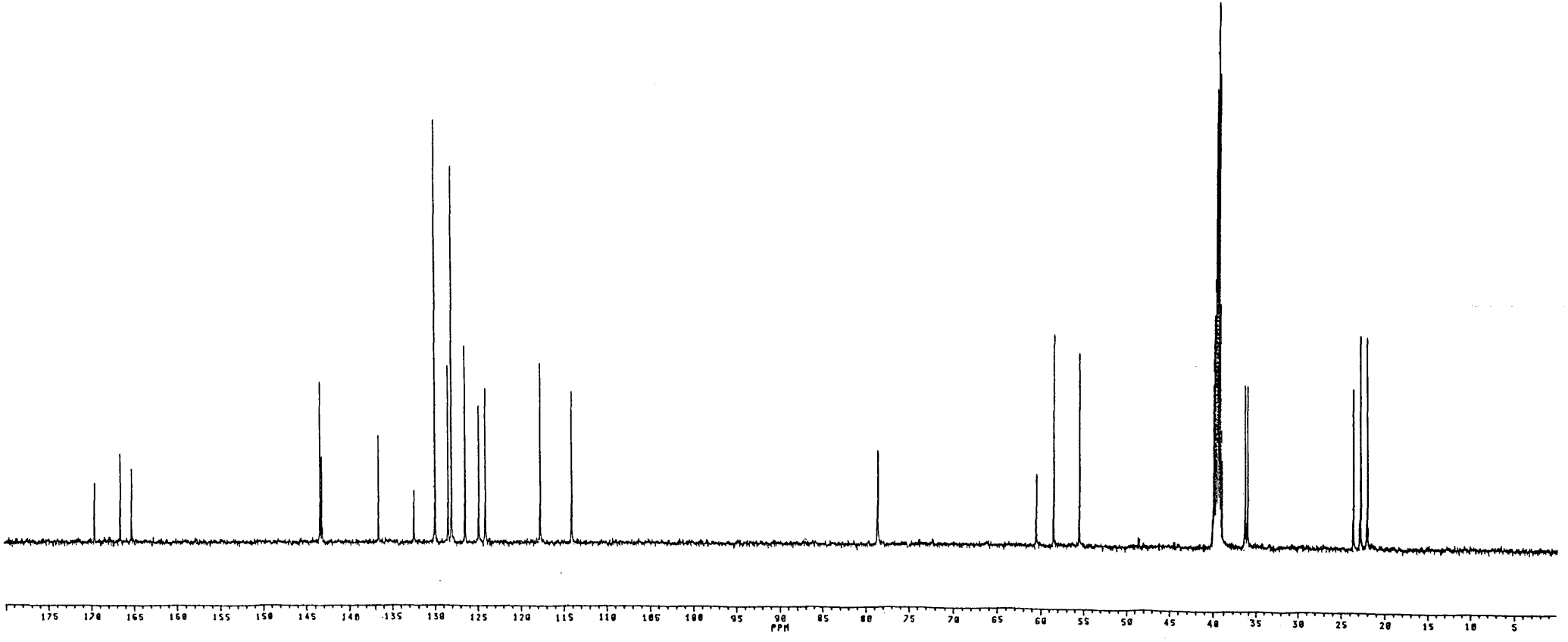
3.7.4.1 ^{1}H-^{13}C-NMR spectroscopy of the novel diketopiperazine.

A ^{1}H-^{13}C-heteronuclear shift correlation experiment was performed to identify protons coupled to specific carbon atoms and the resultant spectrum is shown (Fig. 55).

3.7.4.2 Carbon assignments of the novel diketopiperazine.

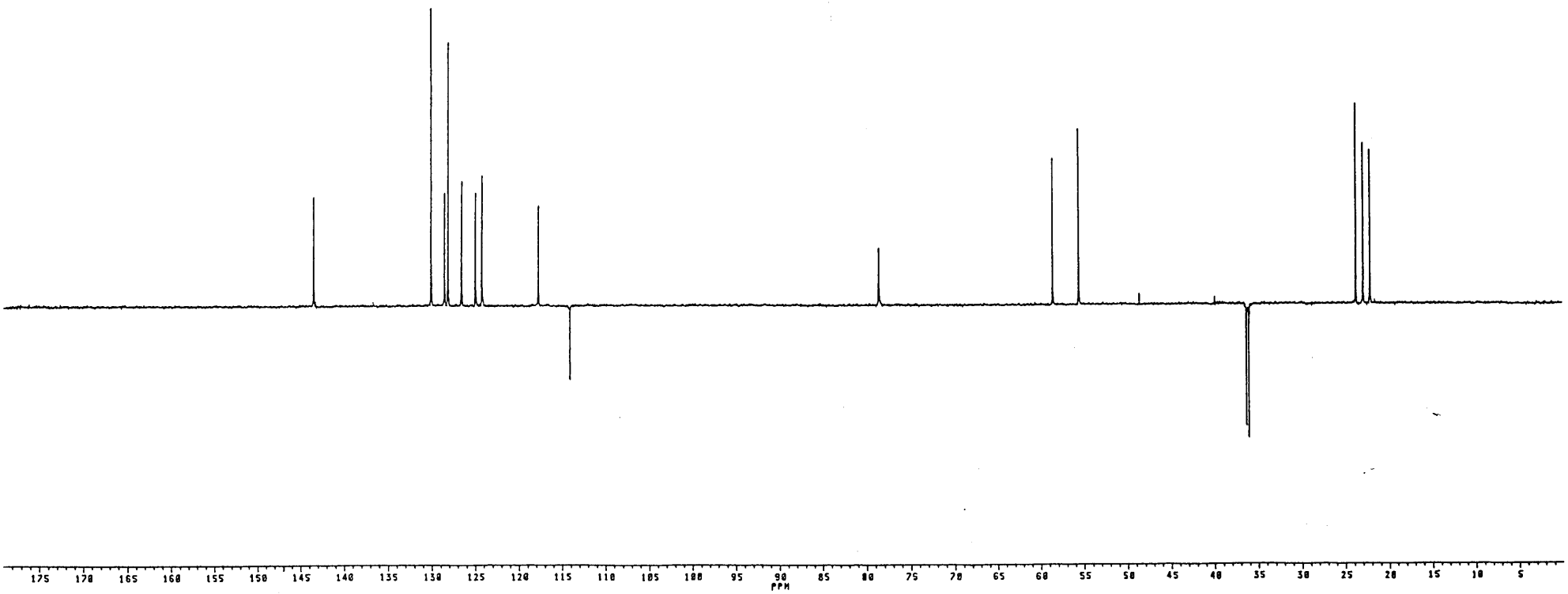
The carbon assignments of the novel diketopiperazine in D_6-DMSO are presented in Table 19. Assignments compared favourably with those obtained for verrucofortine (Hodge *et al.*, 1988), LL-S490β (Kimura *et al.*, 1982) and ditryptophenaline (Maes *et al.*, 1986).

Fig. 53 ^{13}C-NMR spectrum of the novel diketopiperazine in D_6-DMSO.



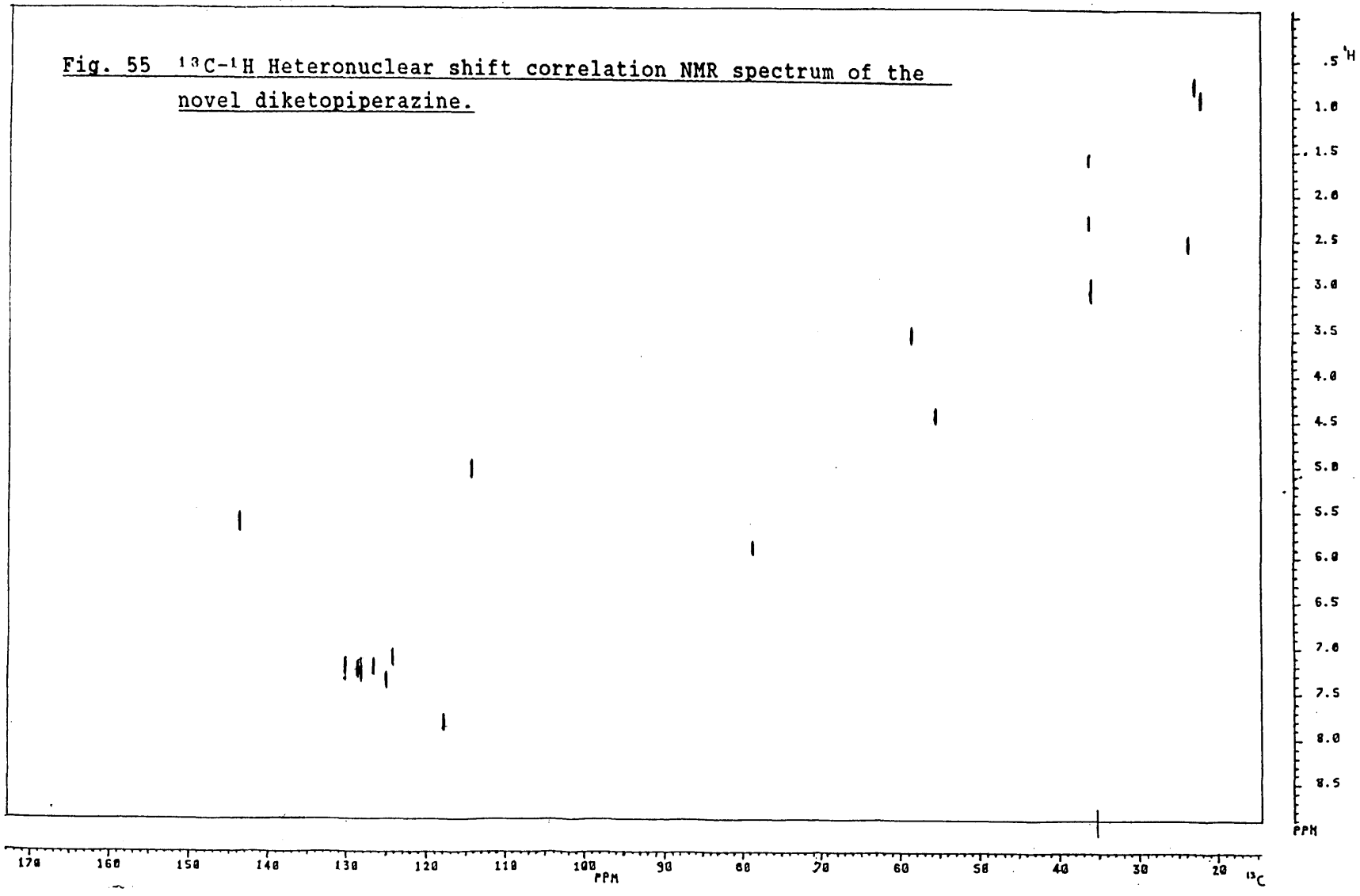
Chemical shift (ppm)

Fig. 54 Factorised ^{13}C-NMR spectrum of the novel diketopiperazine in D_6-DMSO (DEPT experiment).



Chemical shift (ppm)

Fig. 55 ^{13}C-^{1}H Heteronuclear shift correlation NMR spectrum of the novel diketopiperazine.



^{1}H Chemical shift (ppm)

^{13}C Chemical shift (ppm)

Table 19 Carbon assignments of the novel diketopiperazine.

Chemical shift (ppm)	Assignment	Position in proposed structure (Fig. 48)
169.52	C=O [a]	24
166.57	C=O]	4, 7
165.20	C=O]	
143.39	=CH	29
143.20	Q [b]	16
136.58	Q	18
132.47	Q [b]	11
129.97	2 =CH [c]	19, 23
128.44	=CH	14
128.06	2 =CH [c]	20, 22
126.50	=CH [c]	21
124.91	=CH	12
124.13	=CH	13
117.72	=CH	15
114.06	$=CH_2$	30
78.61	CH	2
60.55	Q [d]	10
58.53	CH	8
55.54	CH	5
39.94	Q [e]	26
36.32	CH_2 [f]	9
36.02	CH_2 [f]	17
23.67	CH_3 [g]	25
22.86	CH_3]	27, 28
22.09	CH_3]	

Q = quarternary carbon

[a] Verrucofortine had a value of 170.1 ppm in $CDCl_3$ for the acetyl C=O (C-24) (Hodge *et al.*, 1988). LL-S490β had a value of 171.7 ppm in $CDCl_3$ for C-24 (Kimura *et al.*, 1982). This carbonyl exhibited the greatest downfield shift in both molecules.

[b] In verrucofortine, C-16 and C-11 resonated at 143.3 and 132.1 ppm respectively in $CDCl_3$ (Hodge *et al.*, 1988).

[c] ^{13}C-NMR spectroscopy of a cyclic dipeptide containing L-phenylalanine gave values for Phe ortho-, meta- and para-aromatic protons of 130.42, 128.67 and 127.44 ppm respectively in $CDCl_3$ (Lucente *et al.*, 1981). Values of 131.8, 129.5 and 128.0 ppm were obtained for ortho-, meta-, and para-aromatics respectively of Phe in cyclo-(L-Phe-L-Val) in DMSO (Deslauriers *et al.*, 1975). Corresponding

continued on page 155.

Table 19 continued.

values in ditryptophenaline were 129.19, 129.23 and 127.81 ppm for ortho-, meta- and para-aromatics in $CDCl_3$.

[d] In LL-S490β, C-10 resonated at 60.7 ppm in $CDCl_3$ (Kimura *et al.*, 1982). In verrucofortine, the C-10 signal occurred at 60.8 ppm (Hodge *et al.*, 1988).

[e] This signal was obscured by the DMSO resonances. In LL-S490β the corresponding carbon resonated at 41.0 ppm in $CDCl_3$ (Kimura *et al.*, 1982). In verrucofortine this signal occurred at 40.4 ppm (Hodge *et al.*, 1988).

[f] The assignment of the two β-CH_2 groups of Phe (C-17) and Trp (C-9) was determined by a two-dimensional ^{13}C-^{1}H heteronuclear shift correlation. The protons of C-17 were more equivalent than those of C-9. The Phe β-protons had a characteristic splitting pattern in the proton spectrum.

[g] In NOE experiments when the N-acetyl CH_3 protons (on C-25) were irradiated, an aromatic doublet response was observed which corresponded to H-15. This result, together with evidence from the ^{13}C-^{1}H correlation enabled the C-15 atom to be assigned.

3.8 ISOLATION OF A PUTATIVE OXIDATIVE DEGRADATION PRODUCT OF THE NOVEL DIKETOPIPERAZINE FROM *PENICILLIUM AURANTIOGRISEUM.*

The mycelial extract of *P. aurantiogriseum* contained at least three compounds which co-migrated with the diketopiperazine in thin-layer chromatography. The compounds were resolved by reverse phase HPLC, using solvent conditions B (2.3.3.5). One compound, eluting within 2-3 min (in contrast to the 12 min retention time of the diketopiperazine) was collected and analysed by mass spectrometry.

The mass spectrum (Fig. 56) revealed a molecular ion at m/z 284. Accurate mass measurement of certain fragments was performed (Table 20). There was unfortunately insufficient material for proton-NMR spectroscopic analysis, but mass spectrometric evidence supported a tentative structure (Fig. 17, S.115) for the compound. This molecule might indicate oxidative transformation of the diketopiperazine by *P. aurantiogriseum.*

Other compounds co-migrating with the diketopiperazine in TLC were subjected to preliminary mass spectrometric analysis which indicated fragment ions characteristic of anthranilic acid-containing molecules. Although the presence of additional anthranilate-containig compounds in the mycelium of *P. aurantiogriseum* remains to be substantiated, their occurrence could possibly account for the observed ^{14}C-anthranilate label in this region of the chromatogram (Fig. 16).

Fig. 56 Electron impact mass spectrum of a compound (Fig. 17, S.115), related to the novel diketopiperazine, isolated from a 60 litre fermentation of *P. aurantiogriseum.*

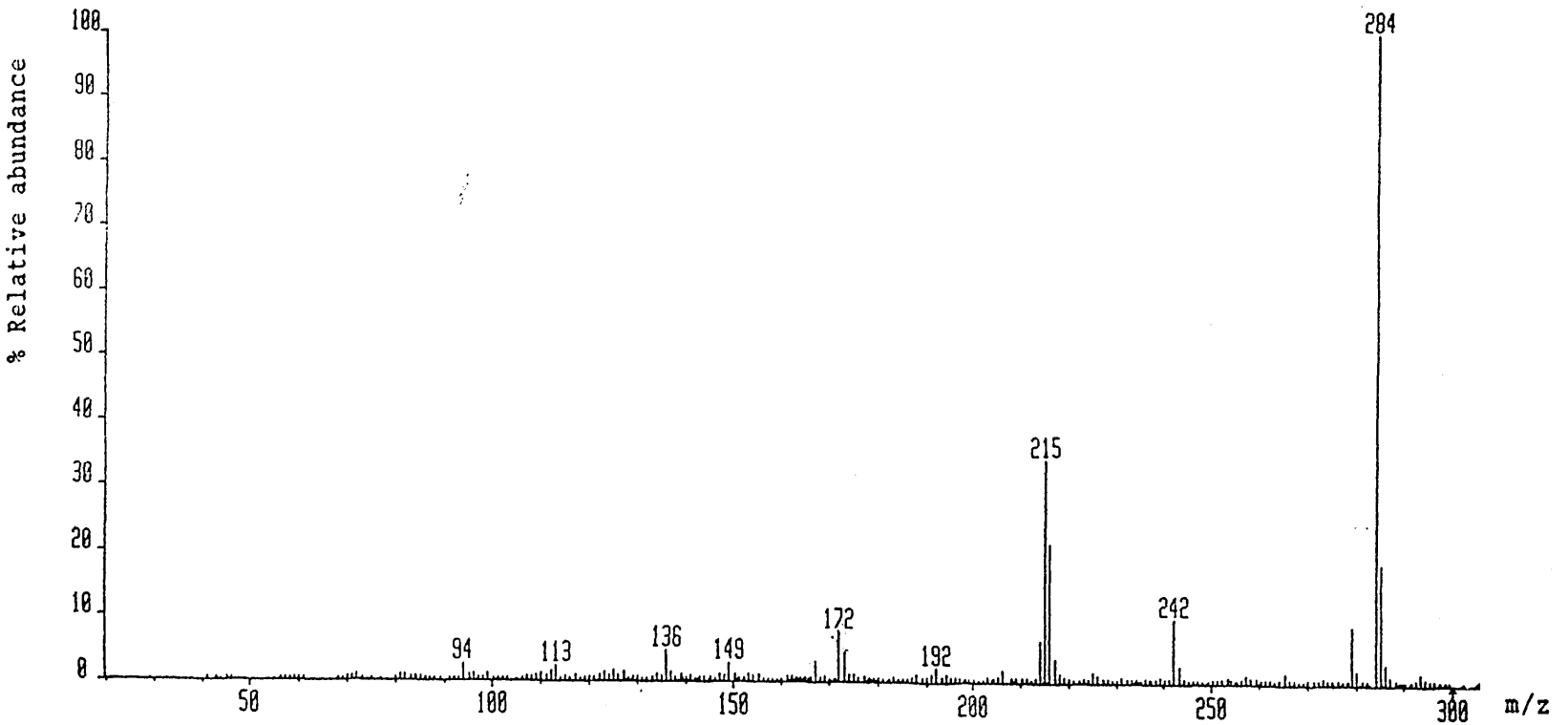


Table 20 Accurate mass measurements of a putative transformation product of the novel diketopiperazine.

Fragment ion (m/z)	Accurate mass (a.m.u.)	Calculated mass (a.m.u.)	Molecular formula
284 (M^+)	284.1533	284.1525	$C_{17}H_{20}N_2O_2$
173	173.0724	173.0715	$C_{10}H_9N_2O$
130	130.0655	130.0657	C_9H_8N

3.9 ASPECTS OF ALKALOID PRODUCTION IN *CLAVICEPS* FUNGI.

3.9.1 Biosynthesis of alkaloids in parasitic tissue of *C. purpurea* strains KL1 and 12-2.

In selecting a suitable isolate of *Claviceps purpurea* for use in the projected study of the enzyme catalysing the first step in the alkaloid biosynthetic pathway, dimethylallyl-tryptophan (DMAT) synthetase, the ergot alkaloid content of strain KL1 was examined, when grown as a parasite and in axenic culture.

Another strain parasitising rye, *C. purpurea* 12-2, was used as a control alongside strain KL1 since this organism had previously been shown to perform only the first step of ergoline biosynthesis, namely conversion of tryptophan to DMAT (Willingale *et al.*, 1983). Moreover, strain 12-2 was able to overcome the biosynthetic block when supplied with exogenous agroclavine, but an additional biosynthetic block further down the pathway was implied since the usual cyclic tripeptide ergot alkaloids were not formed. Alkaloid biosynthesis was instead diverted to lysergic acid amide (LAA). The intention of the present study was to demonstrate, for the first time, similar diversion of exogenously supplied elymoclavine to LAA by *C. purpurea* 12-2.

3.9.1.1 Alkaloid content of sclerotia of *C. purpurea* strains KL1 and 12-2.

Extraction of alkaloid bases from sclerotia of strain KL1 revealed the presence of ergotamine and a trace of ergotaminine. Strain 12-2 contained no indolic alkaloids in the basic fraction in accord with the characteristic lack of ergoline alkaloids in this mutant (Corbett *et al.*, 1974).

Thin-layer chromatography of methanol-soluble amphoteric alkaloids extracted subsequently from sclerotia of KL1 and 12-2 revealed the

presence of tryptophan (R_f 0.1) and DMAT (R_f 0.2) when resolved in chloroform:methanol:ammonia (8:2:1), giving blue and blue-green colours respectively with Ehrlich's reagent.

3.9.1.2 Incubation of parasitic tissue preparations of *C. purpurea* strain 12-2 with precursors of the ergoline alkaloid biosynthetic pathway.

[^{14}C-methylene]-tryptophan, agroclavine and elymoclavine had been administered to separate aliquots of a parasitic tissue preparation of strain 12-2 (see section 2.4.5):

Incubation with [^{14}C-methylene]-tryptophan.

Slightly more than half the added radiolabel was taken up by sclerotial tissue preparations of strain 12-2 (Table 21). 54 day old tissue exhibited a diminished uptake of exogenous tryptophan as compared with 40 day old, which was particularly notable since, in the experiment with older tissue, more than twice the mass of tissue was used (Table 21). At the end of the incubation the radioactivity in the supernatants was consistently attributable to unchanged ^{14}C-tryptophan.

In accord with the evidence of a greater uptake of exogenous radiolabel by 40 day old tissue than by 54 day old sclerotial tissue of strain 12-2, an increased metabolic demand for tryptophan in younger tissue was indicated (Table 21).

54 day old tissue was initially considered to be interesting as it caused a greater diversion of free ^{14}C-tryptophan to methanol-extractable products. However, autoradiography of the TLC-resolved methanol extracts of 12-2 revealed that the ^{14}C-activity was still mainly due to untransformed ^{14}C-tryptophan (Fig. 57). Since a trace of unlabelled DMAT was evident on the TLC plate, DMAT-synthetase had obviously been operational but was not functioning during the incubation.

TLC of the basic fraction (ether extract) confirmed the absence of indole alkaloids in strain 12-2 as expected (Corbett *et al.*, 1974).

Incubation with agroclavine and elymoclavine.

The activity of enzymes in strain 12-2 catalysing steps further along the alkaloid pathway, beyond the biosynthetic block (N-methylase) was examined.

A little under half the added agroclavine remained in the supernatant at the end of the incubation, indicating that a significant amount of the precursor had become cell-associated (Table 22). Elymoclavine was taken up slightly less efficiently than agroclavine by 12-2 sclerotial tissue of a corresponding age (Table 22). TLC of basic compounds extracted with ether from incubated tissues showed no transformation of precursors. Agroclavine (R_f 0.64) and elymoclavine (R_f 0.37) were the sole Ehrlich-positive compounds present. The anticipated demonstration of transformation of elymoclavine to lysergic acid amide (LAA) could not be expected since even the reported conversion of agroclavine to LAA had not occurred during the incubation. It was concluded that the tissues were probably too old, as evidenced in the preceding experiment by the non-functional DMAT-synthetase, and that possibly the production of alkaloid biosynthetic enzymes had been switched off.

Table 21 Administration of [^{14}C-methylene]-tryptophan to parasitic tissue preparations of *C. purpurea* strains 12-2 and KL1.

Age of sclerotia (days)	Weight of sclerotial tissue (g)	A Amount of ^{14}C-Trp fed (µCi)	B Amount of ^{14}C-activity in supernatant at end of incubation (µCi)	(A - B) Calculated amount of ^{14}C-activity in cells (µCi) *(% of added)	Amount of ^{14}C-activity in MeOH extract (µCi) *(% of cell-associated label)	Amount of ^{14}C-activity in ether extract (µCi)
C. purpurea 12-2						
40	1.25	1	0.36	0.64 *(64%)	0.06 *(10%)	0.02[a]
54	2.84	5	2.30	2.70 *(54%)	0.59 *(22%)	N.D.[b]
C. purpurea KL1						
64	1.40	1	0.44	0.56 *(56%)	0.07 *(12%)	negligible[c]

[a] TLC of ether extract revealed that the ^{14}C-activity was due to ^{14}C-tryptophan, presumably carried across in the extraction.
[b] Scintillation counting was not performed. TLC and autoradiography revealed no basic alkaloids.
[c] Less than 0.1 nCi was detected by scintillation counting.

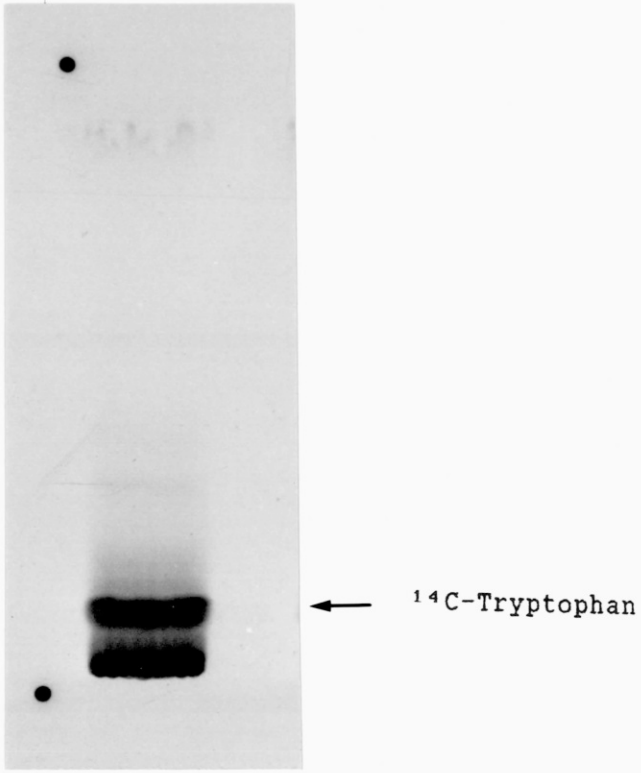


Fig. 57 Autoradiograph of TLC-resolved methanol-extract of 54 day old sclerotial tissue of *C. purpurea* strain 12-2 which had been incubated with [^{14}C-methylene]-tryptophan.

Autoradiograph exposure time, 3 months.

Table 22 Administration of agroclavine and elymoclavine to parasitic tissue preparations of *C. purpurea* 12-2.

Age of sclerotia (days)	Weight of sclerotial tissue (g)	A Amount of precursor fed (mg)	B Amount of precursor in supernatant at end of incubation (mg)	(A - B) Calculated amount of precursor in cells (mg) #(% of added)
		AGROCLAVINE		
40	1.73	6.5	3.0	3.5 #(54%)
54	3.41	6.1	2.9	3.2 #(52%)
		ELYMOCLAVINE		
54	3.15	6.0	3.3	2.7 #(45%)

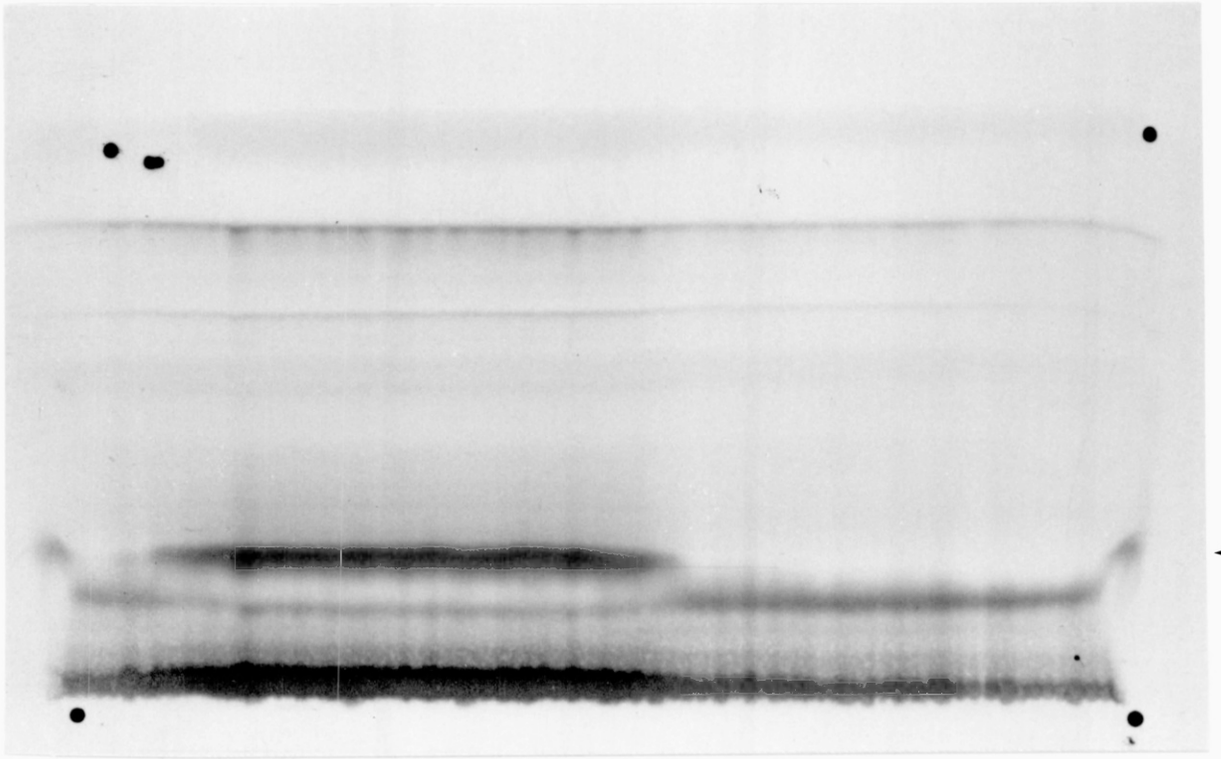
3.9.1.3 Incubation of a parasitic tissue preparation of *C. purpurea* strain KL1 with [^{14}C-methylene]-tryptophan.

The tissue preparation (see section 2.4.5) of 64 day old sclerotia of *C. purpurea* strain KL1 assimilated slightly over half the added ^{14}C-tryptophan (Table 21). Methanol-extractable compounds accounted for 12% of the cell-associated radiolabel (Table 21).

The autoradiograph of the TLC-resolved methanol extract of strain KL1 at first sight appeared to indicate the presence of two radiolabelled compounds which had migrated from the origin (Fig. 58). However, this autoradiograph required careful interpretation. Authentic unlabelled tryptophan migrated to the positions indicated (Fig. 58). Some ^{14}C-tryptophan was retarded in part of the chromatogram consequent on resolving a mixture of components. The apparent additional prominently radiolabelled band on the left hand side of the plate, where the sample was more heavily loaded, also was assumed to be tryptophan. Splitting of the tryptophan component here must have been the consequence of an overloaded origin, where the interparticulate capacity of the silica had become saturated. Some indication of the potential consequence of overloading is evident at the origin, where a radiolabelled component with a high affinity for the silica had been sufficiently in excess of capacity that it had moved to bind to the upper edge of the region.

Although a trace amount of non-radiolabelled DMAT was present in the methanol extract, the DMAT synthetase was unfortunately not functioning during the incubation. Incorporation of ^{14}C-tryptophan into ergotamine also was not evident (Table 21).

Fig. 58 Autoradiograph of TLC-resolved methanol-extract of 64 day old sclerotial tissue of *C. purpurea* strain KL1 which had been incubated with [^{14}C-methylene]-tryptophan.



Position of non-radiolabelled tryptophan →

Autoradiograph exposure time, 3 months.

3.9.2 Alkaloid production in axenic culture of *Claviceps purpurea* strain KL1 .

Growth and alkaloid production of *C. purpurea* KL1 in surface and submerged culture in SCSL and Medium T liquid media were compared to select for optimum DMAT-synthetase activity. Growth of *C. purpurea* KL1 as a surface culture on SCSL medium resulted in a thick, deeply convoluted mycelial mat with extensive dark purple areas, bordered by cream coloured tissue in contrast to the thin, grey-purple mycelium arising from cultivation of the fungus on medium T. However, there was no appreciable difference in alkaloid titre when the organism was cultivated as a surface culture on Medium T and SCSL medium (Table 23). Submerged cultures of strain KL1 in SCSL did not produce alkaloids.

The TLC-resolved extract of SCSL culture filtrate contained no fluorescent compounds, but a densely quenching band (R_f 0.4), apparent against background fluoresence when the chromatogram was examined under UV light (254 nm), became blue after spraying the chromatogram with Ehrlich's reagent. The metabolite was tentatively identified as chanoclavine by co-chromatography with an authentic sample, and this was confirmed by low electon-voltage EI mass-spectrometry (Fig. 59), which revealed a base-peak at 256 mass/charge corresponding to the molecular ion of chanoclavine. Chanoclavine was, therefore, the sole Van Urk-positive alkaloid contributing to the titre of culture filtrate.

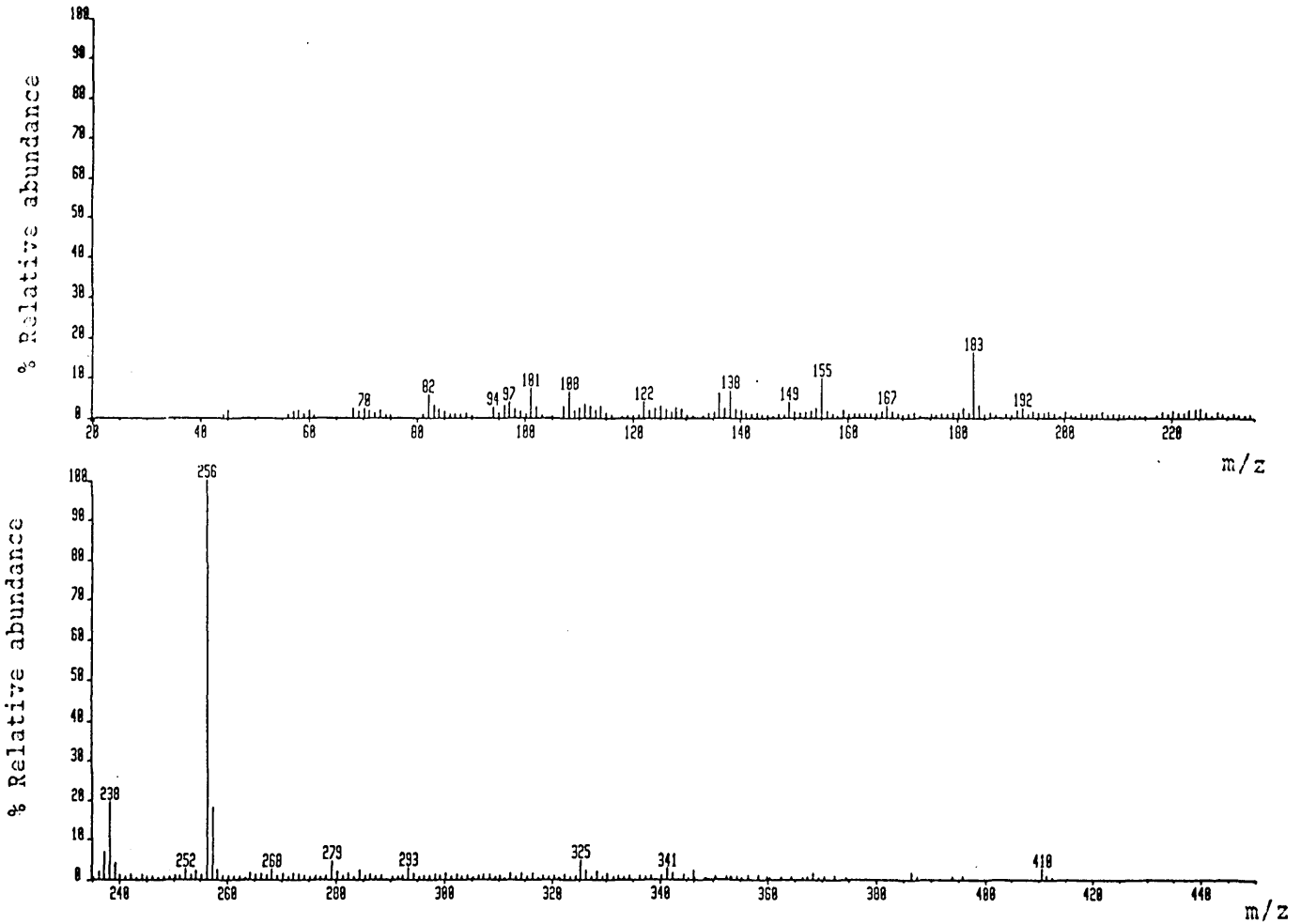
Cultivation of strain KL1 as a surface culture on SCSL medium was therefore considered as a suitable source of DMAT-synthetase to achieve the ultimate aim of this part of the research, namely to isolate the enzyme catalysing the first step of the alklaloid biosynthetic pathway in *Claviceps purpurea*. Isolation of DMAT-synthetase from an ergopeptide-producing strain of *C. purpurea* has not been reported yet. Although saprophytic cultures of *C. purpurea*

strain KL1 did not progress alkaloid biosynthesis beyond chanoclavine, this did not detract from their suitability for isolation of DMAT-synthetase. However, it was considered prudent initially to study the classical purification of DMAT-synthetase from a strain of *C. fusiformis* as achieved by Lee *et al.* (1976).

Table 23 Alkaloid production in surface cultures of *Claviceps purpurea* strain KL 1.

Medium	Volume of medium (ml)	Age of culture at harvest (days)	pH of spent broth at harvest	Alkaloid content of culture filtrate (mg)
Med. T	100	24	6.1	29
SCSL	200	20	4.8	65

Fig. 59 Low elctron voltage electron impact mass spectrum of chanoclavine, isolated from culture filtrate of *C. purpurea* strain KL1.



3.9.3 Alkaloid production in *C. fusiformis* as an indicator of DMAT-synthetase activity.

In the course of establishing the point at which alkaloid production commenced in *C. fusiformis*, as an indicator of DMAT-synthetase activity, a subsidiary experiment was performed to show the effect on final alkaloid titre of ethionine addition to actively producing cultures. Ethionine has been shown to inhibit the biosynthesis of clavine alkaloids by interfering with the N-methylation of DMAT (Agurell and Lindgren, 1968). Ethionine has been used in this way to isolate biosynthetic intermediates in several fungi and was crucial in the isolation of the DMAT metabolite, clavicipitic acid (Robbers and Floss, 1968). Constraint on end-product formation in the clavine alkaloid pathway might be expected to avoid end-product regulation of the first enzyme, namely DMAT-synthetase.

Alkaloid production increased rapidly between the first and third day of fermentation (Fig. 60) implying an optimal amount of DMAT-synthetase during this period. Ethionine treatment at approximately the mid-point in alkaloid production inhibited the titre to two-thirds of that of control cultures at the time of harvest (Fig. 60).

3.9.3.1 Fate of [^{14}C-methyl]-methionine in ethionine-inhibited and control cultures of *C. fusiformis*.

The aim of this experiment was to establish whether or not a preferential diversion of the ^{14}C-methionine pool to clavine alkaloid biosynthesis was occurring under the inhibitive influence of ethionine. Since ethionine was expected to have a deleterious effect on other methylation processes within the cells, this might have been manifested as an accumulating pool of free methionine which would be available for preferential incorporation into alkaloid biosynthesis if its methylation step was less sensitive to ethionine.

[^{14}C-methyl]-methionine was added to both control and treated cultures on day 5, 24 hours after ethionine had been administered. On day 8, ^{14}C-activity measured directly in culture filtrates was the same in both treated and control flasks (Table 24, column C). However, the sum of residual and extracted ^{14}C-activities (Table 24, column A+B) was less in the ethionine-treated flask. These results were difficult to reconcile but values in column C (Table 24) may be unreliable as a high multiplication factor was used in their determination.

Autoradiography of TLC-resolved bases indicated that the major radiolabelled compound was ^{14}C-agroclavine (Fig. 61). Some other more weakly radiolabelled bands were evident which revealed slight differences in the pattern for the ethionine-inhibited and the control culture and which presumably are closely-related alkaloids *e.g.* penniclavine.

Basic compounds extracted from the culture filtrate accounted for 23% and 6% of the added radiolabel, respectively, in control and ethionine-treated cultures (Table 24) and this was regarded as a significant difference. The specific activity of the extracted bases (mainly agroclavine) from control and ethionine-treated flasks at the end of the fermentation was, respectively, 0.018 and 0.008 $\mu Ci\ mg^{-1}$. A possibly more directly representative calculation, using the amount of agroclavine biosynthesised since the time of ethionine addition (day 4), gave specific activities of 0.035 and 0.040 $\mu Ci\ mg^{-1}$ for the control and ethionine-inhibited cultures, respectively. It was concluded that there was no preferential diversion of ^{14}C-methionine into or away from alkaloid biosynthesis under the influence of ethionine.

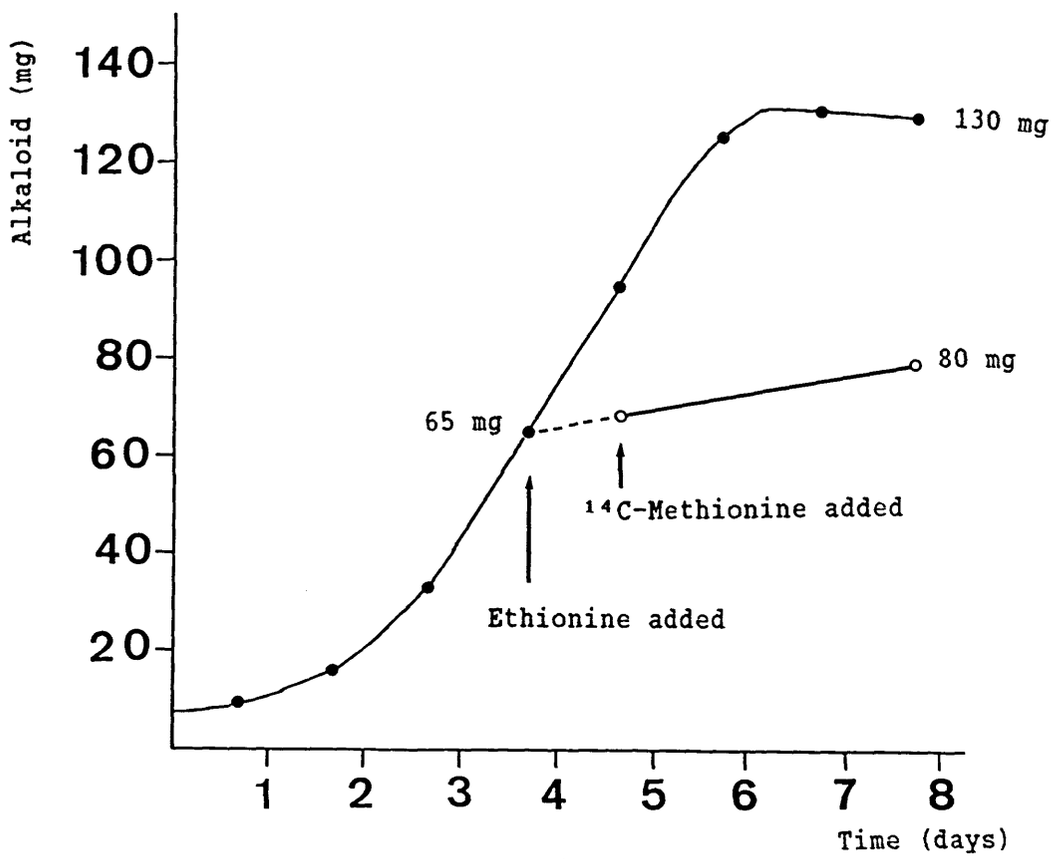


Fig. 60 Alkaloid production in ethionine-inhibited and control cultures of *C. fusiformis.*

●—● , Control; o—o , Ethionine-treated

Table 24 Addition and fate of [^{14}C-methyl]-methionine in ethionine-treated and control cultures of *Claviceps fusiformis* CAS, assessed at day 8.

Amount of ^{14}-methionine added, 10 µCi.

A ^{14}C-activity in $CHCl_3$ extract of filtrate (µCi) *(% of added)	B ^{14}C-activity remaining in base-extracted culture filtrate (µCi)	A + B Calculated ^{14}C-activity in whole culture filtrate (µCi)	C Measured ^{14}C-activity in whole culture filtrate (µCi)	D Amount of cell-associated ^{14}C-activity (µCi) Values obtained as 10-[A+B] and 10-[C]. See text.
CONTROL				
2.3 *(23%)	1.0	3.3	4.8	6.7 or 5.2
ETHIONINE-TREATED				
0.6 *(6%)	1.8	2.4	4.8	7.6 or 5.2

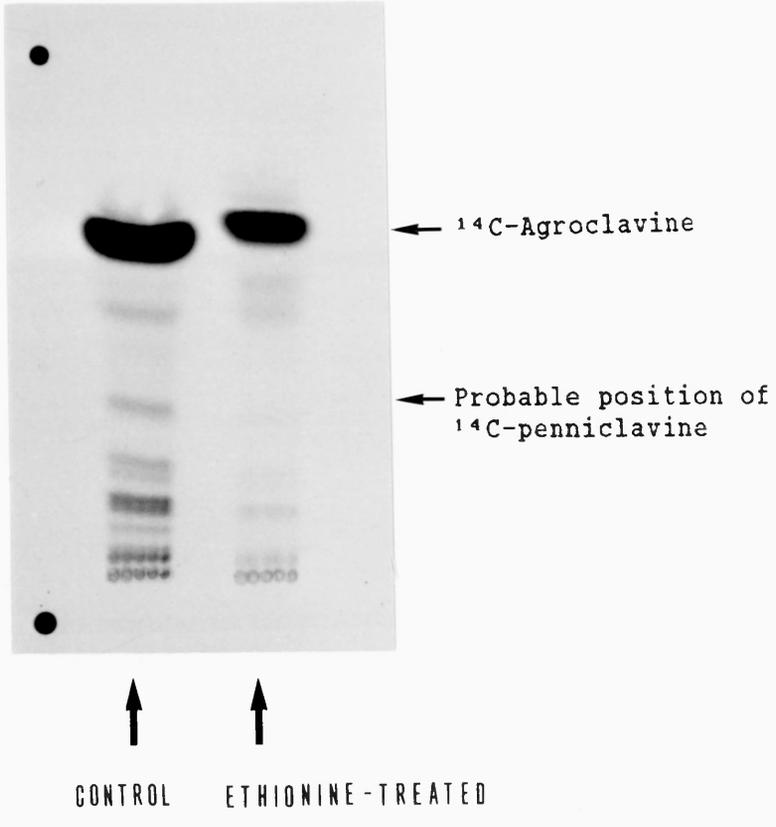


Fig. 61 Autoradiograph of TLC-resolved bases from [^{14}C-methyl]-methionine fed cultures of *C. fusiformis*, with and without ethionine addition.

Autoradiograph exposure time, 2 months.

3.9.3.2 Time-course of incorporation of [^{14}C-methyl]-methionine into agroclavine and amphoteric intermediates of *C. fusiformis.*

The aim of this experiment was to compare incorporation of [^{14}C-methyl]-methionine into agroclavine and N-methyl-DMAT in ethionine-inhibited and uninhibited cultures. In cultures of *C. fusiformis* similar to those of the preceding experiment, ^{14}C-methionine was added to control and ethionine-treated cultures one day after ethionine addition. Aliquots from both series of flasks were removed at intervals to demonstrate the distribution of the radiolabel in the basic and amphoteric fractions of culture filtrate. Uptake of ^{14}C-methionine was apparent within the first 10 minutes after addition, particularly in the ethionine-treated organism (Figs. 62A,B). At the end of the experiment (73 hours after ^{14}C-methionine addition) there was a considerable difference in the amount of ^{14}C-activity in the culture supernatants; the ethionine-treated flask had less than half the value of that in the control (Fig. 62A). This result corroborated the calculated values in the preceding experiment (Table 24, column A+B).

Basic compounds extracted with chloroform from culture supernatants were resolved by TLC (Fig. 63) and autoradiography revealed ^{14}C-agroclavine to be the principal radiolabelled component in both control and ethionine-treated cultures (Fig. 64). Other radiolabelled compounds, probably close structural analogues of agroclavine such as penniclavine, were more apparent in the extract of the control culture supernatant than in that of the supernatant of ethionine-treated culture. The chromatogram (Fig. 63) showed agroclavine to be present in all ethionine-treated samples, confirming that alkaloid biosynthesis had been well under way prior to ethionine addition.

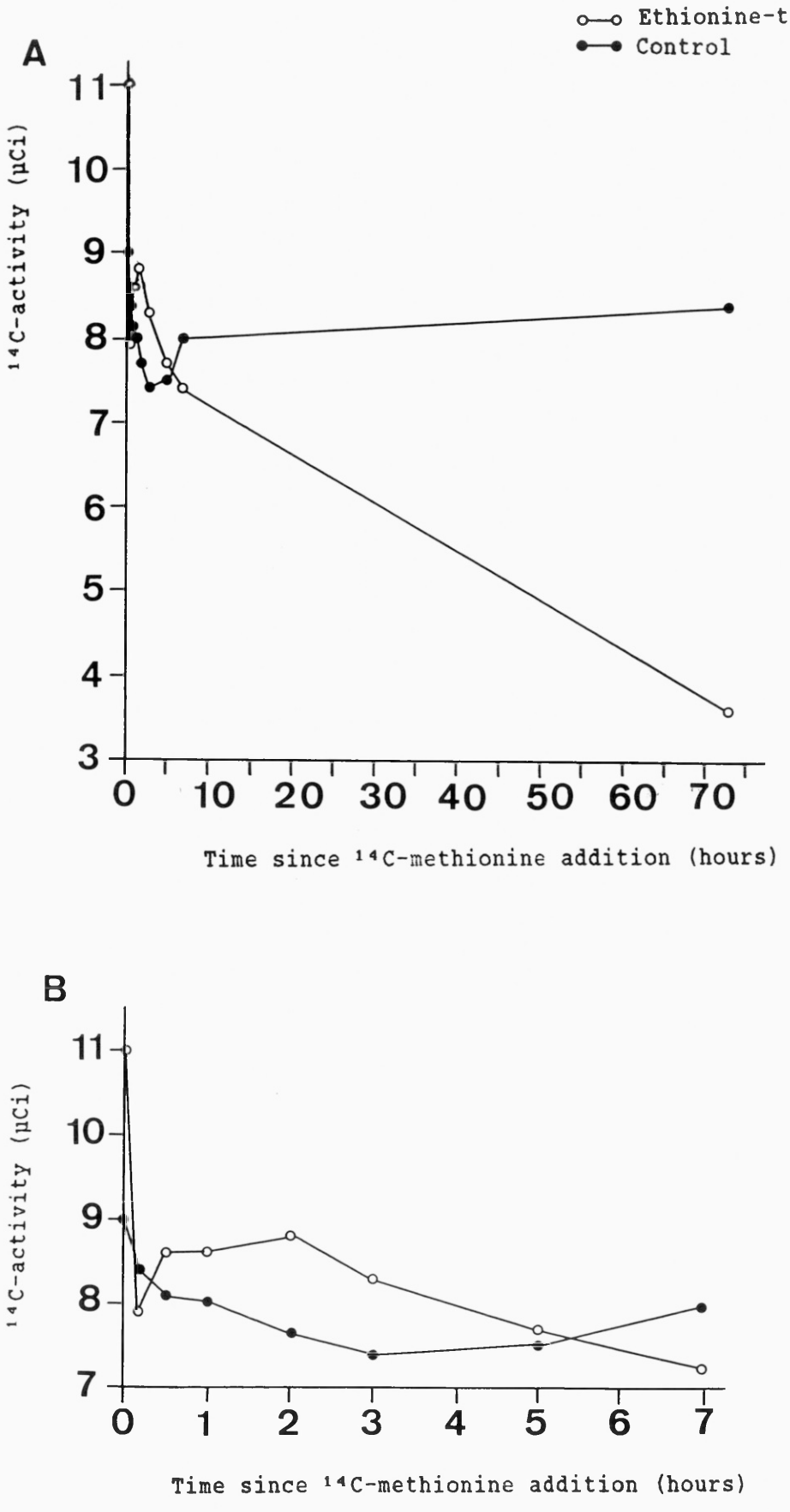


Fig. 62 ^{14}C-activity in culture filtrate after [^{14}C-methyl]-methionine addition to ethionine-treated and control cultures of *C. fusiformis*. Graph B is a scale expansion of the first 7 hours.

Fig. 63 TLC-resolved basic compounds extracted from culture supernatants of ethionine-treated and control cultures of *C. fusiformis*.

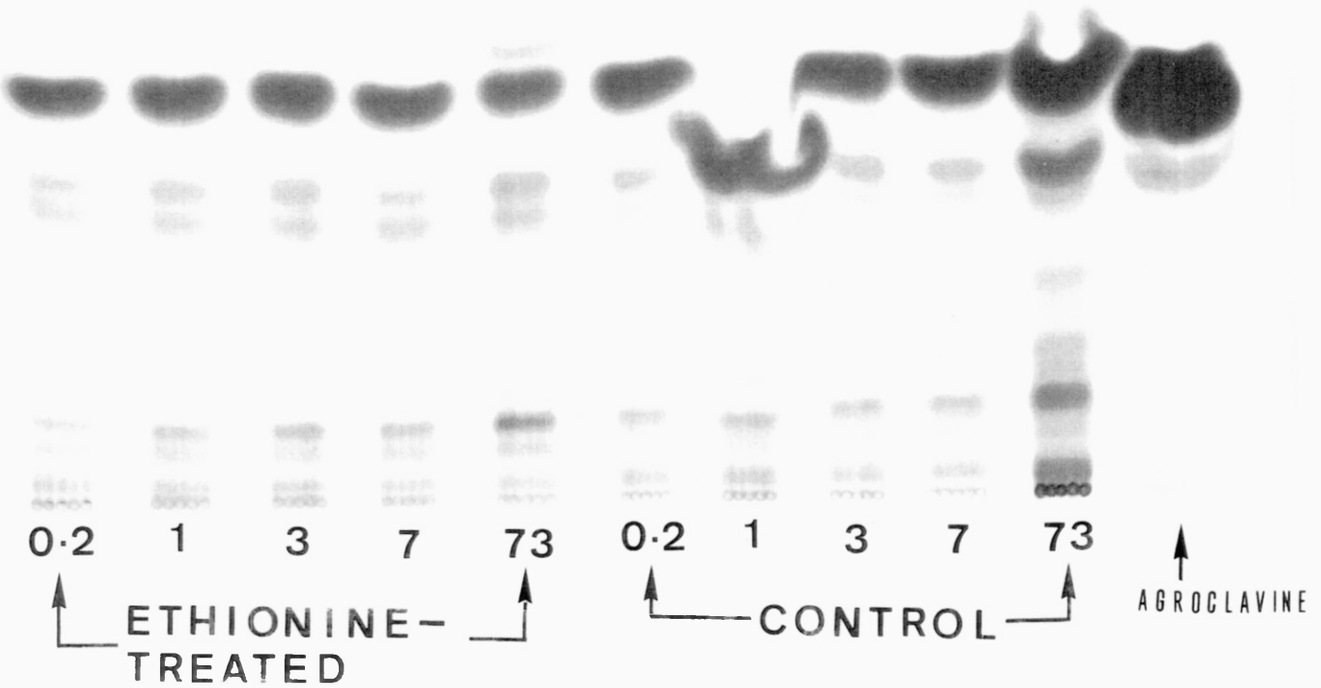


Fig. 64 Autoradiograph of TLC-resolved basic compounds extracted from culture supernatants of [^{14}C-methyl]-methionine-fed control and ethionine-treated cultures of *C. fusiformis*.



^{14}C-activity in the chloroform extracts of culture supernatants increased over the first 7 hours in the control culture to reach a 30% incorporation (Fig. 65). In contrast, there was so little incorporation of ^{14}C-methionine into extracted bases from culture supernatants of ethionine-treated cultures that 7 hours after ^{14}C-methionine addition the incorporation was only about 1% (Fig. 65). Appreciable incorporation of ^{14}C-methionine into agroclavine occurred within only 10 minutes in the control culture; but this value was barely achieved in the ethionine-treated series within 7 hours (Fig. 65).

Fig. 66 shows the relative distribution of ^{14}C-radiolabel in control and ethionine-treated cultures. Amphoteric fractions of the ethionine-treated series analysed by HPLC for ^{14}C-N-methyl-DMAT were found to consist solely of ^{14}C-methionine. It is possible that if sampling had been carried out during the 10-70 hour period, evidence for incorporation of [^{14}C-methyl]-methionine into N-methyl-DMAT would have been obtained.

Without knowledge of the fine details of the effect, on alkaloid biosynthesis, of ethionine addition to cultures of *C. fusiformis* it was considered best not to use an ethionine treatment for cultures from which DMAT-synthetase was to be isolated. Ethionine addition was initially thought to be a good idea as the DMAT-synthetase from *C. fusiformis* SD 58 has been shown to be inhibited by the end-products agroclavine and elymoclavine (Heinstein and Floss, 1976; Lee, 1974).

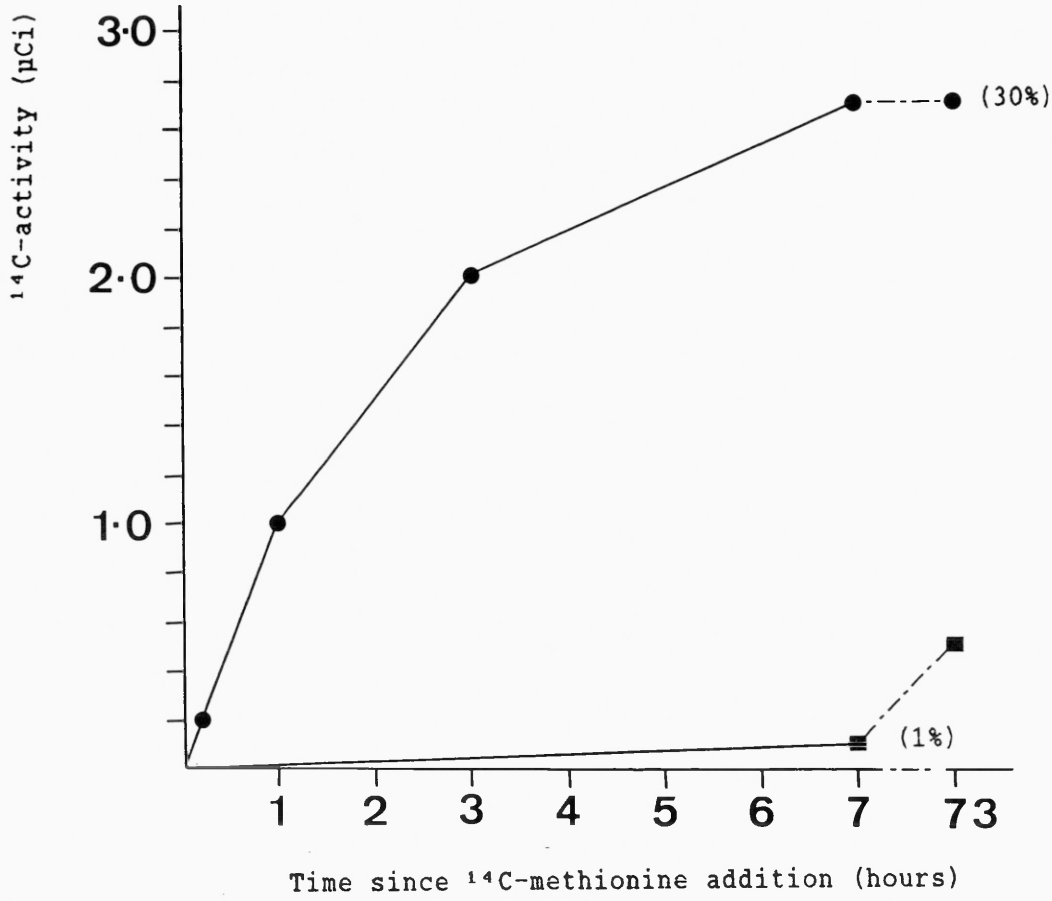
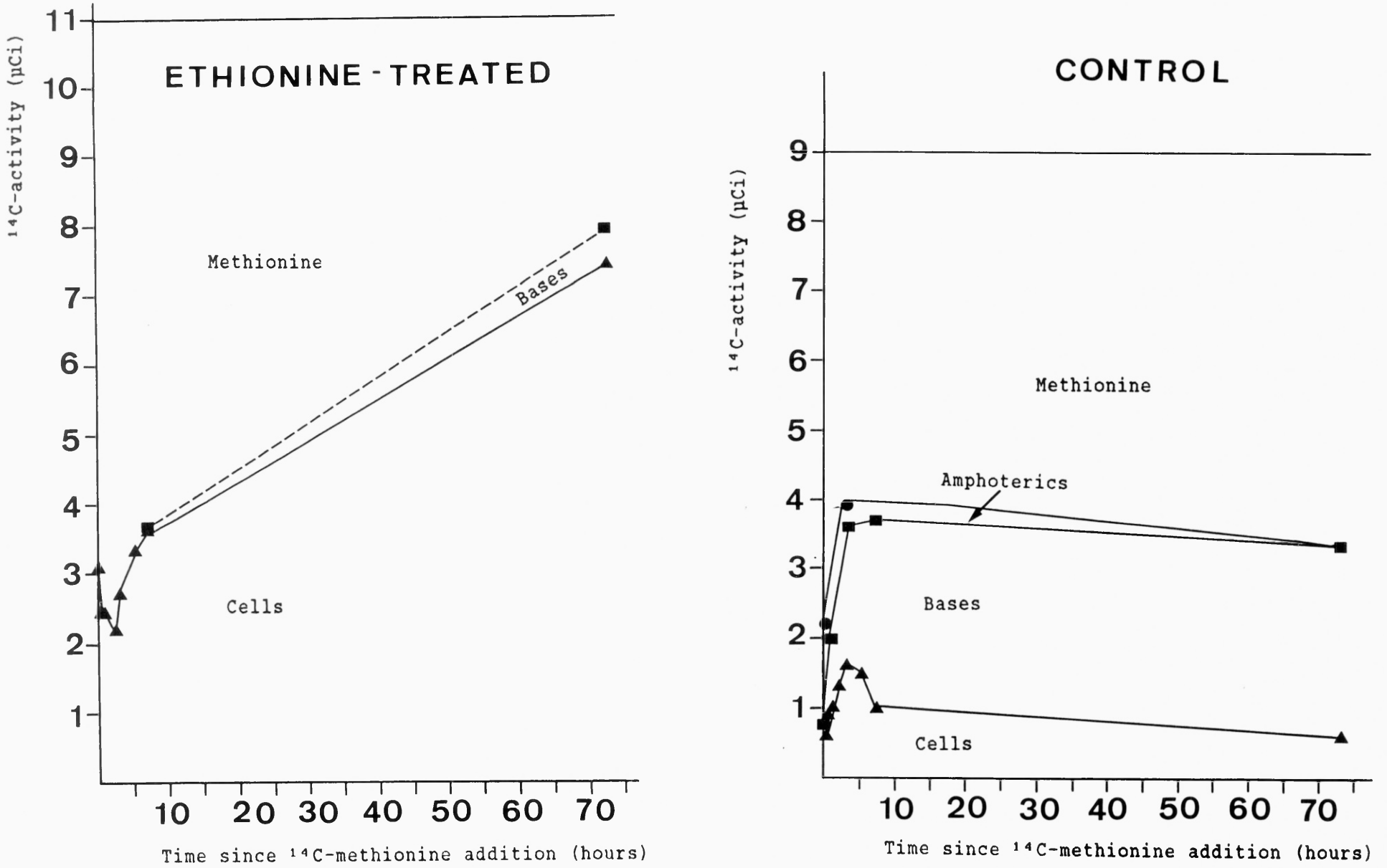


Fig. 65 ^{14}C-activity in chloroform-extracted culture filtrate of ethionine-treated (■——■) and control (●——●) cultures of *C. fusiformis.*

Fig. 66 Distribution of [^{14}C-methyl]-methionine-labelled compounds in control and ethionine-treated cultures of *C. fusiformis*.



3.9.4 Optimisation of cell-free enzyme preparations of *C. fusiformis.*

A cell-free system of *C. fusiformis* demonstrating DMAT-synthetase activity was developed. Various methods of cell disruption were explored and a suitable incubation time was determined. Autoradiography of TLC-resolved cationic species in incubation mixtures provided a qualitative assay for DMAT-synthetase activity. HPLC of the cationic compounds resolved one of the substrates of the reaction, [^{14}C-methylene]-tryptophan from the radiolabelled product DMAT (Fig. 67).

Conversion of [^{14}C-methylene]-tryptophan and dimethylallyl-pyrophosphate to radiolabelled DMAT was achieved with the supernatant fraction of an X-pressed preparation of young (2-3 days) *C. fusiformis* cells. Partial resolution of this fraction by ammonium sulphate precipitation was achieved and the fraction precipitating at 30-45% saturation showed DMAT-synthetase activity (Fig. 68) estimated as about half that of the crude preparation (Table 25). Preliminary analysis of the ammonium sulphate precipitated fraction by gel-electrophoresis showed it to consist of 12 protein bands.

The predicted superiority with respect to DMAT-synthetase activity of 2-3 day old cultures from previous evidence (Fig. 60) was corroborated in these experiments. Cell-free preparations of 7 day old cultures resulted in a 10-fold diminution in the conversion of ^{14}C-tryptophan to DMAT as compared with that from 2-3 day old cells. The X-press method for cell disruption was preferred and an incubation time of 3 hours was found to be long enough for significant transformation.

Unfortunately time did not allow an attempt to isolate DMAT-synthetase from *C. purpurea* KL1.

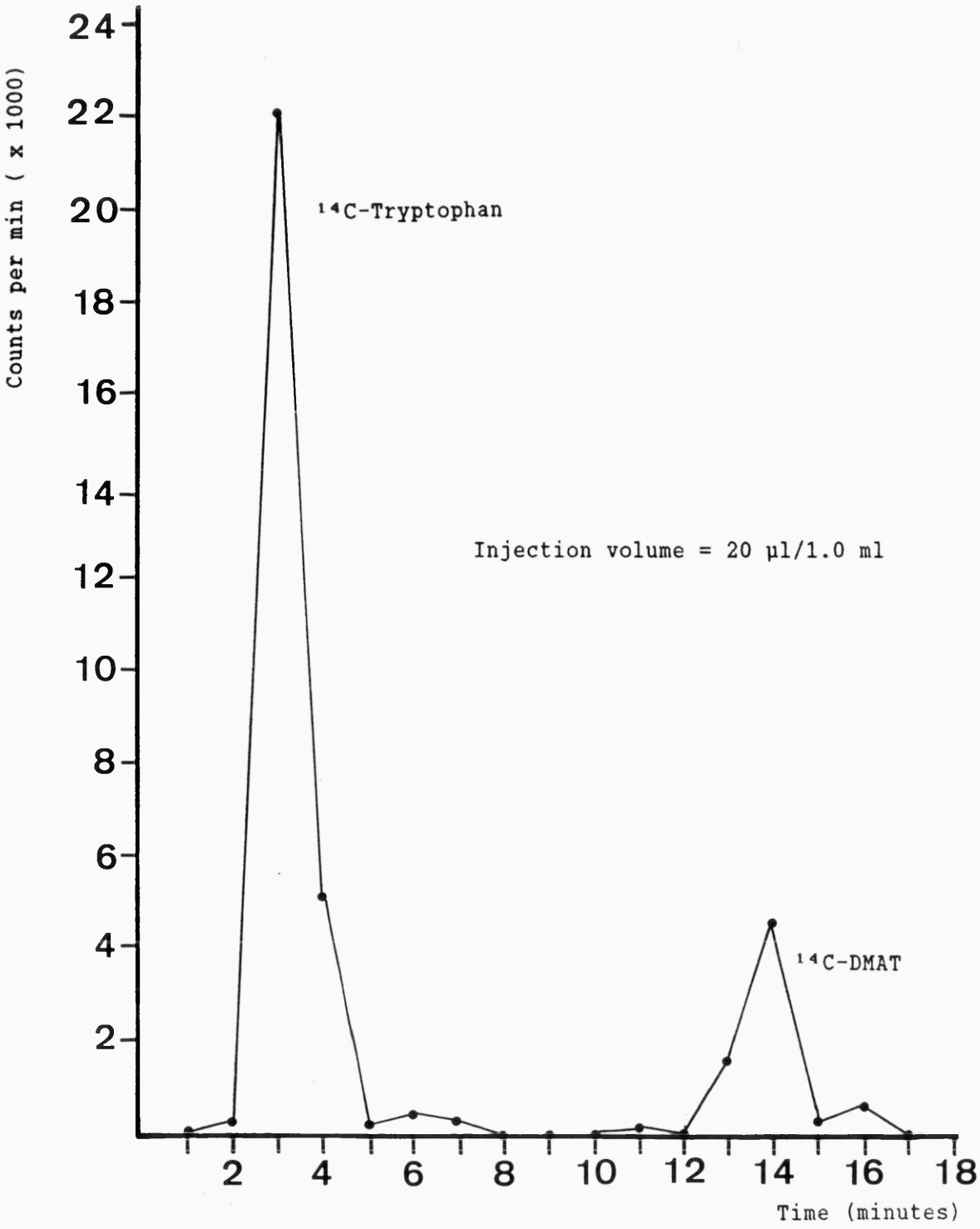


Fig. 67 HPLC resolution of cationic compounds in a cell-free incubation mixture.

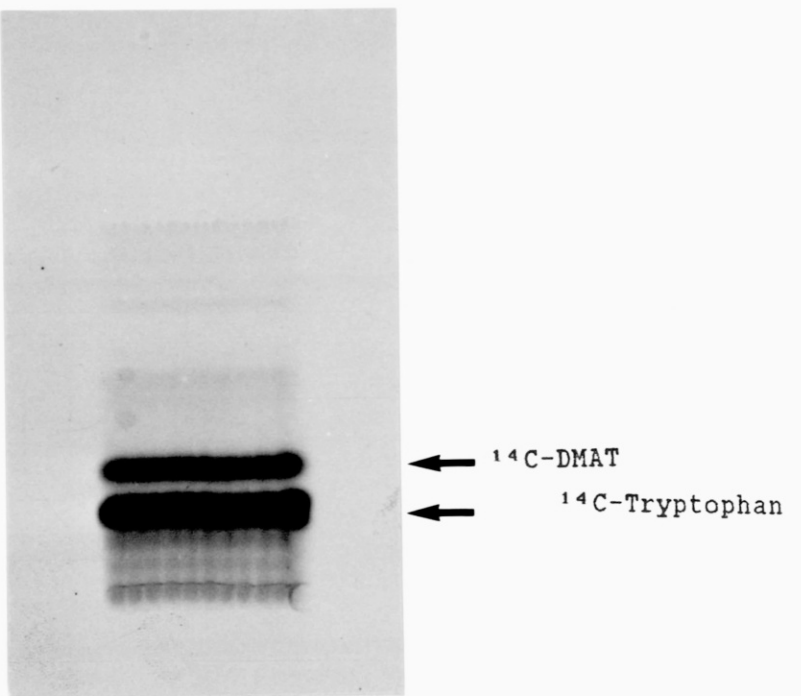


Fig. 68 Autoradiograph showing conversion of [^{14}C-methylene]-tryptophan to ^{14}C-DMAT by a partially purified protein fraction of *C. fusiformis*.

Autoradiograph exposure time, 17 days.

Table 25 Transformation of [^{14}C-methylene]-tryptophan to ^{14}C-DMAT by cell-free preparations of *C. fusiformis* CAS.

Purification stage	Incubation time (hours)	^{14}C-activity (µCi) (% of total eluting in brackets)	
		DMAT	Tryptophan
Supernatant	3	0.2 (20)	0.8 (80)
Supernatant	18	0.2 (22)	0.7 (78)
$NH_4(SO_4)_2$ (30-45%)	3	0.1 (8)	1.2 (92)

4. DISCUSSION.

4.1 STUDIES WITH *PENICILLIUM AURANTIOGRISEUM.*

The isolation and structure elucidation of two novel alkaloid metabolites from *Penicillium aurantiogriseum* was achieved during the research period. Use of [^{14}C-carbonyl]-anthranilic acid as a biosynthetic probe in the present study led to the discovery of a novel benzodiazepine (Fig. 17, S.111). The same approach had been employed in the earlier discovery of the fungal benzodiazepine auranthine (Yeulet and Mantle, 1987). Auranthine was isolated from a fungus originally described as *Penicillium verrucosum var. cyclopium* (Barnes *et al.*, 1977) but which is now assigned as *P. commune* (Macgeorge and Mantle, 1990). The structure of auranthine was elucidated from spectral evidence and biosynthetic reasoning (Yeulet *et al.*, 1986). Preliminary experiments on the dynamics of auranthine production when the fungus was cultivated as a sporing surface mat, revealed that the metabolite first became evident on about day 5, at which point maximum biomass had been achieved (Yeulet and Mantle, 1987).

Demonstration of significant incorporation of radiolabelled anthranilic acid into the novel benzodiazepine confirmed the direct involvement of this precursor in the biosynthesis. The percentage incorporation was numerically rather small (0.36%), though similar to the 0.3% obtained for the incorporation of [^{14}C-carbonyl]-anthranilic acid into auranthine (Yeulet and Mantle, 1987) and into cyclopenin (Laws and Mantle, 1989).

There was substantial dissipation of the carbonyl-^{14}C by surface cultures of *P. aurantiogriseum* in the present study, even when the radiolabel was fed after the trophophase. This implied that the enzymes of the tryptophan biosynthetic pathway, including notably indole-3-glycerolphosphate synthetase which is involved in the

decarboxylation of anthranilate, were still remarkably active although the biomass accumulation in surface culture was complete by about day 5. In submerged fermentation the novel benzodiazepine also behaved as a classical secondary metabolite since accelerated production occurred after the period of active growth of the organism.

The novel metabolite contains anthranilic acid and leucine, which together form the 1,4-benzodiazepine ring, and a cyclic glutamine residue as a substituent. It is of interest that the antibiotic anthramycin (Fig. 69, S.120) is a 1,4-benzodiazepine derivative which showed substantial *in vitro* anti-microbial activity, particularly against Gram-positive bacteria and, to a lesser extent, against Gram-negative organisms and fungi. Anthramycin, produced by a thermophilic Actinomycete, was also shown to exhibit *in vivo* antitumour activity (Leimgruber *et al.*, 1965a).

1,4-benzodiazepines have been particularly intensively studied since the early 1960's because of their value in psychotherapy. Some aspects of structure-activity relationships of 1,4-benzodiazepines have been described (Sternbach, 1971). A substituent on C-7 was found to be of paramount importance, but also substituents on N-1 and in a 5-phenyl ring had a profound effect on the activity. Electron withdrawing substituents, such as a chlorine atom, on C-7 generally imparted high activity, whereas electron donors had the opposite effect. The studies by Sternbach (1971) resulted in the production of seven 1,4-benzodiazepine derivatives which are now in clinical use as tranquilisers or sleep inducers. Fig. 70 shows the structures, the generic names and also the names of the drugs containing these products.

Fig. 69 Structure of anthramycin.

S.120 Anthramycin

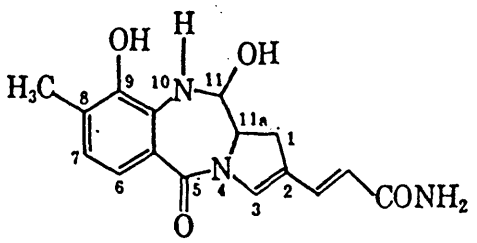
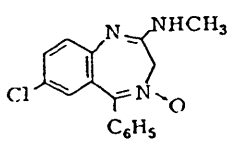
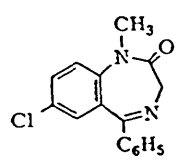


Fig. 70 1,4-Benzodiazepines in clinical use.

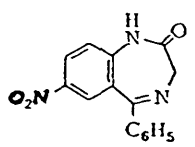
Chlordiazepoxide
(Librium™)



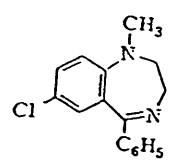
Diazepam
(Valium™)



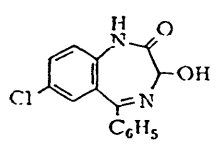
Nitrazepam
(Mogadon™)



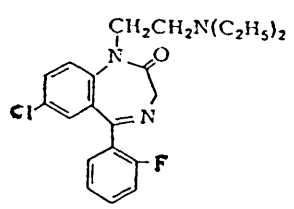
Medazepam
(Nobrium™)



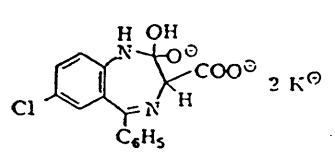
Oxazepam
(Serax™)



Flurazepam
(Dalmane™)



Chlorazepate
(Tranxène™, Tranxilium™)



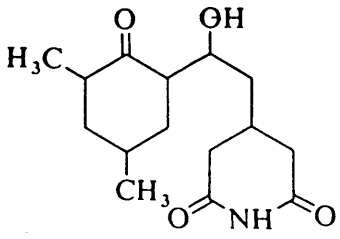
The novel benzodiazepine, however, did not possess structural characteristics representative of these compounds. It is nevertheless an interesting molecule since it contains a glutarimide functionality in addition to the 1,4-benzodiazepine ring. Although uncommon in nature, the glutarimide functionality is a feature of cycloheximide (Fig. 71, S.121), an agent effective against several yeasts, mammalian cells and tumour cells in culture. Cycloheximide was initially isolated from *Streptomyces griseus* by Ford and Leach (1948) at the Upjohn Research Laboratories and its structure was determined by Kornfeld *et al.* (1949).

Another antibiotic (Fig. 71, S.122), un-named in the original research paper, belonging to the cycloheximide family was isolated from *Aspergillus flavipes* (Casinovi *et al.*, 1968). The discovery of this compound was the first time in which a member of the cycloheximide family of metabolites was produced by a fungus. The glutarimide ring of the *A. flavipes* antibiotic contains a double bond and it is interesting to note that this metabolite has no activity against *Saccharomyces cerevisiae* or *Candida albicans* (Casinovi *et al.*, 1968). The glutarimide ring system is also an important feature of cycloheximide-related glutarimide antibiotics, namely, streptovitacin A, streptimidone, inactone and actiphenol (Fig. 71). These compounds have been shown to inhibit peptide synthesis in eucaryotic systems which utilise ribosomes of the 80 S type (Obrig *et al.*, 1971).

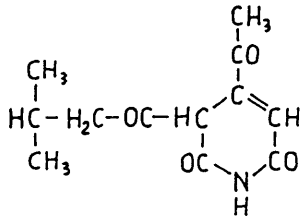
The glutarimide bemegride (Fig. 71, S.127), otherwise prescribed for respiratory depression or circulatory failure, has a limited therapeutic use in restoring depressed function of the CNS medullary centres after an overdose of a depressant drug. The natural product strychnine was formerly used for the treatment of barbiturate poisoning but was very dangerous. Bemegride is a better agent for this purpose (Landquist, 1984).

Fig. 71 Structures of glutarimide-containing compounds.

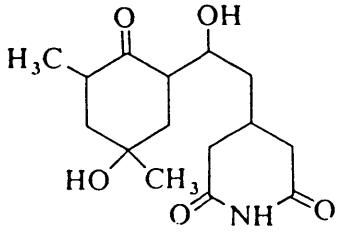
S.121 Cycloheximide (actidione)



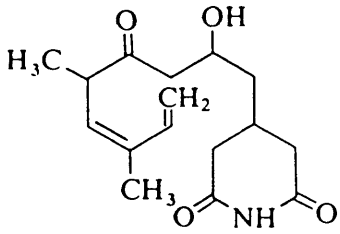
S.122 *Aspergillus* antibiotic



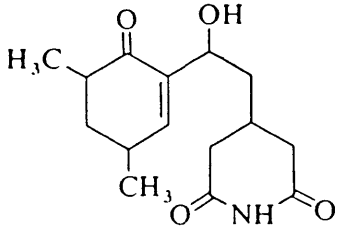
S.123 Streptovitacin A



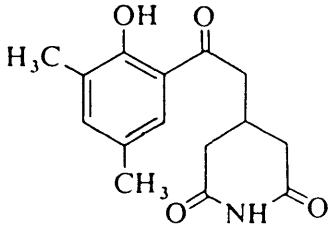
S.124 Streptimidone



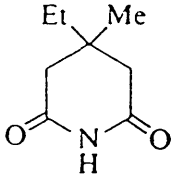
S.125 Inactone



S.126 Actiphenol



S.127 Bemegride



The possession by the novel benzodiazepine of two functionalities with perceivable biological activity, a 1,4-benzodiazepine ring and a cyclic glutarimide, has generated great interest in this compound. As there is a paucity of anti-fungal agents, generally, it may be important to exploit the glutarimide-benzodiazepine isolated in the present study as it bears structural analogies to cycloheximide, one of a limited number of effective agents.

Some interesting questions were raised during structural interpretation of NMR spectra of the novel benzodiazepine, including the relative stability of the two chiral centres of the molecule. Initially epimerisation at the α-centre of the glutarimide moiety was considered to be more likely than racemisation of leucine on the basis of chemical precedent. However, after detailed examination of the proton-NMR spectrum of the heat-transformed benzodiazepine it was concluded that the α-centre of leucine had racemised. The loss of the N-H to α-CH coupling in the leucine moiety of the diastereoisomer and also the greater non-equivalence of the β-CH_2 protons in this molecule could therefore be explained. An alternative argument to corroborate these observations would require explanation of how a change in stereochemistry at the α-centre of the glutarimide moiety could be transmitted through to the leucine β-CH_2 protons.

The glutarimide moiety of the novel benzodiazepine is chemically very interesting. A literature search revealed curious side reactions of terminal glutamine residues in laboratory peptide synthesis, one of which was the formation of pyroglutamic acid on heating (Bodanszky and Martinez, 1981). Although this speculative product was not formed on pyrolysis of the novel benzodiazepine, the benzodiazepine was sensitive to the trace amounts of HCl in deutero-chloroform, used initially as a solvent for NMR studies, but instead formed a tautomeric species which possibly could be a feasible intermediate in the synthesis of a pyroglutaminyl derivative. Therefore, the combined

conditions of high temperature and H^+ ions possibly might favour formation of a more stable 5-membered pyroglutaminyl derivative.

The novel benzodiazepine was tested for biological activity in a limited pharmaceutical screen* but was shown to be inactive in benzodiazepine receptor-binding assays. It is possible that this molecule might express activity if studied more extensively using a variety of tissue preparations or in other biological screens. Furthermore, the novel benzodiazepine might lend itself to structural modification with a view to enhancing any conceivable activity.

The use of a biosynthetic probe in the isolation of a potentially biologically active benzodiazepine has been demonstrated in this reasearch work. However, it must be appreciated that great care is required in the interpretation of results using radiolabelled precursors. For example, in the present study in autoradiography of TLC-separated extract components, a trace amount of a compound with a high specific activity (possibly a minor contaminant of the ^{14}C-anthranilate) was found to co-chromatograph with a large quantity of an unlabelled metabolite. Such an indicator could have resulted in the potentially unrewarding purification of an irrelevant compound. The diketopiperazine described in this thesis was isolated initially in this way because it was thought to be a ^{14}C-anthranilate-containing compound. However, in this case isolation proved to be rewarding since the diketopiperazine discovered was a novel molecule.

The novel diketopiperazine (Fig. 17, S.114) was biosynthesised from tryptophan and phenylalanine, unusually during the period of active growth of *P. aurantiogriseum* and so it is not, therefore, typical of a classical secondary metabolite. A feature of the novel diketopiperazine (DKP) is the rare acetylation at the indole nitrogen atom, observed only in the *Penicillium* metabolite verrucofortine (Hodge *et al.*, 1988) and in the *Aspergillus* metabolite LL-S490β (Ellestad *et al.*, 1973). A second interesting feature of the novel

* MRC Neurobiology Unit, Cambridge University.

DKP is the inverted prenyl group at the C-3 position of the molecule, extending this rather unusual conformation which is characteristic of verrucofortine, LL-S490β, oxaline, roquefortine and nigrifortine. As mentioned in the Introduction, some prenylated indole derivatives exhibit tremorgenic properties and it would therefore be useful to assay the novel diketopiperazine in suitable biological systems.

The question is raised as to the mode of inverted incorporation of the C_5 unit onto the indole ring. A precedent in chemical synthesis is found in the work of Bycroft and Landon (1970) in which the inverted C_5 grouping at C-3 was incorporated by a rearrangement of a dimethylallyl-2-indolyl sulphonium salt. It is of interest that a number of related metabolites such as echinulin (Allen, 1972), the brevianamides (Birch and Wright, 1970) and austamide (Steyn, 1971) contain the inverted prenyl group at the C-2 position of the indoline ring.

The mechanism of isoprenylation of the aromatic ring in tryptophan-containing metabolites is quite curious and there is an interest in the enzymes catalysing such reactions. A crude enzyme preparation which converted cyclo-L-alanyl-L-tryptophan into a monoprenylated derivative with the inverted prenyl group at C-2 has been isolated from an *Aspergillus* species (Allen *et al.*, 1979).

Future work considerations might include the isolation of an enzyme preparation possessing a prenylating activity from *Penicillium aurantiogriseum*. It would be interesting to feed putative precursors of the novel diketopiperazine to whole cultures and cell-free preparations of *P. aurantiogriseum*. More detailed studies to establish the order of biosynthesis by double-labelling techniques could be undertaken. This approach has been used in the study of the dynamics of verruculogen biosynthesis (Mantle and Shipston, 1987). DL-[2-^{3}H]-mevalonic acid and L-[U-^{14}C]-proline were fed to surface cultures of *P. simplicissimum* and the ratio of ^{3}H to ^{14}C in

radiolabelled verrruculogen was found to increase over the period of administration of the radiolabelled precursors. This was interpreted by the authors as a temporal separation of the formation of the diketopiperazine moiety and its subsequent prenylation. If there had been concurrent biosynthetic condensation of the amino acid components of verruculogen with electrophilic substitution of isoprene moieties, similar ^{3}H:^{14}C ratios in the product irrespective of the time of isolation would have been expected. Progressively increasing specific activities of each nuclide, each representing a key primary metabolite, might have been expected as the radiolabelled precursors were added at stages through the fermentation, especially as the demands of replicatory processes such as protein and sterol synthesis would decline. Interestingly, against this expectation the decline in incorporation of radiolabelled proline when growth had ceased was reported by the authors to be indicative of a decline in diketopiperazine synthesis even though much of the verruculogen had yet to be biosynthesised. A similar temporal separation of initial diketopiperazine formation from subsequent prenylation was demonstrated in the biosynthesis of the diketopiperazine roquefortine in *P. crustosum* (Laws and Mantle, 1989).

It would be interesting also to study in more detail the biosynthesis of the novel benzodiazepine. For example, to know whether the benzodiazepine ring is biosynthesised first and the glutarimide ring added subsequently and whether the glutarimide ring is attached intact as a cyclic species or if it is initially substituted as an open chain with cyclisation occurring later.

It is interesting to consider the competing biosynthetic pathways which would have been operating in *P. aurantiogriseum* in the present study. The diketopiperazine was biosynthesised before the benzodiazepine. Anthranilic acid, therefore, was under regulated sequential competition from these secondary metabolite biosynthetic

pathways throughout this batch fermentation. The anthranilate moiety of the benzodiazepine is derived directly from anthranilic acid as demonstrated in section 3.2. However, an alternative biosynthetic pathway for the anthranilyl moiety *via* tryptophan, although not in any way indicated by the present studies, must also be entertained. This alternative biosynthetic route has been implied in the formation of the 1,4-benzodiazepine anthramycin-type antibiotics. Feeding experiments with [^{14}C-benzene-ring]-tryptophan followed by chemical degradation of the biosynthetically labelled anthramycin provided proof that the radioactivity from the tryptophan was found exclusively in the anthranilate unit (Hurley, 1980).

The novel benzodiazepine might possibly utilise free anthranilic acid generated by catabolism of the novel diketopiperazine which was hardly a stable fermentation product. Evidence in the present study for the occurrence of a partially transformed product could be interpreted as relating to a catabolic transformation of the novel diketopiperazine (DKP). An additional competitive dimension in the biosynthesis of the novel DKP is the competition for chorismate by the phenylalanine and tryptophan moieties.

4.2 STUDIES WITH *CLAVICEPS* FUNGI.

Mutant micro-organisms, deficient in a particular biosynthetic enzyme, have proved useful in the investigation of secondary biosynthetic pathways. Their successful application to yield enhancement and generation of novel secondary metabolites, notably antibiotics, clearly demonstrates this point (see, for example, Queener *et al.*, 1978). The complex steps in aflatoxin biosynthesis in *Aspergillus parasiticus* were only revealed by using blocked mutants (Singh and Hsieh, 1977).

Variants of *Claviceps purpurea* which fail to produce alkaloids during parasitism, but which otherwise exhibit typical

characteristics of *C. purpurea*, are rare. However, one such strain designated 12-2, consistently fails to elaborate alkaloids whilst parasitising rye and wheat (Corbett *et al.*, 1974). Willingale *et al.* (1983) found that the first pathway-specific step in ergot alkaloid biosynthesis, namely conversion of tryptophan to dimethylallyl-tryptophan (DMAT), was operational in this mutant. Accumulation of trace amounts of DMAT indicated that the biosynthetic block occurred beyond this step. Willingale *et al.* (1983) demonstrated, using ^{14}C-radiolabelled methionine, that N-methylation of DMAT (the succeeding step in the pathway) was not operational. This indicated that the mutant strain 12-2 was deficient in N-methyl transferase, the enzyme analagous to that detected in *C. fusiformis* (Otsuka *et al.*, 1980). The apparent inactivity of N-methyl transferase is most probably the result of mutational change rather than expression of conventional end-product inhibition, observed to operate on DMAT-synthetase in *C. fusiformis* (Cheng *et al.*, 1980). It is probably not as a result of repression either since there is no accumulation of end-products.

Despite being deficient in alkaloid production the mutant 12-2 strain was capable of parasitising rye. Failure to produce ergot alkaloids therefore does not appear to impair the pathogenicity of the fungus. The success of the alkaloid deficient mutant, 12-2, as a pathogen in the natural situation tends to conflict with the idea that secondary metabolites are produced to relieve the organism of an excessive build-up of intermediates which would otherwise shut down essential primary processes (Bu'Lock and Barr, 1974).

Willingale *et al.* (1983) found that DMAT-synthetase activity was restricted to maturing parasitic sclerotial tissue which had probably ceased cell-division. The present study found there to be negligible *in vitro* DMAT-synthetase activity in *C. purpurea* strain 12-2 on account of non-conversion of ^{14}C-tryptophan to DMAT. This is possibly

because the tissue was too old when taken for experiment, but it emphasises the point that alkaloid biosynthesis is not necessarily a persistent property of maturing sclerotial tissue.

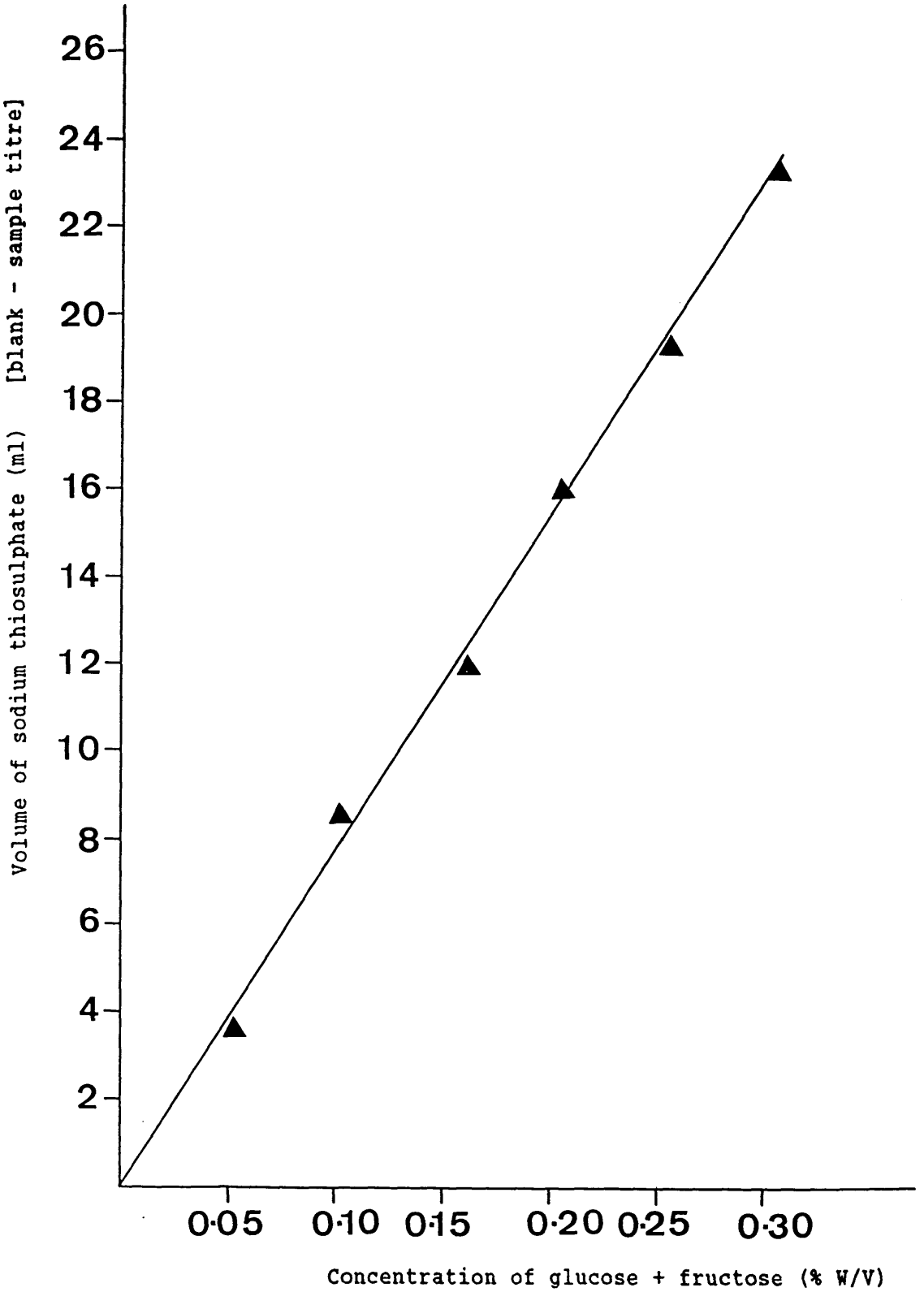
The biosynthesis of cyclic tripeptide alkaloids in axenic culture by a *C. purpurea* mutant having a block beyond chanoclavine-I-aldehyde has been reported (Maier *et al.*, 1980a,b). This mutant strain produced the same alkaloids, ergometrine and ergotoxine, as the parent strain when fed agroclavine, elymoclavine and lysergic acid as precursors.

Mutant organisms are of great use in dissecting biosynthetic pathways as they often accumulate intermediates which can be isolated and characterised. The order of biosynthetic events can sometimes be established by precursor feeding experiments and often the organism is deficient in only a single enzymic activity. Natural mutants of *Claviceps* spp. are rare, however, and it is not easy to obtain variants displaying a specifically altered genomic locus from an artificially mutagenised culture sample.

A greater understanding of secondary biosynthesis in natural ergot fungi will be achieved once the enzymes governing these pathways are more fully characterised. Moreover, isoprenylating enzymes isolated from natural microbial sources possibly could be used to accomplish challenging chemical reactions in the design of novel biologically-active molecules from precursors produced by other fungal genera.

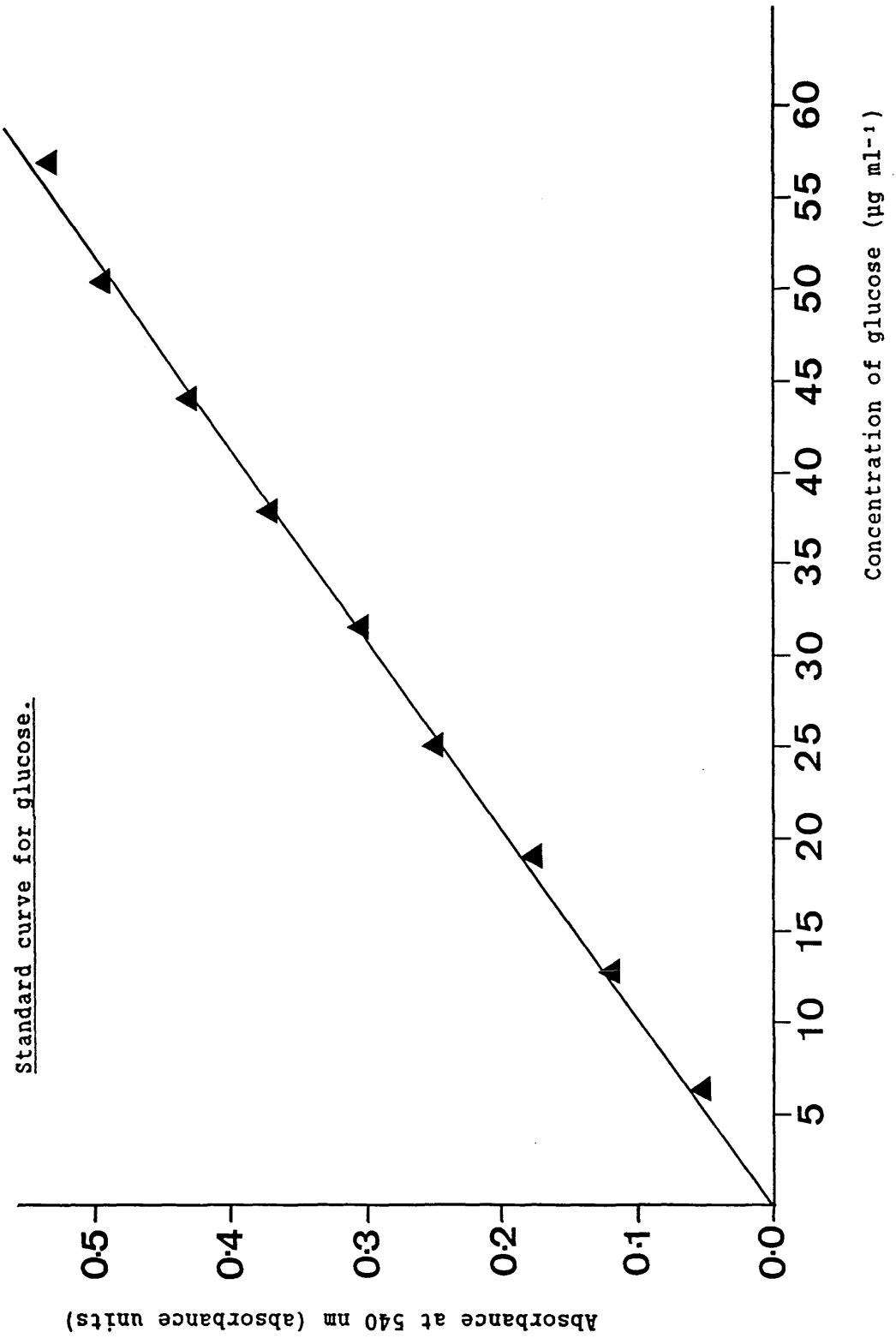
Finally, it is worth reflecting whether the various properties of secondary metabolites are of ecological significance or whether a mistaken function is assigned to a compound because it exhibits qualities that affect our personal interests. Some aspects of the production of alkaloids in *Penicillium* and *Claviceps* fungi have been highlighted in this study, but microbial secondary metabolism is such an interesting and far-reaching subject area that it will no doubt keep researchers puzzling for many years to come.

Appendix I.



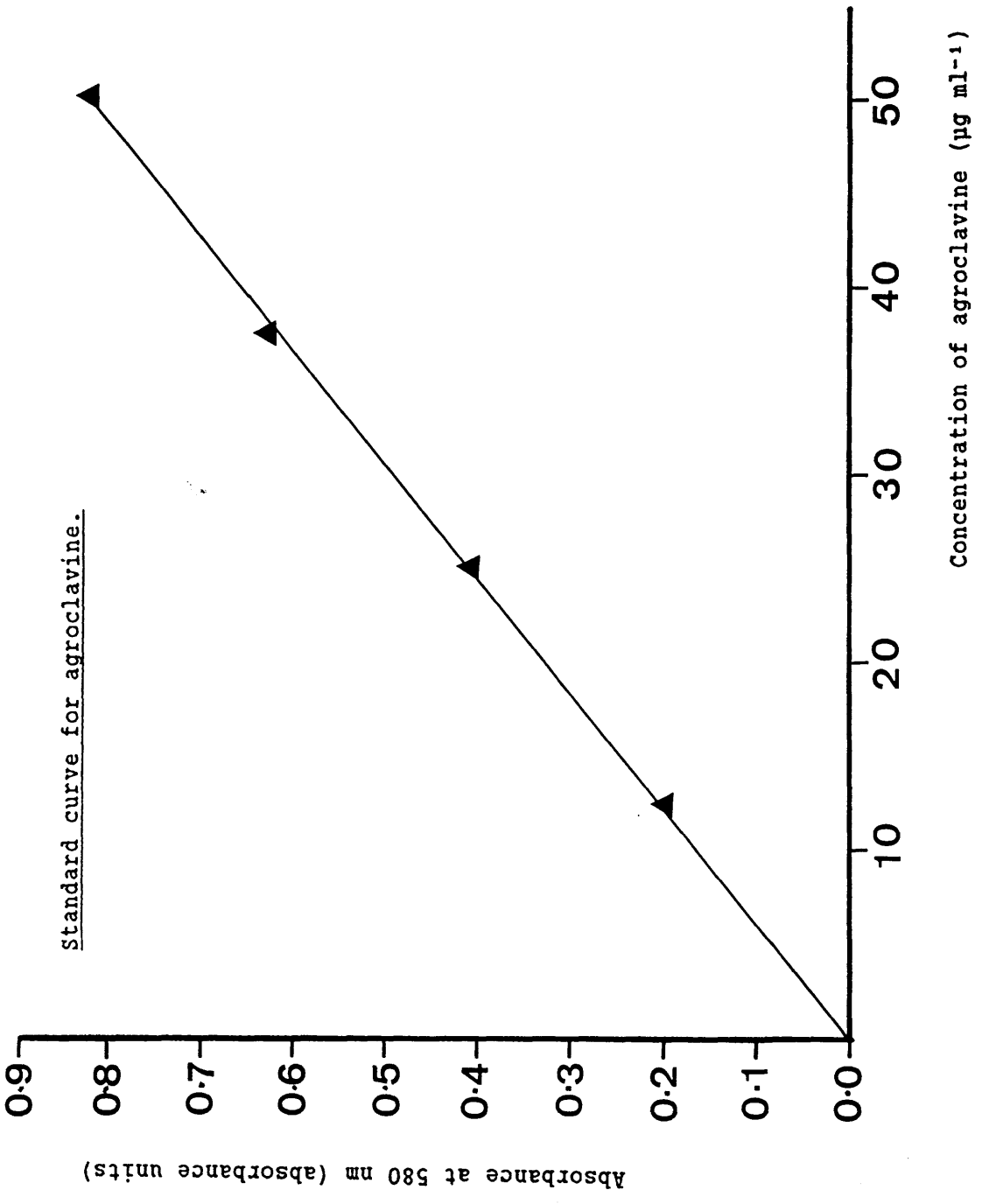
Standard curve of an equimolar mixture of glucose and fructose.

Appendix II.



Standard curve for glucose.

Appendix III.



Ammonium Sulphate % Saturation Table.

Amount of ammonium sulphate (g) to be added to 1 litre

From S_1% ↘ To S_1%→	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	27	55	84	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5		27	56	85	115	146	179	212	246	282	319	357	397	439	481	526	572	621	671	723
10			28	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
	15			28	58	88	119	151	185	219	255	292	331	371	413	456	501	548	596	647
		20			29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
			25			29	60	91	123	157	191	227	265	304	344	386	429	475	522	571
				30			30	61	92	126	160	195	232	270	309	351	393	438	485	533
					35			30	62	94	128	163	199	236	275	316	358	402	447	495
						40			31	63	96	130	166	202	241	281	322	365	410	457
							45			31	64	97	132	169	206	245	286	329	373	419
								50			32	65	99	135	172	210	250	292	335	381
									55			33	66	101	138	175	215	256	298	343
										60			33	67	103	140	179	219	261	305
											65			34	69	105	143	183	224	266
												70			34	70	107	146	186	228
													75			35	72	110	149	190
														80			36	73	112	152
															85			37	75	114
																90			37	76
																		95		38

6. References.

Abe, M. (1951). Isolation and properties of an alkaloid "agroclavine" produced by ergot fungus in saprophytic culture. *Takeda Kenkyusho Nempo*, 10, 145.

Abe, M. (1971). *Abh. Dtsch. Akad. Wiss. Berlin Kl. Chem. Geol. Biol.*, pp. 411-422.

Abe, M., Yamatodani, S., Yamano, T., Kozu, Y. and Yamada, S. (1967). Production of alkaloids and related substances by fungi I. Examination of filamentous fungi for their ability of producing ergot alkaloids, *J. Agric. Chem. Soc. Jpn.*, 41, 68-71.

Abou-Chaar, C.I., Brady, L.R. and Tyler, V.E. (1961). Occurrence of lysergic acid in saprophytic cultures of *Claviceps*. *Lloydia*, 24, 89-93.

Agurell, S. (1964). Costaclavine from *Penicillium chermesinum*. *Experientia*, 20, 25-26.

Agurell, S. (1966). Biosynthesis of ergot alkaloids in *Claviceps paspali* Part II. Incorporation of labelled agroclavine, elymoclavine, lysergic acid and lysergic acid methyl ester. *Acta Pharm. Suecica*, 3, 23-32.

Agurell, S. and Lindgren, J. (1968). Natural occurrence of 4-dimethyl-allyltryptophan: an ergot alkaloid precursor. *Tetrahedron Lett.*, 5127-5128.

Agurell, S. and Ramstad, E. (1962). Biogenic interrelationsips of ergot alkaloids. *Arch. Biochem. Biophys.*, 98, 457-470.

Alho, H., Costa, E., Ferrero, P., Fujimoto, M., Consenza-Murphy, D. and Guidotti, A. (1985). Diazepam-binding inhibitor: A neuropeptide located in selected neuronal populations of rat brain. *Science*, 229, 179-182.

Allen, C.M. (1972). Biosynthesis of echinulin. Isoprenylation of *cyclo*-L-alanyl-L-tryptopanyl. *Biochemistry*, 11, 2154-2160.

Allen, J.K., Barrow, K.D. and Jones, A.J. (1979). Biosynthesis of echinulin. The stereochemistry of aromatic isoprenylation. *J. Chem. Soc. Chem. Comm.*, pp. 280-282.

Anderson, D.G. and Porter, J.W. (1962). The Biosynthesis of Phytoene and Other Carotenes by Enzymes of Isolated Higher Plant Plastids. *Arch. Biochem. Biophys.*, 97, 509-519.

Bajwa, R.S. and Anderson, J.A. (1975). Conversion of agroclavine to setoclavine and isosetoclavine in cell-free extracts from *Claviceps* sp. SD 58 and in a thioglycolate-iron (II) system. *J. Pharm. Sci.*, 64, 343-344.

Bajwa, R.S., Kohler, R.D., Saini, M.S., Cheng, M. and Anderson, J.A. (1975). Formation of clavicipitic acid in cell-free systems of *Claviceps* sp. *Phytochemistry*, 14, 735-737.

Banks, G.T., Mantle, P.G. and Szczyrbak, C.A. (1974). Large-scale production of clavine alkaloids by *Claviceps fusiformis*. *J. Gen. Microbiol.*, 82, 345-361.

Barger, G. and Carr, F.H. (1907). The alkaloids of ergot. *J. Chem. Soc.*, 91, 337-353.

Barnes, J.M., Carter, R.L., Peristianis, G.C., Austwick, P.K.C., Flynn, F.V. and Aldridge, W.N. (1977). Balkan (endemic) nephropathy and a toxin-producing strain of *Penicillium* var *cyclopium*: An experimental model in rats. *Lancet*, 289, 671-675.

Barrow, K.D. and Quigley, F.R. (1975). Ergot alkaloids III. The isolation of N-methyl-4-dimethylallytryptophan from *Claviceps fusiformis*. *Tetrahedron Lett.*, 48, 4269-4270.

Bassett, E.W. and Tanenbaum, S.W. (1958). The metabolic products of *Penicillium patulum* and their probable interrelation, *Experientia*, 14, 38-40.

Baxter, R.M., Kandel, S.I. and Okany, A. (1961). Studies on the mode of incorporation of mevalonic acid into ergot alkaloids. *Tetrahedron Lett.*, 596-600.

Birch, A.J. and Farrar, K.R. (1963). Studies in relation to biosynthesis XXXIII. Incorporation of tryptophan into echinulin. *J. Chem. Soc.*, 4277-4279.

Birch, A.J. and Wright, J.J. (1969). Brevianamides: A new class of fungal alkaloid. *Chem. Comm.*, 644-645.

Birch, A.J. and Wright, J.J. (1970). Studies in relation to biosynthesis XLII. The structural elucidation and some aspects of the biosynthesis of the brevianamides A and E. *Tetrahedron*, 26, 2329-2344.

Birch, A.J., Mcloughlin, B.J. and Smith, H. (1960). The biosynthesis of the ergot alkaloids. *Tetrahedron Lett.*, 7, 1-3.

Birch, A.J., Blance, G.E., David, S. and Smith, H. (1961). Studies in relation to biosynthesis XXIV. Some remarks on the struture of echinulin. *J. Chem. Soc.*, 3128-3131.

Birch, A.J., Kocor, M. Sheppard, W. and Winter, J. (1962). Studies in relation to biosynthesis. 29. The terpenoid chain of mycelianamide. *J. Chem. Soc.*, 1502-1505.

Bird, B.A. and Campbell, I.M. (1982). Occurrence and biosynthesis of asperphenamate in solid cultures of *Penicillium brevicompactum*. *Phytochemistry*, 21, 2405-2406.

Birkinshaw, J.H. and Mohammed, Y.S. (1962). Studies in the biochemistry of micro-organisms. 111. The production of L-phenylalanine anhydride by *Penicillium nigricans* (Bainier) Thom. *Biochem. J.*, 85, 523-527.

Birkinshaw, J.H., Luckner, M., Mohammed, Y.S., Mothes, K. and Stickings, C.E. (1963). Studies in the biochemistry of micro-organisms. 114. Viridicatol and cyclopenol, metabolites of *Penicillium viridicatum* Westling and *Penicillium cyclopium* Westling. *Biochem. J.*, 89, 196-202.

Birkinshaw, J.H., Raistrick, H. and Ross, D.J. (1952). Studies in the biochemistry of micro-organisms. The molecular constitution of mycophenolic acid, a metabolic product of *Penicillium brevicompactum* Diercx. Part 3. Further observations on the structural formula for mycophenolic acid. *Biochem. J.*, 50, 630-634.

Bodanszky, M. and Martinez, J. (1981). Side reactions in peptide synthesis. *Synthesis*, pp. 333-356.

Brown, J.P., Cartwright, N.J., Robertson, A. and Whalley, W.B. (1949). The Chemistry of Fungi. IV. The constitution of the phenol, $C_{11}H_{16}O_3$, from citrinin. *J. Chem. Soc.*, 859-867.

Büchi, G., Kitaura, Y., Yuan, S-S., Wright, H.E., Clardy, J., Demain, A.L., Glinsukon, T., Hunt, N. and Wogan, G.N. (1973). Structure of cytochalsin E, a toxic metabolite of *Aspergillus clavatus*. *J. Am. Chem. Soc.*, 95, 5423-5425.

Bu'Lock, J.D. (1961). Intermediary metabolism and antibiotic synthesis. *Advances in Applied Microbiology*, 3, p.293.

Bu'Lock, J.D. (1967). Essays in Biosynthesis and Microbial Development, John Wiley, London.

Bu'Lock, J.D. (1975). In: The Filamentous Fungi. Vol. I, Smith, J.E. and Berry, D.R., eds., Arnold, London.

Bu'Lock, J.D. and Barr, J.G. (1974). Secondary metabolism in fungi and its relationship to growth and development. In: Filamentous Fungi Vol. I. Smith, J.E. and Berry, D.R., eds., Arnold, London.

Bu'Lock, J.D. and Powell, A.J. (1965). Secondary metabolism. An explanation in terms of induced enzyme mechanism. *Experientia*, 21, 55.

Bycroft, B.W. and Landon, W. (1970). Thio-Claisen rearrangements of dimethylallyl 2-indolyl sulphonium salts: Possible implications in indole alkaloid biosynthesis. *Chem. Comm.*, 967-968.

Casinovi, C.G., Grandolini, G., Mercantini, R., Oddo, N., Olivieri, R. and Tonolo, A. (1968). A new antibiotic produced by a strain of *Aspergillus flavipes*. *Tetrahedron Lett.*, 27, 3175-3178.

Casnati, G. and Pochini, A. (1970). Rearrangement of 3-alkyl-1-allyl-indoles: a model reaction for the biogenesis of echinulin-type compounds. *J. Chem. Soc. Chem. Comm.*, 1328-1329.

Casnati, G., Marchielli, R. and Ponchini, A. (1974). Rearrangement of 3-alkyl-1-allylindoles: A model reaction for the biogenesis of echinulin-type compounds. *J. Chem. Soc. Perkin Trans. I*, 754-757.

Cavender, F.L. and Anderson, J.A. (1970). The cell-free biosynthesis of clavine alkaloids. *Biochem. Biophys. Acta.*, 208, 345-348.

Cheng, L-J., Robbers, J.E. and Floss, H.G. (1980). End-product regulation of ergot alkaloid formation in intact cells and protoplasts of *Claviceps* strain SD-58. *Lloydia*, 43, 329.

Clardy, J., Springer, J.P., Büchi, G., Matsuo, K. and Wightman, R. (1975). Tryptoquivaline and tryptoquivalone, two tremorgenic metabolites of *Aspergillus clavatus*. *J. Am. Chem. Soc.*, 97, 663-665.

Cole, R.J., Kirksey, J.W., Moore, J.H., Blankenship, J.R, Diener, U.L. and Davis, N.D. (1972). Tremorgenic toxin from *Penicillium verruculosum*. *Appl. Microbiol.*, 24, 248-256.

Cole, R.J., Dorner, J.W., Lansden, J.A., Cox, R.H., Pape, C., Cunfer, B., Nicholson, S.S. and Bedell, D.M. (1977). Paspalum staggers: Isolation and identification of tremorgenic metabolites from sclerotia of *C. paspali*. *J. Agric. Food Chem.*, 25, 1197-1201.

Cole, R.J., Dorner, J.W., Springer, J.P. and Cox, R.H. (1981). Indole metabolites from a strain of *Aspergillus flavus*. *J. Agric. Food Chem.*, 29, 293-295.

Corbett, K., Dickerson, A.G., and Mantle, P.G. (1974). Metabolic studies on *Claviceps purpurea* during parasitic development on rye. *J. Gen. Microbiol.*, 84, 39-58.

Cornforth, R.H and Popjak, G. (1969). Chemical synthesis of substrates of sterol biosynthesis. *Methods in Enzymology*, 15, 359-390.

Cress, W.A., Chayet, L.T. and Rilling, H.C. (1981). Crystallization and partial characterization of dimethylallylpyrophosphate:L-tryptophan dimethylallyltransferase from *Claviceps* sp. SD 58. *J. Biol. Chem.*, 256, 10917-10923.

Davies, D.B. and Khaled, M.A. (1976). Conformations of peptides in solution by nuclear magnetic resonance spectroscopy. Part III. Cyclic dipeptide ring conformations. *J. Chem. Soc. Perkin Trans II*, 1238-44.

De Jesus, A.E., Steyn, P.S., Van Heerden, F.R., Vleggaar, R., Wessels, P.L. and Hull, W.E. (1983). Tremorgenic mycotoxins from *Penicillium crustosum*: Isolation of penitrems A-F and the structure elucidation and absolute configuration of penitrem A. *J. Chem. Soc. Perkin Trans. I*, 1847-1856.

De Jesus, A.E., Steyn, P.S., Van Heerden, F.R. and Vleggaar, R. (1984). Structure elucidation of the janthitrems, novel tremorgenic mycotoxins from *Penicillium janthinellum*. *J. Chem. Soc. Perkin Trans. I*, 697-701.

Deslauriers, R., Grzonka, Z., Schaumberg, K., Shiba, T. and Walter, R. (1975). Carbon-13 nuclear magnetic resonance studies of the conformation of cyclic dipeptides. *J. Am. Chem. Soc.*, 97, 5093-5100.

Dickerson, A.G., Mantle, P.G. and Szczyrbak, C.A. (1970). Autolysis Of Extra-Cellular Glucans Produced *In Vitro* By A Strain Of *Claviceps fusiformis*, *J. Gen. Microbiol.*, 60, 403.

Dolphin, D. and Wick, A.E. (1977). Tabulation of Infra-Red Spectral Data. John Wiley & Sons, pp. 269-277.

Dreyfuss, M. Härri, E., Hofmann, H., Kobel, H., Pache, W. and Tscherter, H. (1976). *Eur. J. Appl. Microbiol.*, *3*, *125*.

Dunn, G. Gallagher, J.J., Newbold, G.T. and Spring, F.S. (1949a). Aspergillic acid. Part I. *J. Chem. Soc.*, 126-131.

Dunn, G., Newbold, G.T. and Spring, F.S. (1949b). Aspergillic acid Part II. The conversion of aspergillic acid into leucine and isoleucine (or alloiosleucine). *J. Chem. Soc.*, 131-133.

Ellestad, G.A., Mirando, P. and Kunstmann, M.P. (1973). Structure of the metabolite LL-S490β from an unidentified *Aspergillus* species. *J. Org. Chem.*, 38, 4204-4205.

Evans, B.E., Bock, M.G., Rittle, K.E., Dipardo, R.M., Whitter, W.L., Veber, D.F., Anderson, P.S. and Freidinger, R.M. (1986). Design of potent, orally effective, non-peptidal antagonists of the peptide hormone cholecystokinin. *Proc. Natl. Acad. Sci. USA*, 83, 4918-4922.

Fiedler, E., Fiedler, H.P., Gerhard, A., Keller-Schierlein, W., Koenig, W.A. and Zaehner, H. (1976). Metabolic products of micro-organisms. 156. Synthesis and biosynthesis of substituted tryptanthrins. *Arch. Microbiol.*, 107, 249-256.

Floss, H.G. (1976). Biosynthesis of ergot alkaloids and related compounds. *Tetrahedron*, 32, 873-912.

Floss, H.G. and Anderson, J.A. (1980). Biosynthesis of ergot toxins. In: The biosynthesis of mycotoxins. A study in secondary metabolism. Steyn, P.S., ed. Academic Press.

Floss, H.G. and Gröger, D. (1963). Über die einbau von N-methyl-tryptophan und N-methyl-tryptamine in mutterkornalkaloide von clavin-type. *Z. Naturforsch. Teil B.*, 18, 519-522.

Floss, H.G. and Gröger, D. (1964). Entmethylierung von N-α-methyl-tryptophan durch *Claviceps* sp. *Z. Naturforsch. Teil B.*. 19, 393-395.

Floss, H.G., Mothes, U. and Günther, H. (1964). Uber den Einbau der Tryptophan-Seittenkette und den Mechanismus der reaktion am α-C-Atom. *Z. Naturforsch. Teil B.*, 19, 784-788.

Floss, H.G., Tcheng-Lin, M., Chang, C.J., Naidoo, B., Blair, G.E., Abou-Chaar, C. and Cassady, J.M. (1974). Biosynthesis of ergot alkaloids. Studies on the mechanism of conversion of chanoclavine I into tetracyclic ergolines. *J. Am. Chem. Soc.*, 96, 1898-1909.

Ford, J.H. and Leach, B.E. (1948). Actidione, an antibiotic from *Streptomyces griseus*. *J. Am. Chem. Soc.*, 70, 1223-1225.

Foster, J.W. (1949). Chemical Activities of Fungi. Academic Press.

Frank, R.L. and McPherson, J.B. (1949). Decarboxylation and cyclisation reactions of some pimelic acid derivatives. *J. Am. Chem. Soc.*, 71, 1387-1391.

Gatti, G., Cardillo, R. and Fuganti, C. (1978). Molecular structure of cryptoechinulin G, an isoprenylated dehydrotryptophan metabolite isolated from *Aspergillus ruber*. *Tetrahedron Lett.*, 2605-2606.

Goetz, M.A., Lopez, M., Monaghan, R.L., Chang, R.S.L., Lotti, V.J. and Chen, T.B. (1985). Asperlicin: a novel non-peptidal cholecystokinin antagonist from *Aspergillus alliaceus*. Fermentation, isolation and biological properties. *J. Antibiotics*, 38, 1633-1637.

Gough, D.P., Kirby, A.L., Richards, J.B. and Hemming, F.W. (1970). The characterization of undecaprenol of *Lactobacillus plantarum*. *Biochem. J.*, 118, 167-170.

Gray, R.A., Gauger, G.W. Dulaney, E.L., Kaczka, E.A. and Woodruff, H.B. (1964). Hadacidin, a new plant growth inhibitor produced by fermentation. *Plant Physiol.* 39, 204-207.

Guidotti, A., Furchetti, C.M., Corda, M.G., Konkel, D., Bennett, C.D. and Costa, E. (1983). Isolation, characterisation, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *Proc. Natl. Acad. Sci. USA*, 80, 3531-3535.

Gröger, D., Wendt, H.J., Mothes, K. and Weygand, F. (1959). Untersuchungen zur biosynthese der mutterkornalkaloide. *Z. Naturforsch Teil B.*, 14, 355-358.

Gröger, D., Mothes, K., Simon, H., Floss, H.G. and Weygand, F. (1960). Uber den einbau von mevalonsaure in das ergolinsystem der clavin-alkaloide. *Z. Naturforsch. Teil B.*, 15, 141-143.

Heinstein, P. and Floss, H.G. (1976). In: Secondary Metabolism and Coevolution. Luckner, M., Mothes, K. and Nover, L., eds. Nova Acta Leopoldina, Vol. 7, Suppl. pp. 299-310.

Heinstein, P.F., Lee, S-L. and Floss, H.G. (1971). Isolation of dimethylallylpyrophosphate:tryptophan dimethylallyl transferase from the ergot fungus (*Claviceps* sp.). *Biochem. Biophys. Res. Comm.*, 44, 1244-1251.

Heller, K. and Röschenthaler, R. (1978). Inhibition of protein synthesis in *Streptococcus faecalis* by ochratoxin A. *Can. J. Microbiol.*, 24, 466-472.

Hodge, R.P., Harris, C.M. and Harris, T.M. (1988). Verrucofortine, a major metabolilte of *Penicillium verrucosum* var. *cyclopium*, the fungus that produces the mycotoxin verrucosidin. *J. Nat. Prod.*, 51, 66-73.

Hofmann, A. and Tscherter, H. (1960). Isolierung von lysergäure-alkaloiden aus der Mexikanischen zauberdroge "Oloiuqui" (*Rivea corymbosa*). *Experientia*, 16, 414.

Hofmann, A., Ott, H., Griot, R., Stadler, P.A. and Frey, A.J. (1963). Die Synthese und Stereochemie des Ergotamins. *Helv. Chim. Acta*, 46, 2306-2328.

Holzapfel, C.W. (1968). The isolation and structure of cyclopiazonic acid, a toxic metabolite of *Penicillium cyclopium* Westling. *Tetrahedron*, 24, 2101-2119.

Holzapfel, C.W. and Marsh, J.J. (1977). Isolation and structure of viridamine, a new nitrogenous metabolite of *Penicillium viridicatum* Westling. *S. Afr. J. Chem.*, 30, 197-204.

Horák, M and Gut, J. (1960). Nucleic acids components and their analogues. XI. Infra-red spectroscopy of uracil, 6-azauracil and their derivatives in the carbonyl group stretching vibration region. *Collection Czechoslov. Chem. Comm.*, 26, 1680-1693.

Horowitz, N.H. (1965). Evidence for common control of tyrosinase and L-amino acid oxidase in *Neurospora*. *Biochem. Biophys. Res. Comm.*, 18, 686-692.

Hurley, L.H. (1980). Elucidation and formulation of novel biosynthetic pathways leading to the pyrrolo-[1,4]-benzodiazepine antibiotics anthramycin, tomaymycin and sibiromycin. *Acc. Chem. Res.*, 13, 263-269.

Hsu, J.C. and Anderson, J.A. (1971). Agroclavine hydroxylase of *Claviceps purpurea*. *Biochem. Biophys. Acta*, 230, 518-525.

Ibba, M., Taylor, S.J.C., Weedon, C.M. and Mantle, P.G. (1987). Submerged Fermentation of *Penicillium paxilli* Biosynthesising Paxilline, a Process Inhibited by Calcium-induced Sporulation. *J. Gen. Microbiol.*, 133, 3109-3119.

Ishii, K., Ando, Y. and Ueno, Y. (1975). Toxicological approaches to the metabolites of *Fusaria* IX. Isolation of vomiting factor from moldy corn infected with *Fusarium* species. *Chem. Pharm. Bull.*, 23, 2162-64.

Isogai, A., Washizu, M., Murakoshi, S. and Suzuki, A. (1985). A new shikimate derivative, methyl-5-lactyl-shikimate lactone, from *Penicillium* sp. *Agric. Biol. Chem.*, 49, 167-169.

Johnson, J.R., Kidwai, A.R. and Warner, J.S. (1953). Gliotoxin XI. A related antibiotic from *Penicillium terlikowski*: Gliotoxin monoacetate. *J. Am. Chem. Soc.*, 75, 2110-2112.

Kimura, Y., Inoue, T. and Tamura, S. (1973). *Agric. Biol. Chem.*, 37, 2213.

Kimura, Y., Hamasaki, T., Nakajima, H. and Isogai, A. (1982). Structure of aszonalenin, a new metabolite of *Aspergillus zonatus*. *Tetrahedron Lett.*, 23, 225-228.

King, G.S., Mantle, P.G., Szczyrbak, C.A. and Waight, E.S. (1973). A revised structure for clavicipitic acid. *Tetrahedron Lett.*, 3, 215-8.

King, G.S., Waight, E.S., Mantle, P.G. and Szczyrbak, C.A. (1977). The stucture of clavicipitic acid, an azepinoindole derivative from *Claviceps fusiformis*. *J. Chem. Soc. Perkin. Trans. I*, 2099-2103.

Kopple, K.D. and Ohnishi, M. (1969). Conformations of cyclic peptides. II. Side-chain conformation and ring shape in cyclic dipeptides. *J. Am. Chem. Soc.*, 91, 962-970.

Kornfeld, E.C., Jones, R.G. and Parke, T.V. (1949). The stucture and chemistry of actidione, an antibiotic from *Streptomyces griseus*. *J. Am. Chem. Soc.*, 71, 150-159.

Kozakiewicz, Z. (1989). *Aspergillus* species on stored food products. *Mycological Papers*, No. 161, p. 115.

Kozikowski, A.P., Wu, J-P., Shibuya, M. and Floss, H.G. (1988). Probing ergot alkaloid biosynthesis: Identification of advanced intermediates along the biosynthetic pathway. *J. Am. Chem. Soc.*, 110, 1970-1971.

Landquist, J.K. (1984). Application as pharmaceuticals. In: Comprehensive Heterocyclic Chemistry. Katritzky, A.P. and Rees, C.W., eds. Vol I, p. 170.

Laws, I. and Mantle, P.G. (1985). Nigrifortine, a diketopiperazine metabolite of *Penicillium nigricans*. *Phytochemistry*, 24, 1395-1397.

Laws, I. and Mantle, P.G. (1989). Experimental Constraints in the Study of the Biosynthesis of Indole Alkaloids in Fungi. *J. Gen. Microbiol.*, 135, 2679-2692.

Lee, S-L. (1974). Dimethylallyltryptophan synthetase from *Claviceps* sp. strain SD 58: purification, characterization and regulation. Ph.D. thesis, Purdue University, West Lafayette, Indiana.

Lee, S-L., Floss, H.G. and Heinstein, P. (1976). Purification and properties of dimethylallylpyrophosphate:tryptophan dimethylallyl transferase, the first enzyme of ergot alkaloid biosynthesis in *Claviceps* sp. SD 58. *Arch. Biochem. Biophys.*, 177, 84-94.

Leigh and Taylor (1976). In: Mycotoxins and Other Fungal Related Food Problems. Rodericks, J.V., ed. pp. 228-275.

Leimgruber, W., Stefanovic, V., Schenker, F., Karr, A. and Berger, J. (1965a). Isolation and characterization of anthramycin, a new antitumour antibiotic. *J. Am. Chem. Soc.*, 87, 5791-5793.

Leimgruber, W., Batcho, A.D. and Schenker, F. (1965b). The structure of anthramycin. *J. Am. Chem. Soc.*, 87, 5793-5795.

Leisch, J.M., Hensens, O.D., Springer, J.P., Chang, R.S.L. and Lotti, V.J. (1985). Asperlicin, a novel non-peptidal cholecystokinin antagonist from *Aspergillus alliaceus*. Structure elucidation. *J. Antibiotics*, 38, 1638-1641.

Lucente, G., Pinnen, F., Zanotti, G., Cerruni, S., Fedeli, W. and Gavuzzo, E. (1981). Synthesis and X-ray crystal structure of a tripeptidic cyclol. *Tetrahedron Lett.*, 22, 3671-3674.

Luckner, M. and Mothes, K. (1962). On the biosynthesis of 2,3-dihydroxy-4-phenyl-quinalone (viridicatin). *Tetrahedron Lett.*, No. 23, pp. 1035-1039.

MacDonald, J.C. (1961). *J. Biol. Chem.*, 236, 512-514.

MacDonald, J.C. and Slater, G.P. (1966). The utilisation of tryptophan in the biosynthesis of echinulin. *Can. J. Microbiol.*, 12, 455-463.

Macgeorge, K.M. and Mantle, P.G. (1990). Nephrotoxicity of *Penicillium aurantiogriseum* and *P. commune* from an endemic nephropathy area of Yugoslavia. *Mycopathologia*, in press.

Maes, C.M., Potgieteer, M. ans Steyn, P.S. (1986). NMR assignments, conformation and absolute configuration of ditryptophenaline and model dioxopiperazines. *J. Chem. Soc. Perkin Trans. I*, 861-866.

Maier, W. and Gröger, D. (1976). Dimethylallyltryptophan-synthetase in *Claviceps. Biochem. Physiol. Pflanzen*, 170, 9-15.

Maier, W., Erge, D., Schmidt, J. and Gröger, D. (1980a). A blocked mutant of *Claviceps purpurea* accumulating chanoclavine-I-aldehyde. *Experientia*, 36, 1353.

Maier, W., Erge, D. and Gröger, D. (1980b). Mutational biosynthesis in a strain of *Claviceps purpurea*. *Planta Medica*, 40, 104.

Mann, J. (1978). Secondary Metabolites. Oxford University Press.

Mantle, P.G. (1975). The Industrial Exploitation of Ergot Fungi. In: The Filamentous Fungi Vol 1, Smith, J.E. and Berry, D.R. eds., Arnold.

Mantle, P.G. (1987). Secondary metabolites of *Penicillium* and *Acremonium*. In: Biotechnology Handbooks I, Peberdy, J.F. ed., pp. 161-243. Plenum Press.

Mantle, P.G. and Shipston, N.F. (1987). Temporal Separation of Steps in the Biosynthesis of Verruculogen. *Biochemistry International*, 14, 1115-1120.

See p.211 ☆

Marshall, R., Redfield B., Katz, E. and Weissbach, A. (1968). Changes in phenoxazinone synthetase activity during the growth cycle of *Streptomyces antibioticus. Arch. Biochem. Biophys.*, 123, 317.

McCorkindale, N.J. and Baxter, R. L. (1981). Brevigellin, a benzoylated cyclodepsipeptide from *Penicillium brevicompactum. Tetrahedron*, 37, 1795-1801.

McCorkindale, N.J., Roy, T.P. and Hutchinson, S.A. (1967). *Tetrahedron*, 23, 3801-3808.

McGrath, R.M., Steyn, P.S., Ferreira, N.P. and Neethling, D.C. (1976). Biosynthesis of cyclopiazonic acids in *Penicillium cyclopium*. The isolation of dimethylallyl pyrophosphate: cycloacetoacetyltryptophanyl dimethylallyltransferase. *Biorg. Chem.*, 5, 11-23.

Minato, S. (1979). Isolation of anthglutin, an inhibitor of γ-glutamyl transpeptidase from *Penicillium oxalicum. Arch. Biochem. Biophys.*, 192, 235-240.

Mohammed, Y.S. and Luckner, M. (1963). The structure of cyclopenin and cyclopenol, metabolic products from *Penicillium cyclopium* Westling. *Tetrahedron Lett.*, 28, 1953-1958.

Mothes, K., Weygand, F., Gröger, D. and Grisebach, H. (1958). Untersuchungen zur biosynthese der mutterkornalkaloide. *Z. Naturforsch Teil B.*, 13, 41-44.

Nagel, D.W., Pachler, K.G.R., Steyn, P.S., Vleggaar, R. and Wessels, P.L. (1976). The chemistry and ^{13}C NMR assignments of oxaline, a novel alkaloid from *Penicillium oxalicum. Tetrahedron*, 32, 2625-2631.

Nair, P.M. and Vining, L.C. (1964). *Biochemistry*, 42, 1515.

Nair, P.M. and Vining, L.C. (1965). Isophenoxazine synthase from *Pycnoporus coccineus*. *Biochem. Biophys. Acta*, 96, 318-327.

Northolt, M.D., van Egmond, H.P. and Paulsch, W.E. (1979). *J. Food Prot.*, 42, 485.

Nover, L. and Luckner, M. (1974). Expression of secondary metabolism as part of the differentiation processes during the idiophase development of *Penicillium cyclopium* Westling. *Biochem. Physiol. Pflanzen*, 166, 293-305.

Obrig, T.G., Culp, W.J., McKeehan, W.L. and Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J. Biol. Chem.*, 246, 174-181.

Ohashi, T. and Abe, M. (1970). *J. Agric. Chem. Soc.*, 44, 519-526.

Ohashi, T., Aoki, S. and Abe, M. (1970). *J. Agric. Soc.*, 44, 527-531.

Ohashi, T., Shibuya, N. and Abe, M. (1972). Production of alkaloids and related substances by fungi. IX. A biosynthetic route from tryptophan to ergot alkaloids. *Nippon Nogei Kagaku Kaishi*, 46, 207-213.

Otsuka, H., Anderson, J.A. and Floss, H.G. (1979). The stage of N-methylation in ergot alkaloid biosynthesis. *J. Chem. Soc. Chem. Comm.*, 2, 660.

Otsuka, H., Quigley, F.R., Gröger, D., Anderson, J.A. and Floss, H.G. (1980). *In vivo* and *in vitro* evidence for N-methylation as the second pathway-specific step in ergoline biosynthesis. *Planta Medica.*, 40, 109.

Pitt, J.I. (1985). A Laboratory Guide to Common *Penicillium* Species. Commonwealth Scientific and Industrial Rearch Organisation.

See p.211

Plieninger, H., Höbel, M. and Leide, V. (1963). Synthese des 4-Dimethyl-allyl-tryptophans sowie der Tryptophanessigsäure-(4). *Chem. Ber.*, 96, 1618-1629.

Plieninger, H., Fischer, R., and Leide, V. (1964). Untersuchungen zur Biosynthese des Mutterkornalkaloide II. *Justus Liebig's Ann. Chem.*, 672, 223-231.

Plieninger, H. and Immel, H. (1965). Die Darstellung von reinem 3,3-Dimethyl-allylpyrophosphat nach dem Verfahren von F. Cramer. *Chem. Ber.*, 98, 414-418.

Porter, J.K., Bacon, C.W. and Robbins, J.D. (1979). Ergosine, Ergosinine and Chanoclavine I from *Epichloë typhina*. *J. Agric. Food Chem.*, 27, 595-598

Purchase, I.F.H. and Theron, J.J. (1968). The acute toxicity of ochratoxin A to rats. *Food Cosmet. Toxicol.*, 6(4), 479-483.

Queener, S.W., Sebek, D.K. and Vezina, C. (1978). Mutants blocked in antibiotic synthesis. *Ann. Rev. Microbiol.*, 32, 593.

Quilico, A. and Panizzi, L. (1943). Chemische Untersuchungen über *Aspergillus echinulatus*, I. Mitteilung. *Chem. Ber.*, 76, 348-358.

Robbers, J.E. and Floss, H.G. (1968). Biosynthesis of ergot alkaloids: Formation of 4-dimethylallyltryptophan by the ergot fungus. *Arch. Biochem. Biophys.* 126, 967-969.

Robbers, J.E., Robertson, L.W., Hornemann, K.M., Jindra, A. and Floss, H.G. (1972). Physiological studies on ergot: Further studies on the induction of alkaloid synthesis by tryptophan and its inhibition by phosphate. *J. Bacteriol.*, 112, 791-796.

Robbers, J.E., Otsuka, H. and Floss, H.G. (1980). Clavicipitic acid: Its structure, biosynthesis and role in ergot alkaloid formation. *J. Org. Chem.*, 45, 1117-1121.

Rochelmeyer, H. (1958). *Pharm. Ztg.*, 103, 1269-1275.

Rowan, D.D., Hunt, M.B. and Gaynor, D.L. (1986). Peramine, a novel insect feeding deterrent from ryegrass infected with the endophyte *Acremonium loliae*. *J. Chem. Soc. Chem. Comm.*, 935-936.

Saini, M.S., Cheng, M. and Anderson, J.A. (1976). Conversion of 4-dimethylallyltryptophan to clavicipitic acid. Some properties of the enzyme. *Phytochemistry*, 15, 1497-1500.

Saito, M., Enomoto, M. and Tatsuno, T. (1971). Yellowed rice toxins. Luteoskyrin and related compounds, chlorine-containing compounds and citrinin. In: Microbial Toxins, Vol 6, Ciegler, A., Kadis, S. and Ajl, S.J., eds., pp. 299-380. Academic Press, London.

Sammes, P.G. (1975). Naturally occurring 2,5-dioxopiperazines and related compounds. *Fortschritte. Chem. Org. Naturstoffe, 32, 51-118.*

Scott, P.M., Merrien, M.A. and Polonsky, J. (1976). Roquefortine and isofumigaclavine A, metabolites from *Penicillium roquefortii*. *Experientia*, 32, 140-142.

Scott, P.M., van Walbeek, W., Harwig, J. and Fennell, D.I. (1970). Occurrence of a mycotoxin, ochratoxin A, in wheat and isolation of ochratoxin A and citrinin-producing strains of *Penicillium viridicatum*. *Can. J. Plant Sci.*, 50, 583-585.

Singh, R. and Hsieh, D.P.H. (1977). Aflatoxin biosynthetic pathway; elucidation by using blocked mutants of *Aspergillus parasiticus*. *Arch. Biochem. Biophys.*, 178, 285-292.

Spilsbury, S.F. and Wilkinson, S.W. (1961). The isolation of festuclavine and two new clavine alkaloids from *Aspergillus fumigatus* Fres. *J. Chem. Soc.*, 2, 2085-2091.

Springer, J.P., Clardy, J., Wells, J.M., Cole, R.J. and Kirksey, J.W. (1975). The structure of paxilline, a tremorgenic metabolite of *Penicillium paxilli*. *Tetrahedron Lett.*, 30, 2531-2534.

Springer, J.P., Büchi, G., Kobbe, B., Demain, A.L. and Clardy, J. (1977). The structure of ditryptophenaline: A new metabolite of *Aspergillus flavus*. *Tetrahedron Lett.*, 2403-2406.

Sternbach, L.H. (1971). 1,4-Benzodiazepines. Chemistry and some aspects of the structure-activity relationship. *Angew. Chem. Int. Ed.*, 10, 34-43.

Steyn, P.S. (1971). Austamide, a new toxic metabolite from *Aspergillus ustus*. *Tetrahedron Lett.*, 36, 3331-3334.

Steyn, P.S. (1973). The structure of five diketopiperazines from *Aspergillus ustus*. *Tetrahedron*, 29, 107-120.

Stoll, A. (1918). Swiss Patent No. 79879.

Stoll, A. (1945). Über Ergotamin. *Helv. Chim. Acta.*, 28, 1283-1308.

Stüssi, H. and Rast, D.M. (1981). The biosynthesis and possible function of γ-glutaminyl-4-hydroxybenzene in *Agaricus bisporus*. *Phytochemistry*, 20, 2347-2352.

Szczyrbak, C.A. (1972). Production Of Clavine Alkaloids *In Vitro* By *Claviceps fusiformis*. Ph.D. Thesis, University of London.

Taber, W.A. and Vining, L.C. (1959). Tryptophan as a precursor of the ergot alkaloids. *Chem. Ind. N.Y.*, pp. 1218-1219.

Takase, S., Kawai, Y., Uchida, I., Tanaka, H. and Aoki, H. (1985). Structure of amauromine, a new hypotensive vasodilator produced by *Amauroascus* sp. *Tetrahedron*, 41, 3037-3048.

Tanret, C. (1875). *Compt. Rend. Acad. Sci., Paris*, 81/82, 896.

Taylor, E.H. and Ramstad, E. (1960). Biogenesis of lysergic acid in ergot. *Nature, London*, 188, 494-495.

Teuscher, E. (1964). *Flora*, 155, 80.

Thompson, M.R. (1935). *Science*, 81, 636-639.

Turner, W.B. (1971). Fungal Metabolites. Academic Press, London.

Turner, W. B. and Aldridge, D.C. (1983). Fungal Metabolites II. Academic Press, London.

van der Merwe, K.J., Steyn, P.S., Fourie, L., Scott, De B. and Theron. J.J. (1965). Ochratoxin A: a toxic metabolite produced by *Aspergillus ochraceus*. *Nature*, 205, 1112.

Vicar, J., Budesinsky, M. and Blaha, K. (1973). Proton magnetic resonance studies of cyclopeptides containing pipecolic acid, proline and/or 2-azetidine-carboxylic acid. *Collection Czechoslov. Chem. Commun.*, 38, 1940-1956.

Voight, R. (1962). *Pharmazie*, 17, 101-106.

White, E.C. and Hill, J.H. (1943). *J. Bacteriol.*, 45, 433-443.

Willingale, J., Atwell, S.M. and Mantle, P.G. (1983). Unusual Ergot Alkaloid Biosynthesis in Sclerotia of a *Claviceps purpurea* Mutant. *J. Gen. Microbiol.*, 129, 2109-2115.

Wurst, M., Flieger, M. and Rehácek, Z. (1978). Analysis of ergot alkaloids by HPLC. I. Clavines and simple derivatives of lysergic acid. *Journal of Chromatography*, 150, 477-483.

Yamamoto, Y., Nishimura, K. and Kiriyama, N. (1976). Studies on the metabolic products of *Aspergillus terreus*. I. Metabolites from the strain IFO 6123. *Chem. Pharm. Bull.*, 24, 1853-1859.

Yamazaki, M., Suzuki, S. and Miyaki, K. (1971). Tremorgenic toxins from *Aspergillus fumigatus* Fres. *Chem. Pharm. Bull.*, 19, 1739.

Yeulet, S.E. and Mantle, P.G. (1987). Biosynthesis of auranthine by *Penicillium aurantiogriseum*. *FEMS Microbiology Letters*, 41, 207-210.

Yeulet, S.E., Mantle, P.G., Bilton, J.N., Rzepa, H.S. and Sheppard, R.N. (1986). Auranthine, a new benzodiazepinone metabolite of *Penicillium aurantiogriseum*. *J. Chem. Soc. Perkin Trans. I*, 1891-1894.

Young, P.E., Madison, V. and Blout, E.R. (1976). Cyclic peptides. 15. Lanthanide-assisted ^{13}C and ^{1}H NMR analysis of preferred side-chain rotamers in proline-containing cyclic dipeptides. *J. Am. Chem. Soc.*, 98, 5365-5371.

☆Mantle, P.G. and Tonolo, A. (1968). Relationship between the morphology of Claviceps purpurea and the production of alkaloids. Trans. Brit. Mycol. Soc., 51, 499-506.

Pitt, D. and Poole, P.C. (1981). Calcium-induced conidiation in Penicillium notatum in submerged culture. Trans. Brit. Mycol. Soc., 76, 219-230.