METABOLISM OF TERTIARY ARYLALIPHATIC AMINES AND FORMAMIDES IN RATS

by

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ABSTRACT

The metabolites of the basic tertiary arylaliphatic amine N,N,α-trimethyl-γ-phenylbenzenepropanamine (Recipavrin^R) from male Wistar rats were characterized by gas chromatography-mass spectrometry (GCMS). The work was undertaken in an attempt to determine the source of a novel metabolite, N-(1-methyl-3,3-diphenylpropyl) formamide. The formamide metabolite was isolated from the bile of recipavrin dosed rats only after hydrolysis with the enzyme β-glucuronidase, suggesting that it arose from a glucuronide conjugated precursor. Recipavrin was chosen for the study based on structural similarity to the narcotic analgesic methadone which was shown to give rise to a similar metabolite, 6-formamido-4,4-diphenyl-3-heptanone.

The secondary formamide was not a plausible candidate for a β-glucuronidase liberated metabolite of recipavrin, suggesting that a labile aglycone was responsible for the GCMS observation of the formamide metabolite. Labile isomeric compounds, α-methyl-(N-methylene)-γ-phenylbenzenepropanamine N-oxide, N-(α-methyl-γ-phenylbenzenpropylidene) methylamine N-oxide, and 2-(4',4'-diphenyl-but-2'-yl) oxaziridine were synthesized as possible precursors of the formamide. N-hydroxy-α-methyl-γ-phenylbenzenepropanamine, and N-hydroxy-N,α-dimethyl-γ-phenylbenzenepropanamine were synthesized as candidates for labile β-glucuronidase liberated aglycone precursors of the nitrones.

The biliary nonconjugated and conjugated metabolites of recipavrin were characterized in detail. In addition to the formamide, 15 different metabolites representing the N-

dealkylation, oxidative deamination, N-oxidation and phenyl ring oxidation pathways were identified by GCMS. To determine if thermal decomposition of the methylene nitrone in the GC inlet was responsible for the GCMS observation of the formamide metabolite, liquid chromatography-mass spectrometry (LCMS) was used to show that the formamide and not the isomeric methylene nitrone was present in bile prior to GCMS analysis. Although the synthetic methylene nitrone was shown to degrade in the GC inlet to the formamide, the LCMS experiment ruled out the thermal generation of the biliary formamide from a nitrone precursor.

The nonconjugated and conjugated metabolites of the recipavrin metabolite, norrecipavrin were characterized in detail by GCMS. Since the secondary formamide metabolite was observed in the β-glucuronidase hydrolyzed bile extract, norrecipavrin was implicated as an intermediate in the biotransformation of recipavrin to the formamide.

The possibility of solvent mediated formylation or free radical oxidation of desalkyl metabolites to afford the formamides was ruled out.

The methylene nitrone was shown to afford the formamide metabolite under simulated workup conditions. An alkali catalyzed Beckmann rearrangement of nitrone to amide was used to account for this transformation.

The secondary hydroxylamine was shown to give rise to the methylene nitrone under simulated workup conditions.

It was concluded that the oxidation of a β-glucuronidase liberated secondary hydroxylamine metabolite to the methylene

nitrone followed by Beckmann rearrangement of the nitrone to the formamide was the probable source of the formamide observed by GCMS in extracts of bile from recipavrin dosed rats.

The metabolism of N-methyl-N-(1-methyl-3,3-diphenylpropyl) formamide was investigated in detail to determine whether the carbinolamide, N-hydroxymethyl-N-(1-methyl-3,3-diphenylpropyl) formamide was involved in the genesis of the formamide metabolite of recipavrin. The above carbinolamide and N-(1-hydroxy-1-methyl-3,3-diphenylpropyl) formamide were identified by GCMS along with 16 other metabolites representing the metabolic pathways N-deformylation, N-dealkylation, N-oxidation and phenyl ring oxidation. The carbinolamides were not found in bile from recipavrin dosed rats, ruling out the possibility of a carbinolamide glucuronide precursor of the recipavrin formamide metabolite. This was the first report of the isolation of stable dealkylation intermediates of a high molecular weight formamide. The hepatotoxicity of the anticancer agent N-methyl formamide and the solvent dimethylformamide, suggests that the recipavrin formamides could also be metabolized to toxic carbinolamide or glutathione related metabolites.

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Dr. F.S. Abbott, Research Supervisor.

TABLE OF CONTENTS

ABSTRACT.....ii

TABLE OF CONTENTS.....v

LIST OF FIGURES.....xvi

LIST OF TABLES.....xxvi

LIST OF ABBREVIATIONS.....xxviii

ACKNOWLEDGEMENTS.....xxxi

DEDICATION.....xxxii

I. INTRODUCTION.....1

1. OBSERVATION OF A FORMAMIDE METABOLITE OF METHADONE.....1

2. RECIPAVRIN: A STRUCTURAL ANALOGUE OF METHADONE.....2

3. OVERVIEW OF PREVIOUS WORK ON RECIPAVRIN (SLATTER, 1983).....4

4. METABOLISM OF TERTIARY ARYLALIPHATIC AMINES.....8

5. FORMAMIDE METABOLITES OF AMINES AND AMIDES.....9

6. POSSIBLE SOURCES OF FORMAMIDE METABOLITES OF METHADONE (8) AND RECIPAVRIN (9).....11

A. Formamide artifacts arising from metabolite extraction using organic solvents.....11

B. Formamide artifacts arising from oxidation of amine metabolites during sample isolation.....13

C. Formamide metabolites arising by peroxidation of iminium dealkylation intermediates.....13

D. Formamide metabolites arising by decomposition of N-oxidized metabolites.....16

i. Hydroxylamine metabolites as precursors of nitrones.....16

ii. Nitrones as drug metabolites.....17

iii. Nitrones as chemical precursors of formamide metabolites: Rearrangements of Nitrones.....18

a. Behrend Rearrangement.....18

b. Martynoff Rearrangement.....19

c. Beckmann Rearrangement.....19

d. Photochemical rearrangements.....20

E. Formamide metabolites arising from a carbinolamide precursor.....23

i. Stability and occurrence of carbinolamine and carbinolamides intermediates in drug metabolism studies.....23

ii. Metabolism of formamides.....25

iii. Importance of metabolic studies on arylaliphatic formamides.....25

7. OBJECTIVES OF THE THESIS.....26

II. EXPERIMENTAL.....28

1. CHEMICALS AND MATERIALS.....28

2. ANIMAL EXPERIMENTS.....31

3. PREPARATION OF TLC SPRAY REAGENTS.....32

4. INSTRUMENTATION.....33

A. NMR spectra.....33

B. Infrared spectra.....33

C. Ultraviolet spectra.....33

D. Melting points.....33

E. Capillary GCMS.....34

F. Direct insertion probe mass spectra.....35

G. Packed column GCMS analysis.....36

H. Liquid chromatography-mass spectrometry.....37

I. High resolution mass spectrometry.....38

5.METABOLISM EXPERIMENTS.....39

A. Animal Surgery and Drug Administration.....39

B. Bile sample preparation for GCMS.....40

C. Derivatization Methods.....41

6. CHEMICAL SYNTHESES.....42

A. Flash chromatography.....42

B. Synthetic compounds related to methadone.....42

i. General procedure for meta-chloroperbenzoic acid (MCPBA) oxidations of methadone (8) and EDDP (1).....42

ii. GCMS identification of 6-N-methylformamido -4,4-diphenyl-3-heptanone (Methadone tertiary formamide (49)).....44

iii. Synthesis of 2,3-dimethyl-5,5-diphenylcyclopent-2-enone (50).....44

C. Synthetic compounds related to recipavrin.....45

i. 1,1-Diphenyl-3-butanone (14) (CAS registry 5409-60-9) (Slatter, 1983).....45

ii. Syn/anti 1,1-diphenyl-3-butanone oxime (19) (CAS registry 36317-57-4)(Slatter, 1983).....45

iii. N,N,α-trimethyl-γ-phenylbenzenepropanamine hydrochloride (Recipavrin^{R} (9)) (CAS 13957 -55-6)(Slatter, 1983).....46

iv. N,N,α-trimethyl-γ-phenylbenzenepropanamine N-oxide (recipavrin N-oxide (53)).....47

v. α-Methyl-γ-phenylbenzenepropanamine (CAS registry 29869-77-0) (dinorrecipavrin (20)) (Slatter, 1983).....48

vi. N-hydroxy-α-methyl-γ-phenylbenzene-propanamine (primary hydroxylamine (22)) (Slatter, 1983).....48

vii. Spectral characterization of the hydroxyl-amine autoxidation product (56).....50

viii. N,α-dimethyl-γ-phenylbenzene-propanamine (norrecipavrin (15)) (CAS 29869-78-1)(Slatter, 1983).....50

ix. N-hydroxy-N,α-dimethyl-γ-phenylbenzene-propanamine (secondary hydroxylamine (17))(Slatter, 1983).....51

x. α-Methyl-(N-methylene)-γ-phenylbenzene-propanamine N-oxide (methylene nitrone (24))(Slatter, 1983).....52

xi. Cis/trans N-(*a*-methyl-γ-phenylbenzene-propylidene) methylamine-N-oxide (methyl nitrone (44))....53

xii. *a*-Methyl-(N-methylene)-γ-phenylbenzene-propanamine (polymer) (methylene imine (23) as triazane (41))(Slatter, 1983)....54

xiii. 2-(4',4'-Diphenyl-but-2'yl) oxaziridine (25)(Slatter, 1983)....56

xiv. N-(1-methyl-3,3-diphenylpropyl) formamide (12) (Slatter, 1983)....57

xv.Characterization of the Leuckart specific byproduct 4-(2,2-diphenylethyl) pyrimidine (59)....60

xvi. N-(1-methyl-3,3-diphenylpropyl) formohydroxamic acid (61)....60

xvii. N-methyl-N-(1-methyl-3,3-diphenylpropyl) formamide (26)....61

xviii. N-hydroxymethyl-N-(1-methyl-3,3-diphenylpropyl) formamide (carbinolamide (47))....63

xix. N-(1-methyl-3,3-diphenylpropyl) acetamide (63)....64

xx. N-(1-methyl-3,3-diphenylpropyl) ethanimine (64)....64

xxi. 3-Carbylamino-1,1-diphenylbutane (recipavrin isocyanide (65))....65

xxii. N-(1-methyl-3,3-diphenylpropyl) carbamic acid methyl ester (dinorrecipavrin methylcarbamate (66))....65

xxiii. N-(1-methyl-3,3-diphenylpropyl) carbamic acid ethyl ester (dinorrecipavrin ethylcarbamate (67))....66

xxiv. N-methyl-N-(1-methyl-3,3-diphenylpropyl) carbamic acid methyl ester (norrecipavrin methyl carbamate, (68))....67

xxv. N-methyl-N-(1-methyl-3,3-diphenylpropyl) carbamic acid ethyl ester (norrecipavrin ethyl carbamate (69))....68

D. Synthetic Compounds Related to Promethazine....68

i. 10-(2-Propynyl) phenothiazine (70)....69

ii. 10-(2-Propanone) phenothiazine (71).....69

iii. 10-(2-formamidopropyl) phenothiazine (72).....69

iv. Leuckart specific byproduct, 4-(1-(10-phenothiazinyl) methyl) pyrimidine (73).....71

v. 10-(2-Formamidopropyl) phenothiazine N_{10}-oxide (75).....71

vi. 10-(N-methyl-2-formamidopropyl) phenothiazine (74).....73

vii. 10-(N,N-dimethyl-N-oxo-2-aminopropyl) phenothiazine (promethazine N-oxide (76), Clement and Beckett, 1981b).....74

viii. Characterization of promethazine free base (10).....75

E. Synthetic compounds related to amphetamine.....76

i. α-Methyl-(N-methylene)-β-phenylbenzene-ethanamine (78).....76

ii. 2-(3'-phenylprop-2'-yl) oxaziridine (82).....77

iii. N-(1-methyl-2-phenylethyl) formamide (83) (CAS registry 42044-69-9).....78

7. EXPERIMENTS TO DETERMINE THE SOURCE OF FORMAMIDE METABOLITES OF METHADONE AND RECIPAVRIN.....79

A. Solvent related artifact control experiments for methadone metabolites.....79

B. Effect of added synthetic secondary formamide on peak shape and retention time of the recipavrin formamide metabolite (12).....80

C. Experiments to establish that the secondary formamide metabolite (12) of recipavrin arises from a glucuronide precursor.....81

i. Sulfatase hydrolysis of the recipavrin conjugated metabolites.....81

ii. Control incubation of recipavrin conjugated fraction without β-glucuronidase enzyme.....82

D. Free radical oxidation of recipavrin and norrecipavrin as a source of the secondary formamide (12).....82

i. Incubation of recipavrin (9) with blank bile under simulated workup conditions.....82

ii. Incubation of norrecipavrin with blank bile under simulated workup conditions.....83

E. Effect of extraction pH on the observation of the recipavrin secondary formamide metabolite (12).....83

F. Effect of immediate sample preparation and storage on the secondary formamide metabolite of recipavrin.....83

G. Solvent related artifact control experiments for recipavrin metabolites.....84

H. Solid phase extraction (SPE) of recipavrin metabolites.....84

i. Cartridge conditioning and sample elution (J.T. Baker Ltd., 1982).....85

a. Reversed phase octadecyl (RPC_{18}) cartridges.....85

b. Forward phase silica gel ($FPSiO_2$) cartridges.....85

ii. Amberlite XAD-2 column preparation.....86

iii. Preliminary solid phase extraction experiments.....86

a. Elution of synthetic reference compounds from RPC_{18} columns.....86

b. RPC_{18} test elutions using UV and TLC detection of eluates.....87

iv. Fractionation of bile components by SPE methods.....87

a. Attempted fractionation of biliary recipavrin metabolites using RPC_{18} columns.....87

b. Preliminary cleanup and deionization of bile using a XAD-2 column.....88

c. Separation of all metabolites from extremely polar bile constituents.....88

d. Separation of nonconjugated metabolites on $FPSiO_2$ cartridges.....89

I. Attempts to increase secondary formamide (12) production by the addition of formic acid, formaldehyde, or formaldehyde and hydrogen peroxide during sample preparation.....90

J. Attempts to decrease secondary formamide (12) production with the use of antioxidants and a formaldehyde complexing agent.....92

K. Effect of the antioxidant BHT, formic acid and formaldehyde on dinorrecipavrin metabolite profiles.....93

L. Decomposition of recipavrin N-oxide (53) under simulated workup conditions.....93

M. GCMS analysis and decomposition of the methylene nitrone (24).....94

III. RESULTS AND DISCUSSION.....96

1. FINAL EXPERIMENTS CONCERNING THE SYNTHESIS AND POSSIBLE SOURCES OF THE METHADONE FORMAMIDE METABOLITE.....96

A. Mechanisms for the peroxidation of EDDP to an oxaziridine, diketone and related compounds.....96

i. High resolution mass spectrum of the methadone oxaziridine (2).....97

ii. MCPBA oxidation of methadone HCl.....97

iii. Peroxidation of EDDP free base in the presence of suspended K2CO3.....99

iv. Mechanism for the peroxidation of EDDP.....103

B. Solvent control experiments in the isolation of the methadone formamide metabolite.....107

C. Conclusions regarding the source of the methadone formamide metabolite.....110

2. SYNTHESIS AND CHARACTERIZATION OF SYNTHETIC COMPOUNDS RELATED TO RECIPAVRIN.....110

A. Synthesis and Characterization of the amines.....111

B. Synthesis and Characterization of the amides.....111

C. Synthesis and Characterization of oximes, hydroxylamines and their oxidation products.....122

D. Synthesis and Characterization of the recipavrin N-oxide.....127

E. Synthesis and Characterization of the Nitrones.....127

F. Synthesis and Characterization of the methylene imine (23) (polymer) and oxaziridine (25).....128

G. Synthesis and Characterization of the isocyanide (65) and carbamates (66-69).....133

H. Attempted synthesis and characterization of the formohydroxamic acid (61).....134

I. Synthesis and Characterization of the promethazine related formamides and their oxidation products.....137

J. Synthesis and Characterization of the oxaziridine (82), methylene imine (78) and formamide (83) related to amphetamine.....149

3. MASS SPECTROMETRY OF RECIPAVRIN RELATED COMPOUNDS.....154

A. Fragmentation of the aliphatic side chain.....154

B. Fragmentation of the substituted diphenylbutane moiety.....161

4. METABOLISM OF RECIPAVRIN.....163

A. Biliary metabolites of recipavrin.....174

i. GCMS observation of the intact formamide.....174

ii. LCMS Demonstration of the secondary formamide metabolite.....180

iii. Oxidative deamination.....183

iv. N-dealkylation.....184

v. Phenyl ring oxidation.....187

vi. N-oxidation.....188

B. Nonconjugated biliary metabolites of recipavrin.....191

C. Recipavrin metabolism conclusions.....191

5. METABOLISM OF NORRECIPAVRIN.....194

A. Conjugated metabolites.....194

B. Nonconjugated metabolites.....201

C. Conclusions regarding norrecipavrin metabolism 201
6. METABOLISM OF DINORRECIPAVRIN.....204

A. Metabolites of dinorrecipavrin.....204

7. EXPERIMENTS TO DETERMINE THE SOURCE OF THE FORMAMIDE METABOLITE.....209

A. Dilution of bile extracts with the synthetic secondary formamide (12).....209

B. Attempted detection of chloroform generated artifacts.....212

C. Experiments to establish that the secondary formamide metabolite of recipavrin arises from a glucuronide precursor.....214

i. Sulfatase hydrolysis of the conjugated metabolites.....214

ii. Control incubation of the recipavrin metabolite conjugated fraction without β-glucuronidase enzyme.....214

D. Free radical oxidation of recipavrin (9) and norrecipavrin (15) as a source of the formamide (12).....216

i. Incubation of recipavrin with control bile under simulated workup conditions.....216

ii. Incubation of norrecipavrin with blank bile under simulated workup conditions.....217

iii. Effect of extraction pH on the observation by GCMS of the recipavrin secondary formamide metabolite.....218

E. Solid phase extraction of recipavrin metabolites.....218

i. Preliminary experiments.....218

a. Elution of synthetic reference compounds from RPC_{18} columns.....218

b. RPC_{18} test elutions using UV and TLC detection of eluates.....219

ii. Fractionation of bile components by SPE methods.....220

a. Attempted fractionation of biliary recipavrin metabolites using RPC_{18} columns.....220

b. Preliminary cleanup and deionization of bile using a XAD-2 column.....221

c. Fractionation on $FPSiO_2$ columns.....221

d. Separation of nonconjugated metabolites on the $FPSiO_2$ column.....222

e. Conclusions regarding the use of SPE cartridges.....226

F. Attempts to increase secondary formamide (12) production by the addition of formic acid, formaldehyde, or formaldehyde and hydrogen peroxide during sample preparation.....226

i. Nonconjugated fraction.....227

ii. Conjugated fraction.....230

G. Attempts to decrease formamide production with the use of antioxidants and a formaldehyde complexing reagent.....232

8. METABOLISM OF PROMETHAZINE (10): SEARCH FOR FORMAMIDE METABOLITES.....234

9. DECOMPOSITION OF N-OXIDIZED METABOLITES AS A SOURCE OF THE SECONDARY FORMAMIDE (12) ISOLATED FROM RAT BILE.....242

A. Decomposition of recipavrin N-oxide under simulated sample workup conditions.....242

B. GCMS analysis and thermal decomposition of the methylene nitrone.....247

C. Alkali catalyzed rearrangement of nitrones to amides.....255

10. METABOLISM OF THE RECIPAVRIN METHYLENE NITRONE 256

11. METABOLISM OF THE RECIPAVRIN TERTIARY FORMAMIDE (26).....261

A. Mass spectrometry of arylaliphatic formamides and carbinolamides.....270

B. Metabolism of the tertiary arylaliphatic formamide.....278

i. N-deformylation.....278

ii. Phenyl ring oxidation and conjugation.....280

iii. N-dealkylation via carbinolamides to desalkylformamides.....281

12. METABOLISM OF THE SECONDARY FORMAMIDE (12).....283

IV. SUMMARY AND CONCLUSIONS.....292

V. REFERENCES.....298

VI. APPENDIX.....314

LIST OF FIGURES

Figure 1. (top) Products derived from the chemical oxidation of EDDP (1). (bottom) Structure of methadone (8), recipavrin (9), promethazine (10), dimethylamphetamine (11), and the formamide metabolite (12) of recipavrin. 3

Figure 2. Preliminary syntheses of potential N- and alpha C- oxidized metabolites of recipavrin (9). 5

Figure 3. Superimposed LCMS ion chromatograms of the M^++1 m/z 254 ion of the synthetic methylene nitrone (24), oxaziridine (25) and formamide (12). 7

Figure 4. Structures of parent drug and formamide metabolites of prenylamine (27), aminopyrine (28), caffeine (29), amantidine (30), mianserin (31), indecainide (32), pipemidic acid (33), aminoanthraquinone (34), 2-naphthylamine (35), chlortoluon (36), etc. 10

Figure 5. Schematic showing possible metabolic (m) and chemical (c) transformations that could account for a secondary formamide metabolite (12) in the β-glucuronidase hydrolyzed fraction of bile from recipavrin dosed rats. 15

Figure 6. Ionic mechanisms for the cleavage of C-O or N-O bonds in the oxaziridine ring to afford nitrones or amides respectively. 16

Figure 7. Back polarization between two canonical forms of a nitrone. 18

Figure 8. Behrend rearrangement of a N-methyl nitrone to a methylene nitrone. 19

Figure 9a. Mechanism for the Beckmann rearrangement of nitrones to amides catalyzed by acylating agents (Lamchen, 1968). 21

Figure 9b. Mechanism for the Beckmann rearrangement of nitrones to amides catalyzed by alkali (Lamchen, 1968, Zinner, 1978). 21

Figure 10. A modified Beckmann rearrangement of a hypothetical nitrone glucuronide (46) catalyzed by β-glucuronidase (after Lamchen, 1968). 22

Figure 11. Free radical mechanism for the photochemical rearrangement of a nitrone to an oxaziridine (Lamchen, 1968). 23

Figure 12. Metabolism of recipavrin (9) to the secondary formamide (12) via a hypothetical carbinolamide pathway. 24

Figure 13. (top) Composite of high resolution mass spectral results for the methadone oxaziridine (2) (source temperature 120o). (bottom) Mass spectrum of the methadone formamide (6) arising from GCMS analysis of the methadone oxaziridine. 98

Figure 14. Mass spectrum of the tertiary formamide (49) formed by MCPBA oxidation of methadone HCl. M^+ 323 absent. 100

Figure 15. Possible regioisomeric cyclopentenones (50 and 51) expected from the aldol condensation of the diketone. 101

Figure 16. NMR spectrum of the cyclopentenone aldol product 2,3-dimethyl-5,5-diphenylcyclopent-2-enone (50). 102

Figure 17. Bayer-Villiger MCPBA addition to C=N+ bonds in the first step of the peroxidation of EDDP (1). 103

Figure 18. Possible mechanisms for the formation of the oxidation products of EDDP that were observed by GCMS. 105

Figure 19. Mechanism for the formation of DDP (4) from oxidation of the enamine tautomer of EDDP. 106

Figure 20. 400 MHz ^1H NMR spectrum of the secondary formamide (12). 114

Figure 21. 75 MHz broad band decoupled ^{13}C NMR spectrum of the secondary formamide (12). 115

Figure 22. Infrared spectrum of a nujol mull of the secondary formamide (12). NH stretches 3260 (trans), 3200 (shoulder, cis). C=O stretches 1670 (trans), 1650 (cis). 116

Figure 23. (top) Mass spectrum of the secondary formamide (12)(M^+ 253), (bottom) Mass spectrum of the secondary formamide mono TMS derivative (60)(M^+ 325). 117

Figure 24. 270 MHz NMR spectrum of the pyrimidine byproduct (59) of formamide synthesis. 118

Figure 25. 300 MHz ^1H NMR spectrum of the tertiary formamide (26). 119

Figure 26. 75 MHz ^{13}C NMR spectrum of the tertiary formamide (26). Top. Broad band decoupled. Bottom. Attached proton test. 120

Figure 27. Infrared spectrum of a thin film of the tertiary formamide (26). C=O stretch at 1666 cm^{-1}. 121

Figure 28. 300 MHz NMR spectrum of the primary hydroxylamine (22). 124

Figure 29. Infrared spectrum of a thin film of the primary hydroxylamine (22). 125

Figure 30. Mass spectra of the underivatized primary (22 top) and secondary (17 bottom) hydroxylamines. 126

Figure 31. 300 MHz ^{1}H NMR spectrum of the N-methyl nitrone (44). 129

Figure 32. 100 MHz ^{13}C NMR spectrum of the N-methyl nitrone (44). 130

Figure 33. Mass spectrum of the suspected methyl ether of the formohydroxamic acid (61). 136

Figure 34. 400 MHz ^{1}H NMR spectrum of the secondary formamide (72). 140

Figure 35. 100 MHz ^{13}C NMR SFORD spectrum of the secondary formamide (72). 141

Figure 36. FTIR spectrum (film) of secondary formamide (72). 142

Figure 37. 400 MHz ^{1}H NMR spectrum of the secondary formamide N_{10}-oxide (75) showing only one rotamer is present. 143

Figure 38. 100 MHz broad band decoupled ^{13}C NMR spectrum of the secondary formamide N_{10}-oxide (75). 144

Figure 39. FTIR spectrum (film) of the secondary formamide N_{10}-oxide (75). 145

Figure 40. 400 MHz ^{1}H NMR spectrum of the tertiary formamide (74). 146

Figure 41. IR spectrum of a thin film of the tertiary formamide (74). 147

Figure 42. Mass spectrum of the tertiary formamide (74). 148

Figure 43. 80 MHz NMR spectrum of the amphetamine methylene imine derived polymers (79-81). 151

Figure 44. 400 MHz NMR spectrum of the amphetamine oxaziridine (82). 152

Figure 45. 80 MHz NMR spectrum of the amphetamine secondary formamide (83). 153

Figure 46. General mass spectral fragmentations of diarylbutanes: I. Radical site initiation at the saturated heteroatom with alpha cleavage. 156

Figure 47. General mass spectral fragmentations of diarylbutanes: II. Rearrangement of the gamma hydrogen to the saturated hetero atom with adjacent cleavage and charge retention. 157

Figure 48. General mass spectral fragmentations of diarylbutanes: III. Radical site initiation on the aromatic ring with allylic (benzylic) cleavage. 158

Figure 49. General mass spectral fragmentations of diarylbutanes: IV. Rearrangement of gamma hydrogen with benzylic cleavage and loss of a hydrogen radical to afford diagnostic oxonium ions in phenyl ring oxidized metabolites and their derivatives. 158

Figure 50. Mass spectra of recipavrin metabolites illustrating the three major fragmentation schemes in figure 46,47,48. A. Norrecipavrin (15). B. Secondary formamide (12). 159

Figure 50. Mass spectra of recipavrin metabolites illustrating the three major fragmentation schemes in figures 46,47,48. C. anti-diphenylbutanone oxime (19). D. Recipavrin phenol (90). 160

Figure 51. General structures for metabolites: A. Single bonded R1 substituent. B. Double bonded R1 substituent. 164

Figure 52. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.I. Total ion current for all metabolites (above) and their TMS derivatives (below). 167

Figure 53. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.II. Ion chromatogram m/z 167 showing phenyl ring intact metabolites (above) and their TMS derivatives (below). 168

Figure 54. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.III. Ion chromatogram m/z 183 showing intact phenol metabolites (above) and m/z 255 showing TMS derivatized phenols. 169

Figure 55. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.IV. Ion chromatogram m/z 213 showing intact O-methylcatechol metabolites (above) and m/z 285. 170

Figure 56. (top) Mass spectrum of the synthetic secondary formamide (12). (bottom) Mass spectrum of the underivatized secondary formamide metabolite. 176

Figure 57. (top) Chemical ionization mass spectrum of the synthetic secondary formamide (12). (bottom) Chemical ionization mass spectrum of the secondary formamide metabolite. 177

Figure 58. (top) Mass spectrum of the TMAH derivatized secondary formamide. (bottom) Mass spectrum of the unidentified TMS derivative (95). 178

Figure 59. Mass spectrum of the TMS derivative of the amphetamine secondary formamide (84). 179

Figure 60. LCMS mass spectrum of the secondary formamide (12)(top) and the methylene nitrone (24)(bottom) in the acetonitrile/ water solvent system. 181

Figure 61. Superimposed LCMS selected ion monitoring results for A. A β-glucuronidase-hydrolyzed bile extract from a recipavrin dosed rat. B. A mixture of the synthetic methylene nitrone (24) and secondary formamide (12)(10 ng each) standards. 182

Figure 62. Total ion current and selected ion chromatograms for a nonconjugated extract of biliary recipavrin metabolites. (top) Total ion current for all metabolites. (bottom) Ion chromatogram m/z 167 showing phenyl ring intact metabolites. 192

Figure 63. Metabolic pathways for recipavrin based on the metabolites observed by GCMS. 1a. Phenyl ring oxidation. 1b. Oxidation to the catechol and 3'-O-methylation. 2a. Oxidative deamination. 2b. Ketone reduction. 3. N-dealkylation. 4. N-oxidation. 193

Figure 64. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary norrecipavrin metabolites. I. Total ion current for all conjugated norrecipavrin metabolites (above) Ion chromatogram m/z 167 (below). 195

Figure 65. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary norrecipavrin metabolites. II. Ion chromatogram m/z 183 showing conjugated norrecipavrin phenol metabolites (above). Ion chromatogram m/z 213 (below). 196

Figure 66. (top) Mass spectrum of the underivatized secondary formamide metabolite (12) of norrecipavrin (15). 199

Figure 67. Mass spectrum of the apparent nitro compound O-methylcatechol metabolite (98) of norrecipavrin. 200

Figure 68. (top) Total ion current for the extract of β-glucuronidase-hydrolyzed fraction of urine from norrecipavrin dosed rats. (bottom) Mass chromatogram m/z 167 for the same sample. 202

Figure 69. (top) Mass chromatogram m/z 167 for the extract of nonconjugated metabolites of urine from norrecipavrin dosed rats. (bottom) Mass chromatogram m/z 167 for the extract of nonconjugated metabolites of bile. 203

Figure 70. Total ion current for the extract of β-glucuronidase-hydrolyzed fraction of bile from dinorrecipavrin dosed rats. (bottom) Mass chromatogram m/z 167 for the same sample. 207

Figure 71. Mass chromatogram m/z 183 (top) and 213 (bottom) for the extract of β-glucuronidase-hydrolyzed fraction of bile from dinorrecipavrin dosed rats. 208

Figure 72.(top): Superimposed mass chromatograms of m/z 253 in the synthetic formamide (12)(sample 2, 30 ng, tr=15.68), and bile extract formamide (sample 1, tr=15.84). Bottom: Mass chromatograms of the m/z 253 ion in spiked samples 3 and 4. 211

Figure 73. Total ion current and selected ion chromatogram for β-glucuronidase-hydrolyzed chloroform extract of biliary recipavrin metabolites. (top) Total ion current for all metabolites. (bottom) Ion chromatogram m/z 167. 213

Figure. 74. Total ion current (top) and m/z 167 ion chromatogram (bottom) for the sulfatase hydrolyzed extract of bile from rats dosed with recipavrin-D_3. Diphenylbutanone (14) is present at 13.37 min. 215

Figure 75. Total ion current (top) and mass chromatogram m/z 167 (bottom) for a β-glucuronidase-hydrolyzed biliary recipavrin metabolites eluted from the FPSiO2 column with ethanol, hydrolyzed with β-glucuronidase and eluted from a RPC18. 223

Figure 76. (top) Mass spectrum of the secondary formamide metabolite of recipavrin eluted from the FPSiO2 column with ethanol, hydrolyzed with β-glucuronidase and eluted from a RPC18 column with 40% ethanol.(bottom) Mass spectrum of the secondary formamide. 224

Figure 77. Total ion current (top) and mass chromatogram m/z 167 (bottom) for nonconjugated biliary recipavrin metabolites eluted from the FPSiO2 column with 92/8 benzene/ethanol. 225

Figure 78. Ion chromatogram m/z 167 of the control (bottom) and formaldehyde/peroxide treated (top) nonconjugated fraction of bile from recipavrin dosed rats. 229

Figure 79. Figure showing m/z 167 mass chromatograms for the control (top) and peroxide/ formaldehyde treated (bottom) conjugated fraction of bile from recipavrin dosed rats. 231

Figure 80. Structures of promethazine (10), promethazine N-oxide (76), promethazine secondary formamide (72) and its phenol (100) and sulfoxide (101) and N_{10}-oxide (75). 236

Figure 81. (top) Mass spectrum of the synthetic phenothiazine secondary formamide. (bottom) Methylene phenothiazine (m/z 212) and β-carboline (m/z 180) resonance stabilized cations arising from ring directed heterolytic cleavage. 237

Figure 82. (top) Direct insertion probe mass spectrum of the secondary formamide N_{10}-oxide (75). (bottom) Direct insertion probe mass spectrum of the Cope elimination product 10-(2-propenyl)phenothiazine (77). 239

Figure 83. Mass chromatograms for an extract of β-glucuronidase-hydrolyzed conjugated promethazine metabolites (above) Ion chromatogram m/z 58 showing intact secondary amine metabolites (below) Ion chromatogram m/z 72. 241

Figure 84. Total ion current for the control GCMS analysis of recipavrin N-oxide. 246

Figure 85. (top) Capillary GCMS total ion current of freshly synthesized and purified recipavrin methylene nitrone injected at low concentration onto a freshly silanized injection port liner and GC column. (bottom) Mass spectrum of the single peak. 248

Figure 86. (top) Summary of high resolution mass spectrum of the nitrone (24) (Source temperature 120°). (bottom) Mass spectrum of the formamide. 249

Figure 87. Capillary GCMS of the methylene nitrone using a clean injection port liner. (top) Total ion current showing decomposition of 10 ug of freshly purified recipavrin methylene nitrone (24). 250

Figure 88. GCMS analysis of the methylene nitrone using a clean injection port liner. (top) Total ion current of a sample overload (100 ug). (bottom) Close up of the total ion current showing the formamide (12, tr=21.31) and nitrone (24, shoulder. 251

Figure 89. Capillary GCMS of the methylene nitrone (24) using a dirty injection port liner. (top) Total ion current showing decomposition of 10 ug of freshly purified recipavrin methylene nitrone. Close up of the total ion current showing the formamide (bottom). 252

Figure 90. Total ion current chromatogram form GCMS analysis using a clean injection port liner of an extract of control bile spiked with 100 ug methylene nitrone (24). 253

Figure 91. (top) Mass chromatogram m/z 167 showing phenyl ring intact metabolites of the methylene nitrone (24). (bottom) Mass chromatogram m/z 183 showing phenol metabolites of the recipavrin methylene nitrone. 258

Figure 92. (top) Mass chromatogram m/z 213 showing O-methylcatechol metabolites of the methylene nitrone (24). (bottom) Mass spectrum of the secondary formamide (12) arising from GCMS analysis of the nitrone metabolites. 259

Figure 93 (top) Total ion current for the BSTFA derivatized bile extract of nitrone (24) metabolites. (bottom) Mass spectrum of the suspected primary hydroxylamine TMS derivative (102). 260

Figure 94. Composite selected ion chromatograms used to locate metabolites in β-glucuronidase-hydrolyzed extracts of bile from tertiary formamide dosed rats. A. Ion chromatogram m/z 167 showing phenyl ring intact metabolites, m/z 183. 268

Figure 95. Composite selected ion chromatograms used to locate metabolites in β-glucuronidase-hydrolyzed extracts of bile from tertiary formamide dosed rats. B. Ion chromatogram m/z 167 showing phenyl ring intact TMS-derivatized metabolites, m/z 255 showing TMS phenols. 269

Figure 96. Mass spectra of the carbinolamide metabolites. (a) Carbinolamide (47). (b) Carbinolamide TMS (47a). 272

Figure 97. Proposed mass spectral fragmentations of the carbinolamide (47) and TMS derivatives of the carbinolamide (47a), the carbinolamide phenol (107a), and carbinolamide O-methylcatechol (110a). 273

Figure 98. Mass spectra of the carbinolamide metabolites. (c) Carbinolamide phenol di-TMS (107a). (d) Carbinolamide O-methylcatechol di-TMS (110a). 274

Figure 99. Proposed mass spectral fragmentations of the carbinolamide (47) and TMS derivatives of the carbinolamide (47a), the carbinolamide phenol (107a), and carbinolamide O-methylcatechol (110a). 275

Figure 100. Proposed mass spectral fragmentations of the carbinolamide (47) and TMS derivatives of the carbinolamide (47a), the carbinolamide phenol (107a), and carbinolamide O-methylcatechol (110a). 276

Figure 101. Summary of bond cleavages in the mass spectra of the carbinolamide metabolite TMS derivatives (47a, 107a and 110a). R1=TMS, R2=H or OCH3, R3= H or OTMS. 277

Figure 102. Metabolic pathways for the tertiary formamide (26) based on the metabolites observed by GCMS. a. Phenyl ring oxidation. b. Oxidation to the catechol and 3'-O-methylation. c. Oxidative deamination. d. Ketone reduction. e. α-Carbon oxidation. 279

Figure 103. Mass chromatogram m/z 167 for the extract of β-glucuronidase-hydrolyzed fraction of bile from secondary formamide dosed rats. 284

Figure 104. Mass chromatogram m/z 213 for the extract of β-glucuronidase-hydrolyzed fraction of bile from secondary formamide dosed rats. 284

Figure 105. Mass chromatogram m/z 183 (top) and 213 (bottom) for the nonconjugated urine fraction from secondary formamide dosed rats. 285

Figure 106. (top) Mass chromatogram m/z 167 for the nonconjugated bile extract of secondary formamide metabolites Standard GC conditions. (bottom) Mass chromatogram m/z 167 for the nonconjugated urine extract of secondary formamide metabolites. 286

Figure 107.(top) Mass spectrum of the carbinolamide metabolite (111) observed in the conjugated and nonconjugated fraction. (bottom) Mass spectrum of the carbinolamide phenol metabolite (112) observed in the conjugated and nonconjugated fraction. 289

Figure 108. Fragmentation scheme to account for the M^+-18 peak of the carbinolamide metabolite (111). 290

LIST OF TABLES

Table 1. Table showing volumes of bile extract and synthetic formamide (12) used in the dilution experiment. 81

Table 2. Table showing volumes of reagents added to bile from recipavrin dosed rats. 90

Table 3. Recovery of the methadone formamide metabolite (6) using various sample isolation procedures. 108

Table 4. Diagnostic masses of the diarylmethyl cation for phenyl ring oxidized metabolites and their derivatives. 162

Table 5. Names structure and formulae for recipavrin, recipavrin metabolites and synthetic reference compounds. 165

Table 6. Names, structures and formulae for phenol and O-methyl catechol metabolites of recipavrin. 166

Table 7. GCMS data for phenyl ring intact recipavrin metabolites and their derivatives. 171

Table 8. GCMS data for phenolic metabolites of recipavrin and their derivatives. 172

Table 9. GCMS data for O-methyl catechol metabolites of recipavrin and their derivatives. 173

Table 10. Retention times and diagnostic ion abundances for the conjugated norrecipavrin metabolites. 197

Table 11. Retention times and diagnostic ion abundances for the conjugated dinorrecipavrin metabolites. 204

Table 12. The effects of added synthetic secondary formamide standard on peak height ratios, peak shape and retention time of the formamide metabolite present in a β-glucuronidase-hydrolyzed bile extract 210

Table 13. GCMS peak areas of compounds arising from the chemical and/or thermal degradation of recipavrin N-oxide under various simulated workup conditions. 244

Table 14. Names structures and formulae for the tertiary formamide (26) and its metabolites. 262

Table 15. GCMS data for phenyl ring intact metabolites of the tertiary formamide (26) and their derivatives. 264

Table 16. GCMS data for phenolic metabolites of the tertiary formamide (26) and their derivatives. 265

Table 17. GCMS data for O-methylcatechol metabolites of the tertiary formamide (26) and their derivatives. 266

Table 17 (continued). GCMS data for O-methylcatechol metabolites of the tertiary formamide (26) and their derivatives. 267

Table 18. Metabolites and retention times for the secondary formamide metabolites. 287

LIST OF ABBREVIATIONS

1. Common abbreviations

Ac.....acetyl

AMP.....amphetamine

amu.....atomic mass unit

APT.....attached proton test

Ar.....aryl

arom.....aromatic

BB.....broad band decoupled

b.p.....boiling point

BHT.....butylated hydroxy toluene

BSTFA....N,O-bis-(trimethylsilyl)trifluoroacetamide

CAS.....Chemical abstracts service

CI.....chemical ionization

D.....deuterium

d.....doublet

dd.....doublet of doublets

DDP.....N,5-dimethyl-3,3-diphenylpyrrolidine

dimedone.5,5-dimethyl-1,3-cyclohexanedione

DIP.....direct insertion probe

DLI.....direct liquid introduction

DMCS.....dimethyldichlorosilane

DMSO.....dimethylsulfoxide

EDDP.....2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

EI.....electron impact

EMDP.....2-ethyl-5-methyl-3,3-diphenylpyrrolidine

eq.....equivalent

equiv....equivalent

et al....*et alia*

etc.....etcetera

EtOH.....ethanol

$FPSiO_2$...forward phase silica gel SPE cartridge

FT.....fourier transform

GC.....gas chromatograph

GCMS.....gas chromatography-mass spectrometry

GLU.....glucuronic acid

H-bond...hydrogen bond

HPLC.....high pressure liquid chromatography

IEC.....ion exchange chromatography

IR.....infra red spectrometry

J.....coupling constant in Hz

LC.....liquid chromatograph

LCMS.....liquid chromatography-mass spectrometry

Lit.....literature value

M^+.....molecular ion

m.....medium (IR), multiplet (NMR)

min.....minutes

m.p.....melting point

m/z.....mass to charge ratio

MC.....mass chromatogram

MCPBA....metachloroperbenzoic acid

MeOH.....methanol

MOX.....O-methylhydroxylamine hydrochloride

MS.....mass spectrometer

NADP.....nicotinamide adenine dinucleotide phosphate

NMR.....nuclear magnetic resonance spectrometry

NOE.....nuclear overhauser effect

pet.....petroleum

Ph.....phenyl

ppm.....parts per million

PTZ.....phenothiazine

py.....pyridine

q.....quadruplet

rf.....TLC mobility

RPC_{18}....reversed phase octadecyl SPE cartridge

s.....strong (IR), singlet (NMR)

sec.....secondary

SFORD....single frequency off resonance decoupled

SIM.....selected ion monitoring

SPE.....solid phase extraction

str.....stretch

t.....triplet

tert.....tertiary

TLC.....thin layer chromatography

TMAH.....trimethylanilinium hydroxide

TMS.....tetramethylsilane

tr.....retention time

UV.....ultra violet

v.....very

w.....weak

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DEDICATION

To my parents, Nancy and Wally Slatter.

I. INTRODUCTION

1. OBSERVATION OF A FORMAMIDE METABOLITE OF METHADONE

In previous work in this laboratory it was observed that the perchlorate salt of the major pyrrolidine metabolite of methadone ((±)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate, (EDDP), 1) undergoes chemical oxidation to a thermolabile oxaziridine (2). Other oxidation products present included a diketone (3), 1,5-dimethyl-3,3-diphenylpyrrolidone ((DDP), 4), and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline ((EMDP), 5). The oxaziridine (2) decomposed to the isomeric formamide (6) during GCMS analysis (figure 1) (Abbott, Slatter and Kang, 1986). GCMS results for the formamide (6) were identical to a novel metabolite of methadone found in β-glucuronidase-hydrolyzed bile extracts from methadone dosed rats (Abbott, Slatter, Burton and Kang, 1985). The methylene nitrone structure (7) was originally proposed for the methadone metabolite (Kang, 1981) on the basis that similar nitrones were reported to arise from the metabolism of arylaliphatic amines in the amphetamine series (Coutts and Beckett, 1977). No methylene nitrone (7) was isolated from the EDDP oxidation mixture. The possibility that the nitrone was a labile precursor of the formamide metabolite (6) warranted further synthetic investigation.

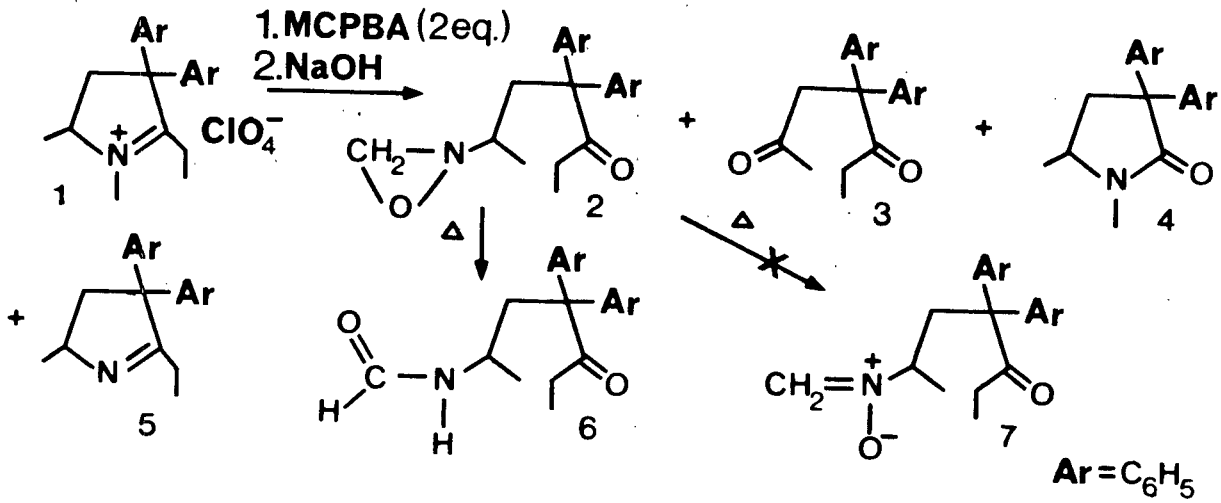
The cyclization of desalkyl metabolites of methadone (8) had been previously documented as the major chemical change in the metabolism of methadone in rats (Pohland, *et al.*, 1971, Sullivan, *et al.*, 1972). The cyclization was assumed to be a

spontaneous addition of the basic secondary amino group to the ethyl ketone side chain of methadone with dehydration to EDDP (1). This cyclization complicated attempts to synthesize the nitrone (7), since open chain primary and secondary hydroxylamine synthetic intermediates underwent the cyclization reaction common to the more basic desalkyl metabolite of methadone (8)(Slatter, 1983).

Since the cyclization of synthetic intermediates complicated the synthesis of the potential nitrone precursor (7) of the methadone formamide (6), and because the chemical oxidation of EDDP (1) was apparently not the source of the formamide metabolite (6), recipavrin (9) was adopted as a model drug to determine the mechanism whereby formamides are observed in conjugated biliary extracts from rats dosed with tertiary arylaliphatic amines.

2. RECIPAVRIN: A STRUCTURAL ANALOGUE OF METHADONE

Recipavrin^{R} is the trade name for (±) N,N,α-trimethyl-γ-phenylbenzenepropanamine hydrochloride (9, CAS Registry 22173-83-70). This drug was first synthesized in the late 1940's (May and Mosettig, 1948, Adamson, 1949). Recipavrin is a basic arylaliphatic amine (pKa=9.48) with anticholinergic activity approximately equal to imipramine or terodiline (Nilvebrant and Sparf, 1983) and an LD_{50} of 80 mg/kg in mice (Eddy, *et al.*, 1950). The anticholinergic properties of recipavrin led to its marketing by A.B. Recip (Stockholm) as a spasmolytic for ulcers and spastic colitis (Unlisted Drugs, 1952), and



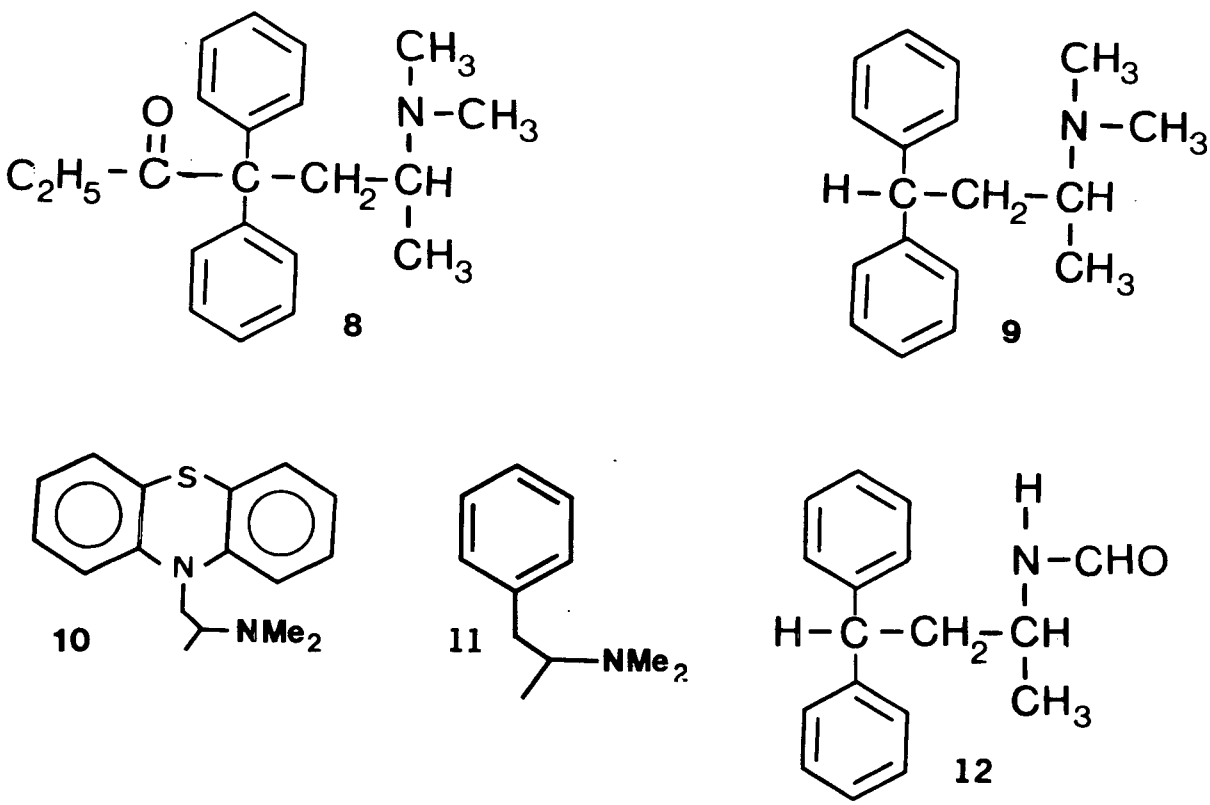


Figure 1. (top) Products derived from the chemical oxidation of EDDP (1). (bottom) Structure of methadone (8), recipavrin (9), promethazine (10), dimethylamphetamine (11), and the formamide metabolite (12) of recipavrin.

later as an ingredient in Preparyl^{R} indicated for vegetative neuroses (Unlisted Drugs, 1953) and Robetyl^{R} ,for anxiety and tremor (Unlisted Drugs, 1960).

Recipavrin was initially synthesized as an analogue of the narcotic analgesic methadone, but was devoid of analgesic activity (Eddy, *et al.*, 1950). Structurally, the only difference was the replacement of the ethyl ketone side chain of methadone (8) with a hydrogen atom. It is this similarity that has led to the use of recipavrin (9) as a model compound in drug derivatization studies (Vessman, *et al.*, 1970, Hartvig and Vessman, 1974, Hartvig, *et al.*, 1972, 1976) and NMR studies directed toward discerning the active conformation of methadone-related analgesics (Casy, 1966). Recipavrin also has structural elements in common with the antihistamine, promethazine (10) and dimethylamphetamine (11).

3. OVERVIEW OF PREVIOUS WORK ON RECIPAVRIN (Slatter, 1983)

The first step in the investigation of recipavrin metabolism was to synthesize recipavrin and its potential metabolites, in particular the oxaziridine (25), nitrone (24) and formamide (12) analogues proposed on the basis of the methadone studies. The synthetic work is summarized in figure 2. With reference compounds in hand it was possible to show that the secondary formamide (12) could be observed by GCMS analysis of extracts of β-glucuronidase-hydrolyzed bile from recipavrin dosed rats.

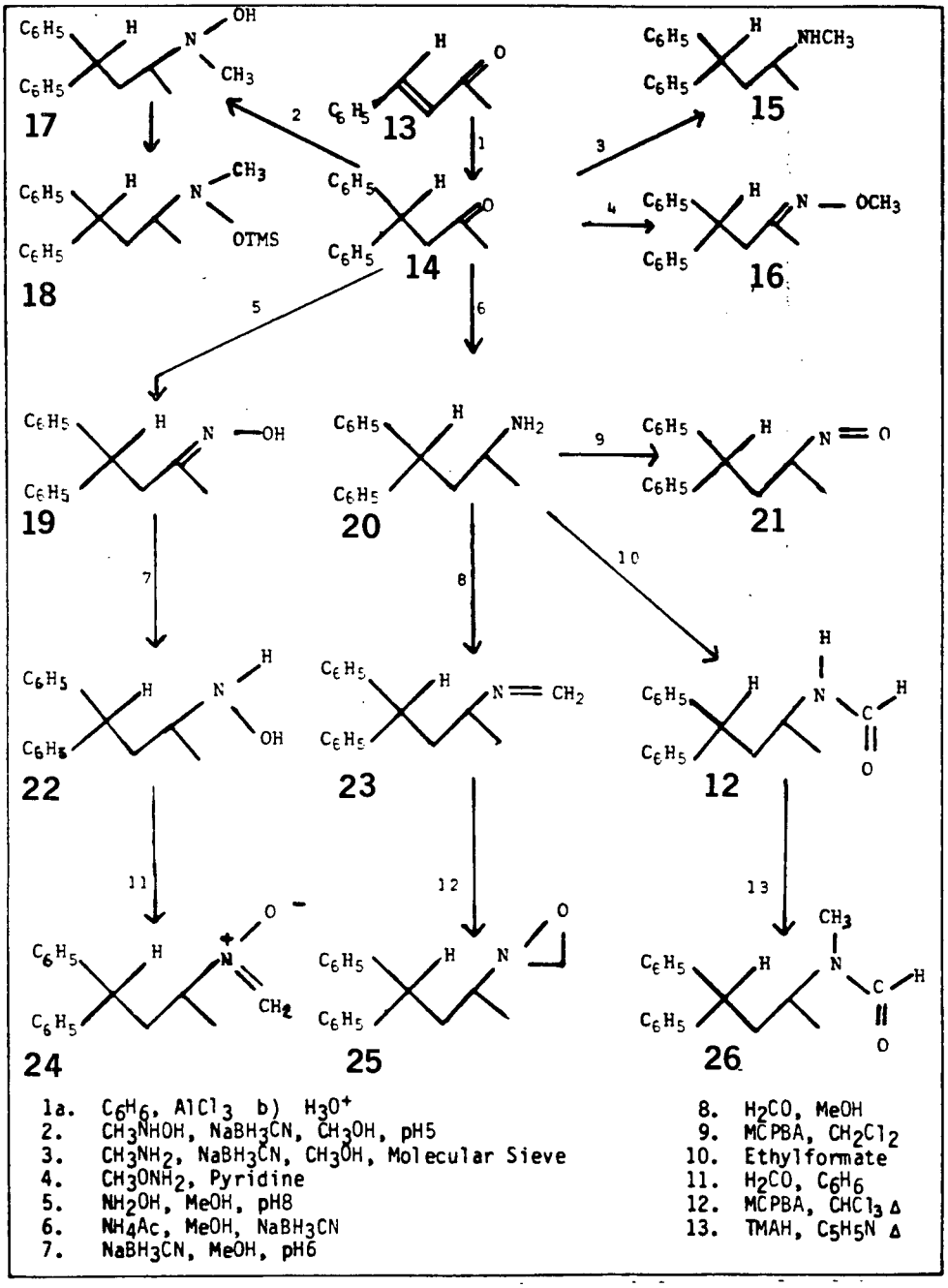


Figure 2. Preliminary syntheses of potential N- and alpha C-oxidized metabolites of recipavrin (9) .

The oxaziridine (25) isomerized in the GC inlet to the secondary formamide (12). The nitrone (24) was thermolabile with decomposition during GCMS analysis to the imine (23), oxime (19) and an unidentified compound which resembled the formamide (12) but chromatographed poorly and had additional ions in the mass spectrum. When bile was spiked with the secondary hydroxylamine (17), and worked up by solvent extraction, the formamide (12) was observed by GCMS. The absence of the imine in metabolite extracts was taken to be evidence against the nitrone (24) as a thermolabile precursor of the formamide (12).

The LCMS behavior of the methylene nitrone (24), formamide (12) and oxaziridine (25) analogues of recipavrin (9) in aqueous methanol revealed that the methylene nitrone decomposes to a minor extent to the secondary formamide (12) on standing at room temperature in methanol solution (figure 3, Slatter, 1983). Thus, the nitrone (24) was also a possible chemical precursor of the formamide metabolite (12).

The initial work on recipavrin (9) concluded that the oxaziridine (25) and nitrone (24) were both possible chemical precursors of the formamide metabolite (12). There were other possible precursors that required synthesis and characterization of the full array of recipavrin metabolites.

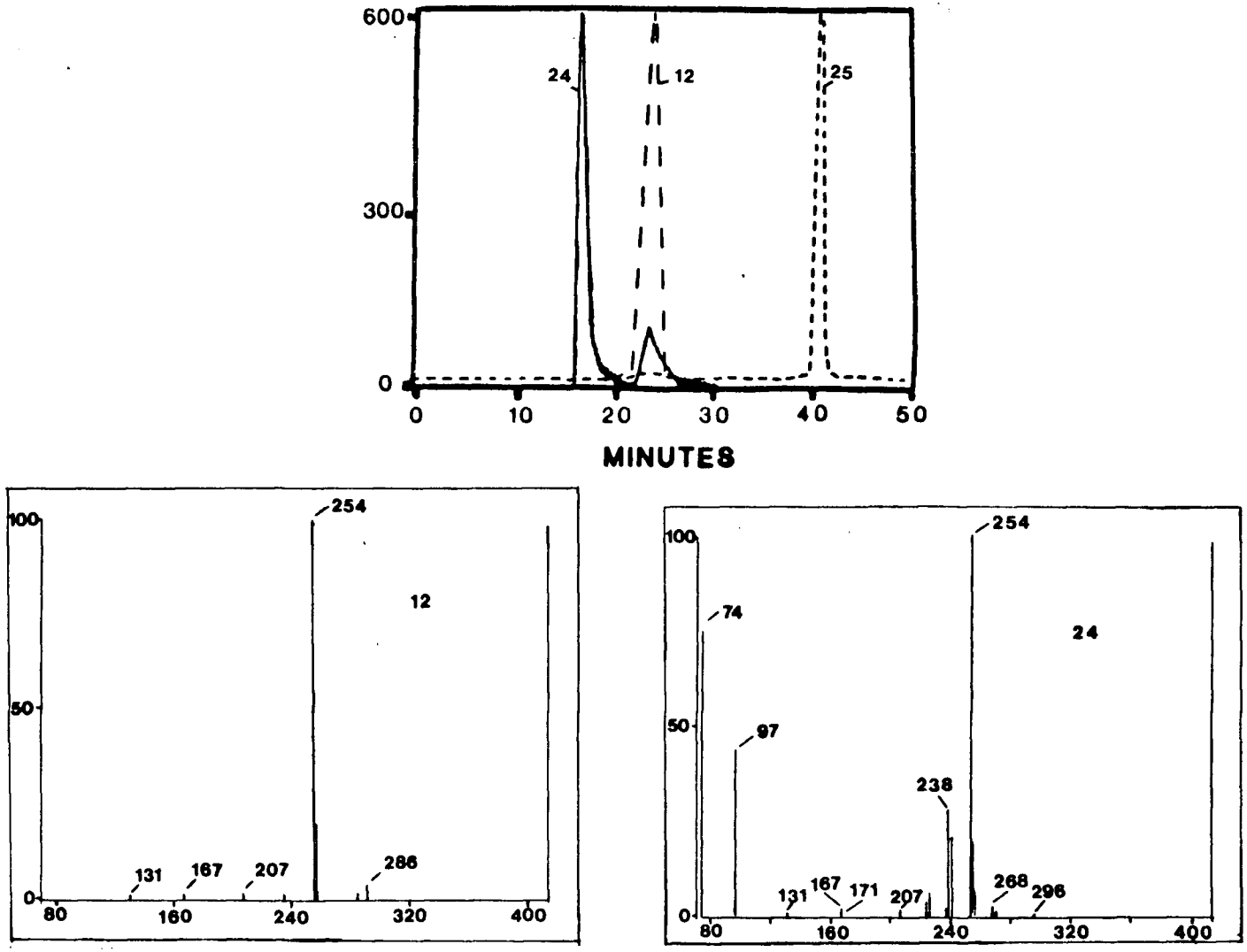


Figure 3. (top) Superimposed LCMS ion chromatograms of the M^++1 m/z 254 ion of the synthetic methylene nitrone (24), oxaziridine (25) and formamide (12) . The formamide was also present as a decomposition product of the nitrone. (Bottom) LCMS mass spectra of the formamide (12) and nitrone (24). Methanol/water solvent system. (Slatter, 1983).

4. METABOLISM OF TERTIARY ARYLALIPHATIC AMINES

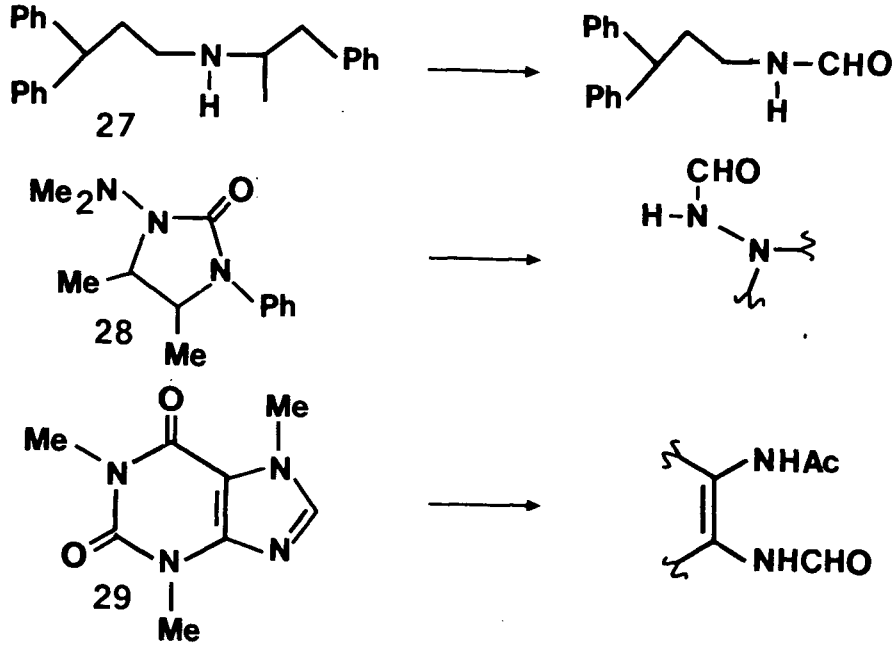
The metabolism of tertiary amines has been reviewed by Rose and Castagnoli (1983). An important pathway is the formation of N-oxides which are thermolabile compounds commonly observed by GCMS as Cope elimination products (Cope, *et al.*, 1951). The tertiary amine functional group is also a possible substrate for N-glucuronidation (Caldwell, 1982).

The similarity of recipavrin (9) to dimethylamphetamine (11) allows the metabolic pathways of N-oxidation, N-dealkylation, oxidative deamination and phenyl ring oxidation to be applied to the recipavrin series. A key difference is that recipavrin (molecular weight 253) is above the minimum molecular weight for biliary excretion in the rat, while dimethylamphetamine (11) is not. The metabolism of amphetamines has been reviewed by Caldwell (1976). N-oxidative metabolism of amphetamines has been reviewed by Coutts and Beckett (1977). The metabolism of dimethylamphetamine (11) has been the subject of a short communication (Beckett and Al Sarraj, 1972). The metabolism of a basic tertiary arylaliphatic amine to a secondary formamide was a novel observation which could not be assigned to any of the common pathways of amphetamine metabolism. The characterization of the formamide metabolites of recipavrin appeared timely as formamide metabolites of other drugs began to appear in the literature at the same time.

5. FORMAMIDE METABOLITES OF AMINES AND AMIDES

Formamide metabolites have been described for prenylamine (27) (Remberg, *et al.*, 1977), aminopyrine (28) (Iguchi, *et al.*, 1975), caffeine (29) (Tang, *et al.*, 1983), amantidine (30) (Koppel and Tenczer, 1985), mianserin (31) (DeJongh, *et al.*, 1981), indecainide (32) (Lindstrom, *et al.*, 1984), pipemidic acid (33) (Kurobe, *et al.*, 1980), aminoanthraquinone (34) (Gothoskar, *et al.*, 1979), 2-naphthylamine (35) (Boyland and Manson, 1966), chlortoluon (36) (Muecke, *et al.*, 1976), N-methylbenzamide (37) (Ross, *et al.*, 1983) and viloxazine (38) (Case, *et al.*, 1975) (figure 4).

The formamide metabolites of arylamines arise by enzymatic formylation mechanisms involving kynurenine formamidase (Santi and Hopsu-Havu, 1968), or in the case of aminopyrine, have been proven to arise by alpha carbon



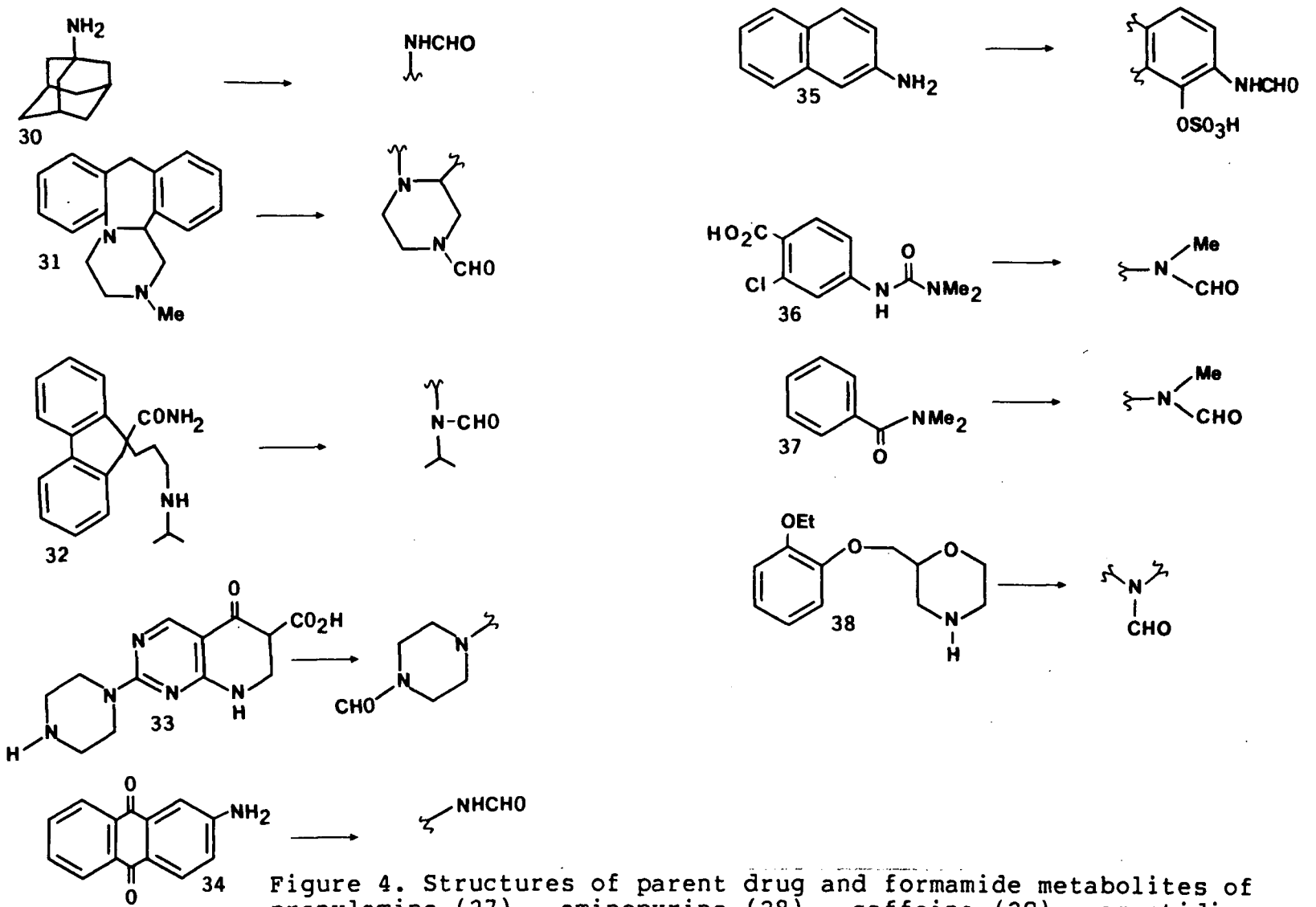


Figure 4. Structures of parent drug and formamide metabolites of prenylamine (27), aminopyrine (28), caffeine (29), amantidine (30), mianserin (31), indecainide (32), pipemidic acid (33), aminoanthraquinone (34), 2-naphthylamine (35), chlortoluon (36), N,N-dimethylbenzamide (37) and viloxazine (38).

6. POSSIBLE SOURCES OF FORMAMIDE METABOLITES OF METHADONE (8) AND RECIPAVRIN (9)

There are several possible combinations of metabolic and chemical changes which could account for the GCMS observation of a secondary arylaliphatic formamide in metabolite extracts derived from β-glucuronidase hydrolyzed bile of tertiary arylaliphatic amine dosed rats. All center on preliminary oxidation of nitrogen and carbon atoms adjacent to nitrogen. The interplay between chemical and metabolic changes in the analysis of labile N-oxidized compounds have been reviewed (Coutts and Beckett, 1977, Lindeke, 1982, Hlavica, 1982). The occurrence of amide metabolites of arylamines from a similar array of sources which include nitrones and oxaziridines is under investigation in another laboratory (Gooderham and Gorrod, 1986). Solvent, amine, oxaziridine, nitrone, and amide mediated generation of the secondary formamide (12) were all possible explanations for the source of the metabolite observed by GCMS.

A. Formamide artifacts arising from metabolite extraction using organic solvents

The reaction of solvent impurities with drugs or their metabolites is a well known pitfall of drug metabolite extraction (Beckett and Cowan, 1978, Chamberlain, 1985). The problem can be minimized by destroying or removing the problem impurity and then distilling the solvent just before use.

Problem solvents such as ether or chloroform should be avoided, however, the volatility, solvating power, cost and ease of purification of these solvents make them good choices for routine metabolite extraction. Problems arise when a trace metabolite such as a formamide could arise from reaction with solvent derived impurities. Formamides are known to arise from reaction of amines with formyl chloride present in chloroform (Stillwell, *et al.*, 1978). Carbamates can arise from the reaction of amines with phosgene in the presence of an alcohol (Wester, *et al.*, 1981, Cone, *et al.*, 1982). Organic peroxides such as diethyl peroxide formed photochemically in ether react with amines. The peroxidation of secondary amines gives rise to formamides. The peroxidation of iminium compounds gives rise to labile oxaziridines, another possible source of formamide. The iminium compounds could in turn arise metabolically or result from condensation of an amine with aldehydes present in the extract or introduced as solvent impurities. All these reactions require a secondary amine or primary amine substrate.

The formamide metabolites of methadone and recipavrin were derived from the β-glucuronidase hydrolyzed fraction. Basic secondary amines are not subject to glucuronide conjugation and should not be present in the hydrolyzed fraction. However, the possible oxidation of the secondary amine metabolites was avoided by protecting samples from light, keeping them on ice, working them up as soon as possible after collection and by using a minimum volume of a freshly purified extraction solvent.

B. Formamide artifacts arising from oxidation of amine metabolites during sample isolation

The free radical oxidation of amines by peroxides (Sayigh and Ulrich, 1963) and by amine autooxidation in aqueous solution (Beckwith *et al.*, 1983) are both known to afford amides from amines. Alkaline conditions and the presence of dissolved oxygen are possible contributors to the formation of tertiary formamide (26) and secondary formamide (12) from recipavrin (9) and the desalkyl metabolite norrecipavrin (15) respectively. However, neither of these amines is likely to be present in the conjugated fraction.

C. Formamide metabolites arising by peroxidation of iminium dealkylation intermediates

Pathway a in figure 5 details a peroxidative route to a secondary formamide (12) mediated by an iminium compound (23) and oxaziridine (25).

Iminium compounds in equilibrium with carbinolamines have been proposed as dealkylation intermediates and as electrophilic reactive metabolites of a number of different amines (Overton, *et al.*, 1985). Peroxidation of iminium compounds affords thermo and chemolabile oxaziridines (Emmons, 1957, Krimm, 1958). Autooxidation of iminium compounds affords isomeric oxaziridines and amides (Auret, *et al.*, 1984). Thus, metabolically generated iminium ions are possible substrates for peroxidation to oxaziridines by endogenous H_2O_2 or organic peroxides introduced during sample preparation.

The isomerization of oxaziridines to amides is well documented (Emmons, 1957, Lattes, *et al.*, 1982). Methylene oxaziridines afford formamides by thermal or Fe^{2+} mediated mechanisms. The driving force for oxaziridine rearrangements is relief of ring strain. Competition between C-O cleavage to give nitrones and N-O cleavage to give amides is determined by substituents on nitrogen and the oxaziridine ring carbon. Electron withdrawing substituents on the ring carbon favor C-O cleavage and nitrone formation. A tertiary carbon substituent on nitrogen stabilizes the oxaziridine ring. The ionic mechanisms for each isomerization are shown in figure 6. Because of the lability of the oxaziridine functional group, sample analysis conditions would result in immediate, quantitative conversion of a methylene oxaziridine to the isomeric formamide.

There is no precedent for oxaziridines as drug metabolites, although the oxaziridine structure has been proposed as an intermediate in the metabolism of some compounds (Gorrod and Manson, 1986 (review); Manson, 1971; Dostert, *et al.*, 1985, Gooderham and Gorrod, 1986).

The lack of a plausible glucuronide precursor is the chief shortcoming of this pathway.

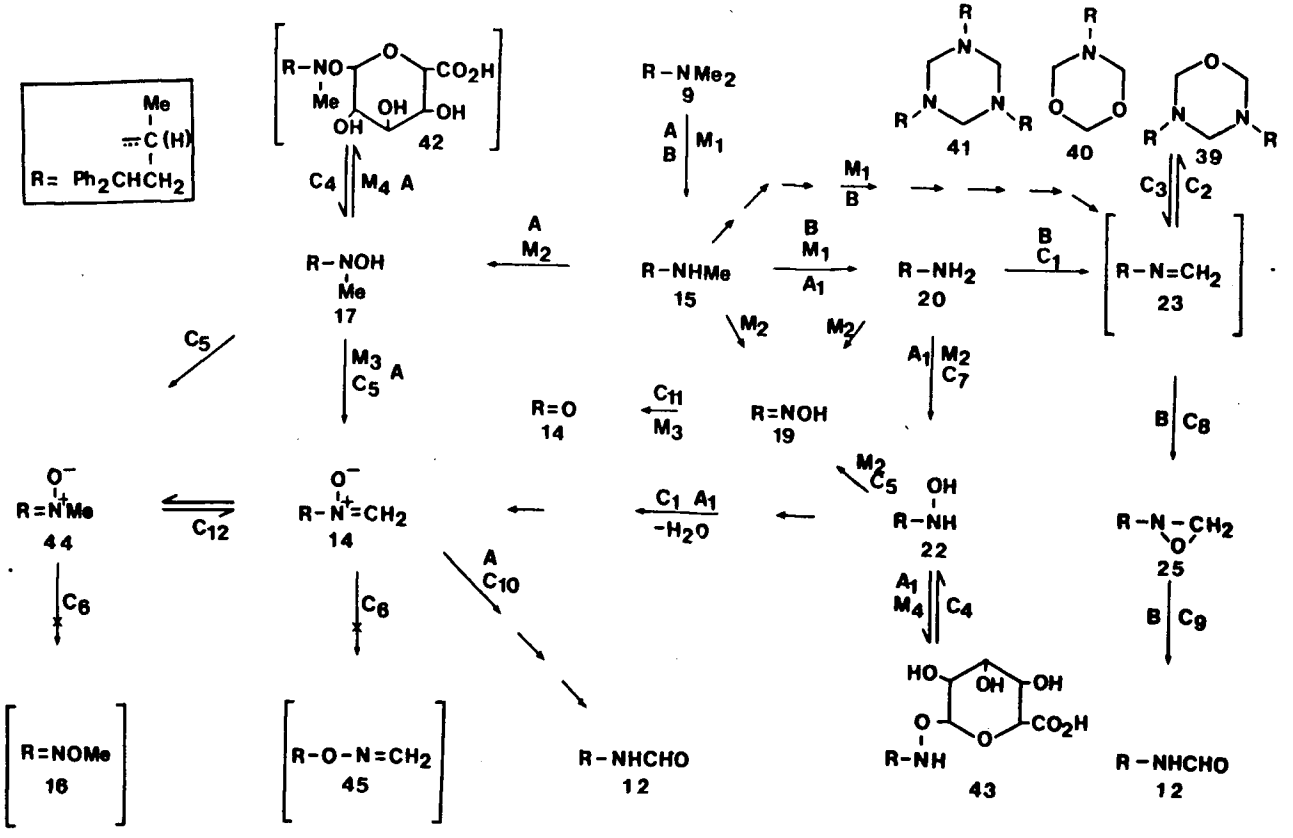


Figure 5. Schematic showing possible metabolic (m) and chemical (c) transformations that could account for a secondary formamide metabolite (12) in the β-glucuronidase hydrolyzed fraction of bile from recipavrin dosed rats.

LEGEND: Pathways to formamide (12):A: Pathway mediated by oxidation of secondary hydroxylamine aglycone (17).A_1: Pathway mediated by formaldehyde condensation with primary hydroxylamine aglycone (22). B. Pathway mediated by peroxidation of iminium compound (23). Metabolic pathways (M_1-M_4): M_1. N-demethylation. M_2. N-oxidation. M_3. Oxidative deamination. M_4. Glucuronidation. Conditions required for chemical changes occuring during isolation of metabolites (C_1-C_{12}): C_1. CH_2O. C_2. Excess CH_2O. C_3. 250°. C_4. β-glucuronidase. C_5. O_2/OH^-. C_6. Martynoff rearrangement. C_7. H_2O_2. C_8. $CH_2O/H_2O_2/H^+$. C_9. Heat or Fe^{2+}. C_{10}. OH^- or acylating agent (Beckmann rearrangement). C_{11}. H_3O^+. C_{12}. Behrend rearrangement.

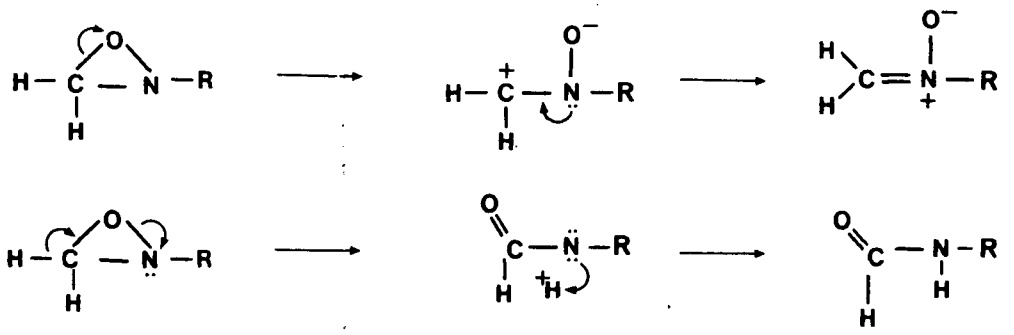


Figure 6. Ionic mechanisms for the cleavage of C-O or N-O bonds in the oxaziridine ring to afford nitrones or amides respectively.

D. Formamide metabolites arising by decomposition of N-oxidized metabolites

Pathway B in figure 5 shows an N-oxidative route to the secondary formamide, wherein the true metabolite is a primary or secondary hydroxylamine O-glucuronide (43 and 42 respectively).

i. Hydroxylamine metabolites as precursors of nitrones

Secondary arylaliphatic hydroxylamines are *in vitro* metabolites of other basic tertiary (Clement and Beckett, 1981, Beckett, *et al.*, 1983) and secondary amines (Coutts and Beckett, 1977). Demonstrating the existence of hydroxylamines *in vivo* is not always possible (Beckett, *et al.*, 1983). The existence of secondary hydroxylamine metabolites as glucuronide conjugates has been inferred from the existence of the hydroxylamine aglycones of amphetamines (Beckett and Al Sarraj, 1972a), phenmetrazine and phendimetrazine (Beckett and Salami, 1972) and chlorphentermine (Beckett and Belanger, 1974, Caldwell, *et al.*, 1975). Other *in vivo* hydroxylamine

metabolites have also been reported for chlorpromazine (Beckett and Essien, 1973) and phentermine (Beckett and Belanger, 1975, 1978).

The reason for the difficulties in characterizing *in vivo* hydroxylamines was presumed to be the ease with which the hydroxylamines undergo oxidation, especially under the aerobic and mildly alkaline conditions commonly employed to extract other more basic metabolites (Beckett, *et al.*, 1977).

The oxidation of the recipavrin secondary hydroxylamine (17) under these conditions could give rise to a mixture of N-methyl (44) and N-methylene (24) nitrones, both isomeric with the secondary formamide metabolite (12).

A primary hydroxylamine aglycone (22) arising from β-glucuronidase hydrolysis of recipavrin metabolites could condense with formaldehyde to afford the methylene nitrone (24). The facile condensation of hydroxylamine metabolites of amphetamines with aldehydes to afford nitrones has been cited as a reason for the difficulty in detecting hydroxylamine metabolites in the amphetamine series (Beckett, *et al.*, 1979).

ii. Nitrones as drug metabolites

The methylene nitrone (24) is analogous to nitrones described as metabolites of other arylaliphatic amines such as methamphetamine and promethazine (10) (Beckett and Coutts, 1977, Clement and Beckett 1981).

Cofactor dependency and generation of the amphetamine nitrone from *in vitro* metabolism of the secondary hydroxylamine has proven that the nitrone does not arise solely from the oxidation of the hydroxylamine during isolation of metabolites (Coutts *et al.*, 1977).

iii.Nitrones as chemical precursors of formamide metabolites: Rearrangements of Nitrones

The chemistry of nitrones has been extensively investigated (Hamer and Macaluso, 1964, Delpierre and Lamchen, 1964, Kliegel, 1977, 1978, (reviews)). The dipolar nature of the nitrone functional group makes it amenable to molecular rearrangement (figure 7) There are several rearrangements described in the literature which make the methylene nitrone (24) an attractive precursor if the secondary formamide observed by GCMS in bile extracts were a chemically generated artifact.

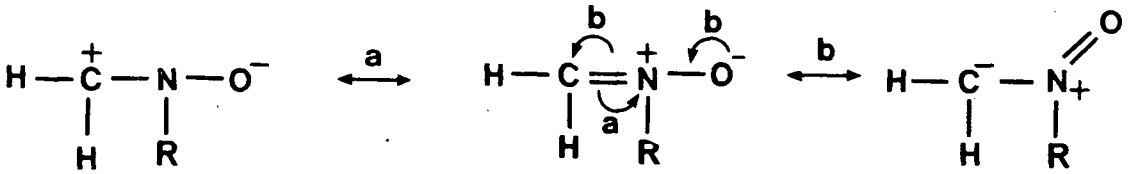


Figure 7. Back polarization between two canonical forms of a nitrone.

a. Behrend Rearrangement

The methyl nitrone (44) (a keto-nitrone) could be converted by a 1,3-prototropic shift (Behrend rearrangement, figure 8) to the methylene isomer (24, an aldo-nitrone) in alkaline media. (Hamer and Macaluso, 1964, Lamchen, 1968, Heistand, 1978, Smith and Gloyer, 1975).

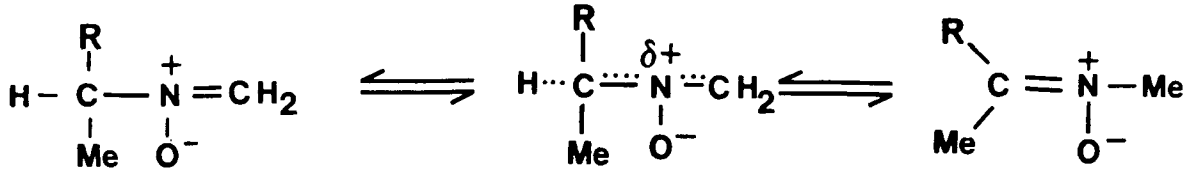


Figure 8. Behrend rearrangement of a N-methyl nitrone to a methylene nitrone.

b. Martynoff Rearrangement

Rearrangement of the nitrones 24 and 44 under thermal conditions should afford the isomeric oxime ethers 16 and 45 respectively. (Villarreal and Grubbs, 1978). Nitrones have also been shown to isomerize to amides when heated. Oxaziridines have been proposed as intermediates in this reaction (Emmons, 1957, Larson, *et al.*, 1970)

GC phase or internal surface effects on the stability of amphetamine nitrones have been observed (Coutts, *et al.*,1978a). On column decomposition of the nitrone substituted benzodiazepines, chlordiazepoxide and demoxepam has caused problems with GCMS analysis (Joyce, *et al.*, 1984).

c. Beckmann Rearrangement

The Beckmann rearrangement of nitrones to amides is catalyzed by a number of acylating agents (Beckmann, 1890, 1893, 1905, 1909). Several mechanisms have been proposed. These have been reviewed and modified by Lamchen (1968) to the acylation mechanism shown in figure 9a.

Basic conditions have also converted nitrones to their isomeric amides (Bigiavi and Marri, 1934, Hamer and Macaluso, 1964, Umezawa, 1960, Zinner, 1978). This rearrangement applied to the methylene nitrone (24) would give rise to the formamide (12). Zinner (1978) has proposed a N-hydroxy-N,O-acetal (nitrone alcoholate) intermediate in the alkali catalyzed rearrangement (figure 9b).

If the UDP-β-Glucuronyl-transferase enzyme were inserted in the Beckmann rearrangement mechanism as the acylating agent, a nitrone N,O- glucuronide internal salt (46), a zwitter ion similar to the N-glucuronides of tertiary amines (Caldwell, 1982) would be formed from a methylene nitrone *in vivo*. In this case the formamide (12) would be liberated by enzymatic hydrolysis of the nitrone glucuronide (figure 10). Hydrolysis of the same conjugate of the N-methyl nitrone (44) would afford diphenylbutanone (14).

d. Photochemical rearrangements

Pathways A and B in Figure 5 could be linked by a photochemical rearrangement of nitrone (24) to oxaziridine (25) (Spence, *et al.*, 1970). The free radical mechanism shown in figure 11 was used by Lamchen (1968) to account for most nitrone to oxaziridine photorearrangements.

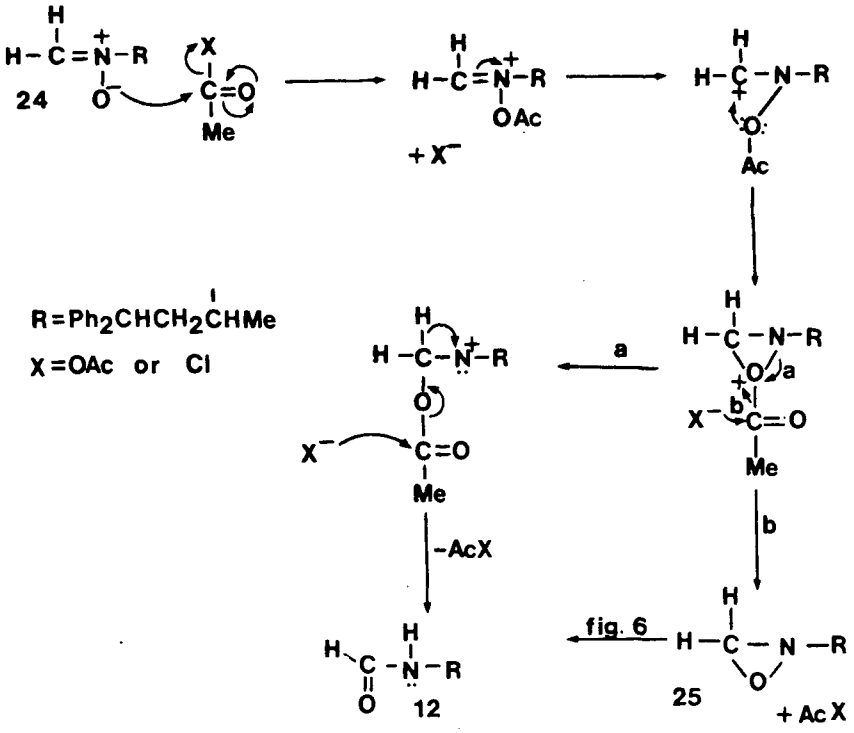


Figure 9a. Mechanism for the Beckmann rearrangement of nitrones to amides catalyzed by acylating agents (Lamchen, 1968).

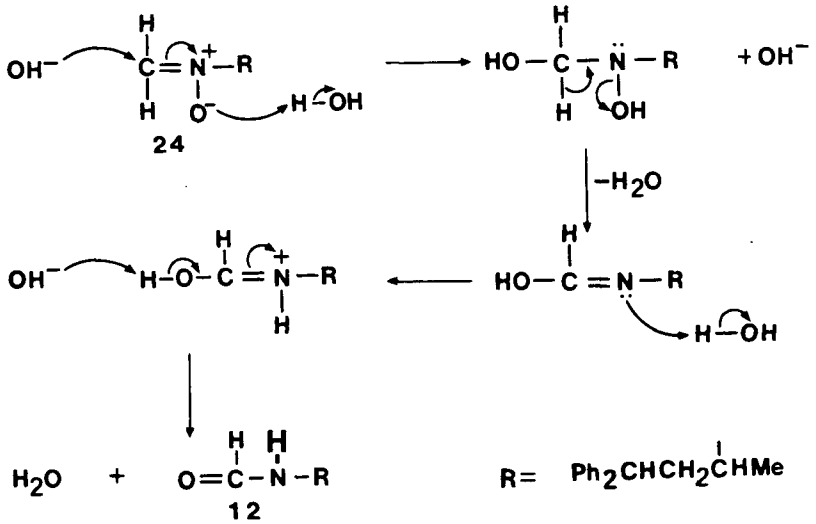


Figure 9b. Mechanism for the Beckmann rearrangement of nitrones to amides catalyzed by alkali (Lamchen, 1968, Zinner, 1978).

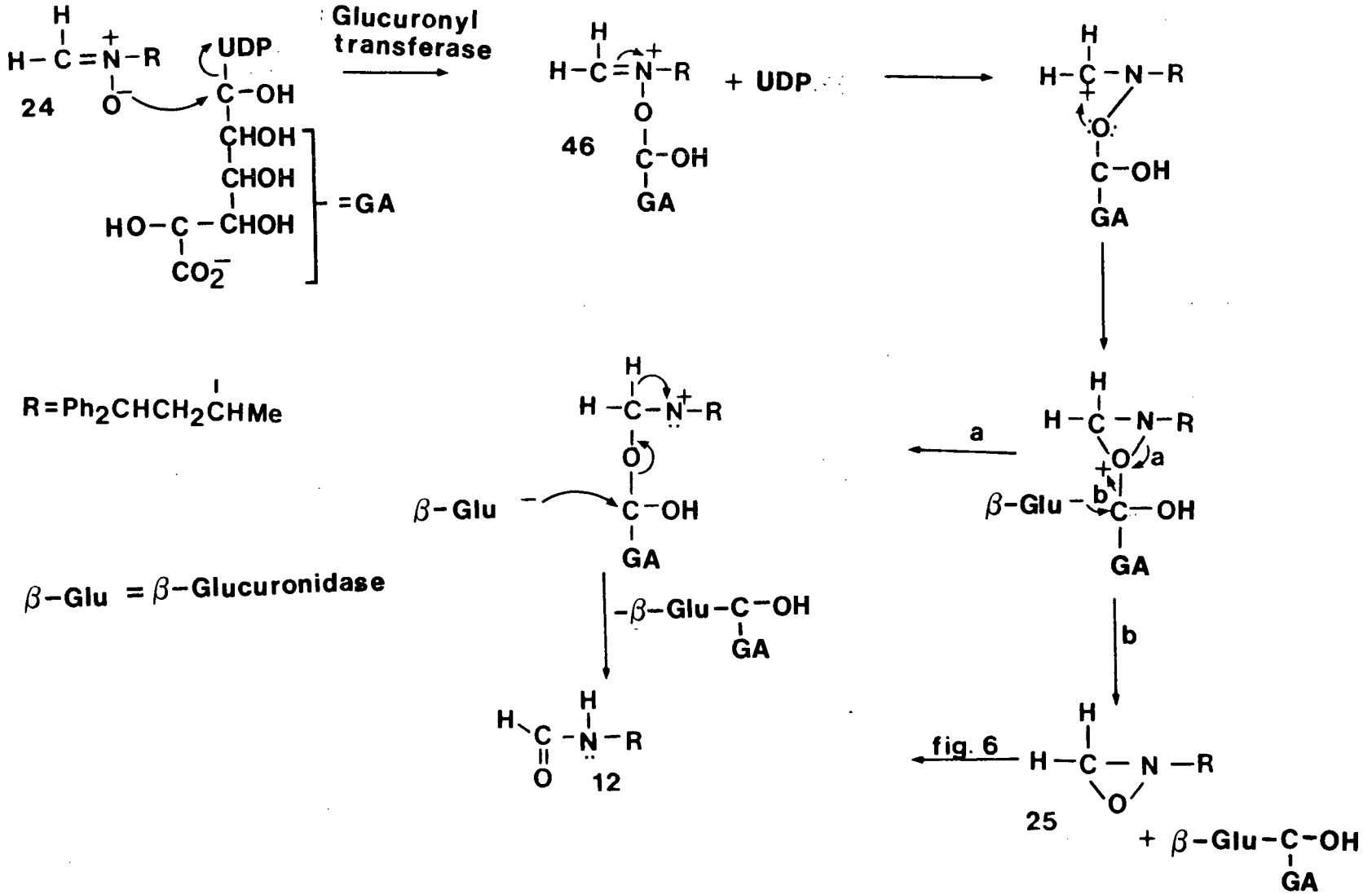


Figure 10. A modified Beckmann rearrangement of a hypothetical nitrone glucuronide (46) catalyzed by β-glucuronidase (after Lamchen, 1968).

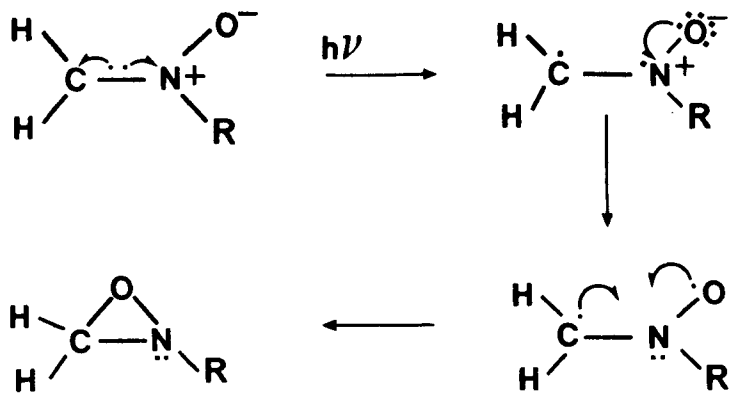


Figure 11. Free radical mechanism for the photochemical rearrangement of a nitrone to an oxaziridine (Lamchen, 1968).

These chemical findings indicate that chemical and thermal isomerizations of the methylene nitrone (24) could result in the observation of a secondary formamide (12) by GCMS.

E. Formamide metabolites arising from a carbinolamide precursor

i. Stability and occurrence of carbinolamine and carbinolamides intermediates in drug metabolism studies

The mechanism shown in figure 12 involves a carbinolamine mediated four electron oxidation of a tertiary amine to a tertiary formamide. In non-basic tertiary amines such as N,N-dimethylaniline and tertiary N-methylamides (Ross, *et al.*, 1983) this is a possibility since the intermediate carbinolamine is relatively stable due to resonance electron delocalization and is long lived enough to undergo further oxidation to the carbinolamide (47) or be conjugated with glucuronic acid (Allen, *et al.*, 1971, McMahon and Sullivan, 1965).

In basic tertiary amines however, it is commonly accepted that the high electron density results in a spontaneous hydrolysis of the carbinolamine to the desalkyl compound plus formaldehyde. Nonetheless, there are exceptions to the spontaneous hydrolysis of basic carbinolamine dealkylation intermediates such as in the metabolic oxidations of pyrrolidine drugs through the carbinolamine to lactams (Hucker, 1973).

The tertiary formamide (26) shown in figure 12 is a non-basic compound, ideally suited to metabolic carbinolamide formation and glucuronide conjugation. Hydrolysis of the carbinolamide glucuronide (48) followed by decomposition of the carbinolamide aglycone (47) during sample extraction would result in the observation of a secondary formamide (12).

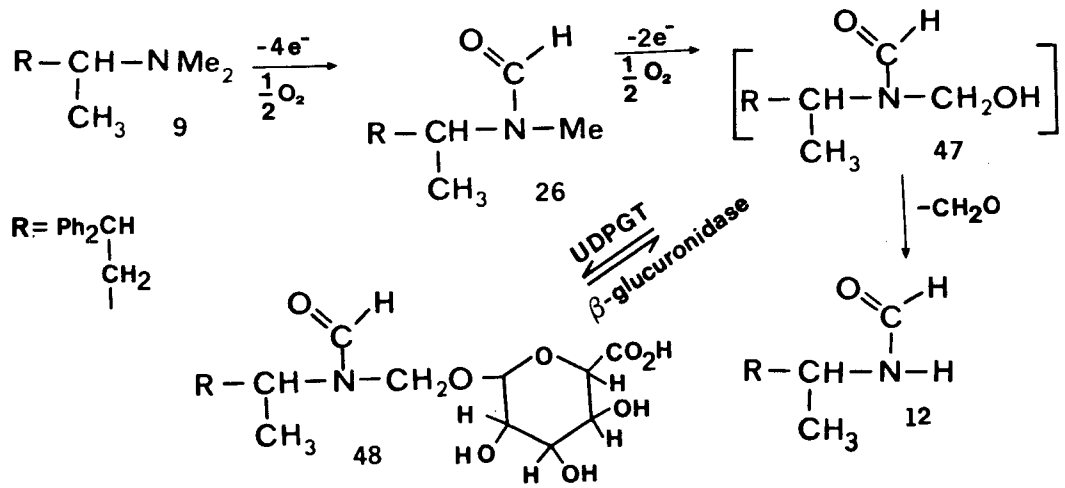


Figure 12. Metabolism of recipavrin (9) to the secondary formamide (12) via a hypothetical carbinolamide pathway.

ii. Metabolism of formamides

Reactive metabolites of the anticancer agent N-methylformamide (NMF) (Pearson, *et al.*, 1987ab) and DMF (Scailteur and Lauwerys, 1987) are known to result in hepatotoxicity. Although the metabolism of these simple aliphatic formamides has been extensively investigated (Kestell, *et al.*, 1985, 1987, Scailteur, *et al.*, 1984, Brindley, *et al.*, 1983), there are very few reports on the metabolism of higher formamides. Borchert *et al.*, (1981) have studied the demethylation rate of a tertiary formamide analogue of methamphetamine. Swaminathan and Bryan (1984) have studied the metabolism of the urinary bladder carcinogen N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT). No other reports on the metabolism of formamides with alkyl chains larger than t-butyl are available.

iii. Importance of metabolic studies on arylaliphatic formamides

Amphetamine, methamphetamine, methylenedioxyamphetamine (MDA) and N-methyl methylenedioxyamphetamine (MMDA) are often illegally manufactured by the Leuckart-Wallach reaction (Frank, 1983) and as a result of incomplete hydrolysis, often contain arylaliphatic formamides as well as other impurities (LeBelle, *et al.*, 1973, Kram, *et al.*, 1977).

The recipavrin formamides 12 and 26 were structurally similar to the amphetamine formamides. Metabolic studies were undertaken to characterize potentially hepatotoxic compounds of importance to amphetamine abuse, and as part of a study on the source of formamide metabolites of the tertiary arylaliphatic amine recipavrin (9) .

7. OBJECTIVES OF THE THESIS

Considering the novel nature and potential toxicity of formamide metabolites, this work set out to clarify the mechanism of formamide generation with the following main objectives.

1. Studies on the source of the methadone formamide metabolite and the peroxidation of EDDP (1) were to be completed. Solvent effects on the recovery of the formamide metabolite, perturbations of the reaction conditions in the EDDP oxidation and the peroxidation of the parent drug methadone were undertaken.

2. Metabolites of recipavrin (9), especially those that may mediate the observation of a secondary formamide metabolite were to be synthesized. Improving the synthesis of the secondary formamide (12) and obtaining better analytical results for the primary hydroxylamine was also necessary.

3. The biliary metabolites of recipavrin (9) were to be characterized in detail to identify any possible precursors of the formamide metabolite (12).

4. The metabolites of norrecipavrin (15) and dinorrecipavrin (20) were to be characterized in detail to determine whether the desalkyl amines were intermediates in the generation of the secondary formamide metabolite (12).

5. Formamide analogues of promethazine (10) were synthesized and metabolic extracts from promethazine dosed rats were screened for formamide metabolites.

6. The possible chemical generation of the secondary formamide metabolite (12) from an isomeric oxaziridine (25) or nitrone (24) was to be investigated.

7. The metabolism of the recipavrin tertiary formamide (26) as an intermediate in the carbinolamide (47) mediated formation of the secondary formamide metabolite (12) of recipavrin was to be investigated.

8. The metabolites of the tertiary formamide (26) and secondary formamide (12) analogues of recipavrin were to characterized as of potentially toxic metabolites of Leuckart specific formamides.

II. EXPERIMENTAL

1. CHEMICALS AND MATERIALS

Chemicals were reagent grade and obtained from the following sources.

Aldrich Chemical Co.(Milwaukee, Wisconsin)

1,3-Dihydronaphthalene, 4-dimethylaminopyridine, aluminum chloride, benzalacetone, calcium chloride, deuterochloroform (gold label), diethylene glycol, diphenylacetonitrile, ethylchloroformate, D-glucuronic acid, iminodibenzyl, lithium aluminum hydride, methylchloroformate, n-butyllithium (1.6 M in hexane), N-methylhydroxylamine hydrochloride, para-nitroperbenzoic acid, phenothiazine, propargyl bromide, sodium hydride, sodium cyanoborohydride, tetrahydrofuran, trichloromethylchloroformate, triethylamine.

Allied Chemical (New York, N.Y.)

Ferrous sulfate, sodium acetate

American Scientific and Chemical Co. (Seattle, Washington)

Formaldehyde (38%) aqueous, hydrochloric acid, potassium hydroxide, sodium hydroxide, sulfuric acid.

Applied Science Laboratories (State College, Pennsylvania)

Dexsil-300 on Gas Chrom Q, amberlite XAD-2 resin.

J.T. Baker Ltd. (Philipsburg, New Jersey)

Flash chromatography supplies and solid phase extraction apparatus.

BDH Chemicals (Toronto, Ontario)

Acetone, acetonitrile, benzene, calcium carbonate, chloroform, ether (anhydrous), ethylformate, hydrochloric acid, hydroxylamine hydrochloride, ligroine (60-80°), magnesium sulfate (anhydrous), pet. ether (30-60°), pyridine, sodium chloride, sodium sulfate (anhydrous), toluene.

Brinkmann Instruments (Toronto, Ontario)

Dragendorf's reagent.

Caledon Laboratories Ltd. (Georgetown, Ontario)

All Caledon products were "distilled-in- glass" grade.

Acetonitrile, dichloromethane, ethyl acetate, methanol (HPLC grade), water (HPLC grade).

Eastman Kodak Co. (Rochester, New York)

Thin layer chromatograms (silica gel 0.2 mm), methylamine (40% aqueous).

Fisher Scientific Co. (Fairlawn, New Jersey)

Magnesium chloride, sodium bicarbonate.

Kabivitrum Ltd. (Stockholm, Sweden)

N-ethyl-N,α-dimethyl-γ-phenylbenzenepropanamine, Recipavrin C^2H_3 (α-trideuteromethyl-N,N-dimethyl-γ-phenylbenzenepropanamine), terodiline HCl

Linde Co. (Union Carbide, Vancouver B.C.)

Molecular sieve Type 4A

Mallinkrodt Chemicals (St. Louis, Missouri)

Potassium carbonate (anhydrous), sodium bicarbonate, sodium sulfate.

Matheson Ltd. (Edmonton, Alberta)

Hydrogen chloride gas.

Merck Ltd. (Rahway, New Jersey)

Yellow mercuric oxide.

Merck Sharpe and Dohme (Isotopes) (Montreal Que.)

Deuterium oxide, Dimethylsulfoxide-D_6 (DMSO-D_6)

Matheson Coleman and Bell Co. (Norward, Ohio)

Dimethylsulfoxide, methylacrylate, potassium carbonate.

Pierce Chemical Co. (Rockford, Illinois)

N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), O-methylhydroxylamine HCl (MOX), Trimethyl anilinium hydroxide (TMAH), acetonitrile (silylation grade).

Sigma Chemical Co. (St. Louis Mi.)

Amphetamine sulfate, glucurase^{R}, sulfatase, phenolphthalein glucuronide.

Supelco Ltd. (Bellafonte, Pa.)

Dimethylchlorosilane.

Stanchem Ltd. (Winnipeg, Manitoba)

Ethanol 95%.

Synthesized in our laboratory (Abbott, *et al.*, 1979)

2,2-Diphenyl-4-dimethylaminovaleronitrile, (±)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate (EDDP), methadone hydrochloride, 4,4-diphenyl-2,5-heptanedione (Abbott *et al.*, 1986).

Terochem (Edmonton, Alberta)

Meta-chloroperbenzoic acid (85%).

2. ANIMAL EXPERIMENTS

Animals and surgical equipment were obtained from the following suppliers.

Animal Care Facility (U.B.C., Vancouver, B.C.)

Wistar male rats (200 - 300g)

Becton Dickinson (Rutherford, New Jersey)

Yale needle 22G, 1 1/2", tuberculin syringe 1 cc

Clay Adams (Parsippany, New Jersey)

Polyethylene tubing PE 10

Ethicon Ltd. (Peterborough, Ontario)

4-0 Silk

Permabond International Div. (Englewood, New Jersey)

910^{R} Adhesive

3. PREPARATION OF TLC SPRAY REAGENTS

A. Naphthoresorcinol spray reagent (Heyns and Kelch, 1953)

1,3-Dihydronaphthalene (200 mg) was dissolved in a solution of 166 ml of 95% ethanol and 34 ml of 85% phosphoric acid.

B. Dragendorf's Reagent

Equal volumes of Dragendorf's A and B solution (Brinkman Instruments, Toronto) were mixed and stored in the refrigerator until used.

C. Tollen's Reagent

Tollen's reagent was prepared according to Vogel (1956) and used immediately. Excess was disposed of immediately due to the explosion hazard.

4. INSTRUMENTATION

A. NMR spectra

NMR spectra were recorded in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard on a Bruker WP-400, WP-80, or Oxford-Nicolet-270 spectrometer at the Department of Chemistry, UBC. All NMR data are reported as follows: NMR (MHz): shift in ppm from TMS (splitting, coupling constant, assignment). For example, NMR (400 MHz): 1.16 (d, J=8Hz, $C\underline{H}_3CH$).

B. Infrared spectra

Infrared spectra were recorded as liquid films or nujol mulls on NaCl plates using a Unicam SP-1000 spectrometer. All infrared data are reported as follows: IR (film, solution (thickness), or mull): frequency in cm^{-1} (intensity, assignment). For example: IR (nujol mull): 1710 (s, C=O str.).

C. Ultraviolet spectra

Ultraviolet spectra were recorded on a Beckmann Model 24 spectrometer. Absorbances are reported as follows: UV (solvent): Lambda max in nm, (Molar extinction coefficient, assignment). For example: UV Spectrum (methanol) 207 (18000, pi-pi* arom.).

D. Melting points

Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected.

E. Capillary GCMS

All mass spectra are reported as follows: GCMS (column)(capillary column assumed unless otherwise specified)): M^+ (% intensity); base peak (100%); next strongest peak (% intensity); etc. Standard operating conditions are assumed unless otherwise specified.

Capillary GCMS was performed on a Hewlett Packard 5987A GCMS system using a crosslinked methylsilicone fused silica capillary column (HP19091-112), 25 meters by 0.32 mm i.d. (capacity approximately 250 ng per component). Under standard conditions, column temperature was programmed from 50° to 150° at 30° per minute , then at 4° per minute to 260° and then held constant for two minutes. Injections (1 uL) were made in splitless mode with helium carrier gas back pressure set at 15 psi. Injection port, interface and source temperatures were 250°. The solvent diverter was left on and data acquisition was delayed until 5 minutes after each injection. The mass range scanned was 40 to 400 AMU with a multiplier voltage of 2300, emission current 300 uA and ionization energy 70 eV.

Modifications of standard GCMS operating conditions:

i. Condition B: Standard operating conditions with helium back pressure set at 8 psi.

ii. Condition C: For promethazine related compounds, Standard operating conditions, with oven temperature programmed from 50° to 200° at 30° per minute and then from 200° to 260° at 4° per minute. Helium back pressure was 15 psi.

iii. Condition D: For preliminary characterization of recipavrin related compounds and spiking experiments a 25 M by 0.2 mm SE-54 column (Hewlett Packard part number 19091-61725) was used under standard operating conditions.

iv. Condition E: For solvent control experiments on methadone, oven temperature was programmed from 50° to 150° at 30° per minute, held constant at 150° for 5 minutes then programmed from 150° to 280° at 10° per minute and held constant at 280° for 12 minutes. Helium back pressure was 15 psi.

F. Direct insertion probe mass spectra

Direct insertion probe mass spectra were done on the Hewlett Packard 5987A with temperature programmed desorption from the probe. The probe crucible was loaded with 1 ul of a 1 ug/ul solution of the analyte. After the methanol had evaporated the probe was inserted and the probe temperature was held constant at 50° for one minute and then programmed to increase at 30° per minute to a maximum of 350°.

G. Packed column GCMS analysis

Packed column GCMS analysis was done on a Hewlett Packard 5700A gas chromatograph interfaced to a Varian MAT 111 mass spectrometer via a variable slit separator. Electron impact mass spectra were recorded at 70 eV, ion source pressure 5x 10^{-6} torr, emission current 300 uA, and source temperature 250°. Computerized background subtractions were made and mass spectra were recorded over a scan range of 40 to 750 amu with one scan every five seconds. Total ion current (TIC) plots were based on m/z 50-500. Mass chromatograms were plotted in scan mode. The data was processed by an online Varian 620L computer system. A silanized glass column (2m x 2mm i.d.) packed with 3% Dexsil 300 on 80/100 mesh Chromosorb W-AW (Supelco, Bellefonte, Pennsylvania) was used with helium carrier gas (20 ml/min.). Under standard conditions column temperature was programmed from 150-280° at 4° per minute and then held constant for 8 minutes. The injection port, line and separator temperatures were held at 250°.

Other GCMS operating conditions:

i. Condition F: As above with column temperature programmed from 200 to 280° at 8° per minute.

ii. Condition G: For amphetamine related compounds, column temperature was held at 150° for 4 minutes, then increased at 4° per minute to 270° and then held constant for 4 minutes.

H. Liquid chromatography-mass spectrometry

HPLC grade acetonitrile and water were passed through a 0.45 u filter, degassed under water aspirator vacuum prior to use, and treated with helium during liquid chromatography.

A Hewlett Packard model 1090 liquid chromatograph with a variable volume automatic loop injector, HP3392 integrator, fixed wavelength (254 nm) detector and HP-85 microprocessor was used. The LC was equipped with a 100 x 2.1 mm Hypersil ODS column (5u particle size, HP catalogue no. 79916). Stock solutions were prepared in acetonitrile (10 ng/ul) and passed through a 13 mm diameter 0.45 u Alpha-450 filter (Gelman, Ann Arbour, Michigan) before injection into the LC. The conjugated bile fraction equivalent to 8 ml of rat bile, was blown free of methanol with nitrogen and reconstituted with 0.5 ml of acetonitrile. The sample was filtered free of solids (0.45 u Alpha-450 filter); the filter was washed with 0.5 ml acetonitrile. The combined filtrates were used for LCMS.

Two ul injections were used with a flow rate of 200 ul / min. (column pressure=44 psi) and solvent ratio 65% water 35% acetonitrile, to optimally resolve standards and interfering peaks in bile samples. The oven temperature was held at 40°. After passing through the UV detector, the eluent entered a sample transfer line and in line filter (HP part no.

3150-0369) connected to a Hewlett Packard direct liquid introduction (DLI) LCMS interface (option number 004). The split ratio in the DLI was determined experimentally to be 13% (26 ul/min. entering the mass spectrometer). For LCMS, the HP 5987A MS system was operated in positive chemical ionization mode with a stable source pressure of 1.3×10^{-4} torr, source temperature 250°, emission current 300 uA, repeller voltage 8 V, multiplier voltage 2400 eV and ionization energy 240 eV. Suitable mass spectra of the secondary formamide and methylene nitrone standards were obtained with 100 ng/ul standard solution injections. Then ion monitoring of the m/z 254 (M^++1), 238 (M^++1-16), and 295 (M^++1+CH_3CN) ions (200 microsecond dwell time on each ion) was done on a 10 ng/ul mixture of the two standards. A blank injection was then made to ensure no carryover occurred from the standard mixture. Then the conjugated bile extract was analyzed by selected ion monitoring LCMS to detect the presence of the nitrone and/or formamide.

I. High resolution mass spectrometry

High resolution mass spectra were recorded on a Kratos MS 50 high performance mass spectrometer in electron impact mode at 70 eV with a source temperature of 120°. The scan range was 31-743 amu.

5.METABOLISM EXPERIMENTS

A. Animal Surgery and Drug Administration

Male Wistar rats were fasted for 18 hours before surgery. The rats were anaesthetized with ether and the bile duct was cannulated with PE-10 tubing. The cannula, secured with silk thread and a small drop of Kodak 901 adhesive, was passed through the abdominal wall and under the skin to the back of the neck where it was exteriorized. The abdomen was closed with interrupted sutures and the rat was placed in a restraint cage. Fifteen minutes after recovery from anaesthesia, the rat was given a subcutaneous injection of recipavrin hydrochloride (10 mg in 0.1 ml of sterile distilled water). Bile and urine were collected for 18 hours. A second dose of drug was given 12 hours after the first dose. The bile cannula was threaded through an 18 gauge needle piercing the rubber cap of a plain Vacutainer^{R} tube. The collection tube was held in an ice bath and protected from light during the collection interval. The 18 hour bile sample (12-18 ml) was diluted to 20 ml, mixed well and worked up immediately or stored frozen at -70^{o}.

The following drugs and doses were administered by subcutaneous (sc) or intraperitoneal (ip) injection to at least 3 bile duct cannulated animals:

Recipavrin HCl: 10 mg/0.1 ml H_2O sc, Recipavrin $^2H_3$ HCl: 10 mg/0.1 ml H_2O sc, Norrecipavrin HCl: 10 mg/0.1 ml H_2O sc, Dinorrecipavrin HCl: 10 mg/0.1 ml H_2O sc, Recipavrin tertiary formamide 10 mg/0.1 ml corn oil sc, Recipavrin secondary formamide: 10 mg/0.1 ml corn oil sc (1 animal only), Promethazine HCl (Phenergan^R): 12.5 mg/0.1 ml H_2O sc (1 animal only), Recipavrin methylene nitrone 10 mg/0.1 ml ethanol:propylene glycol 1:1 sc. (1 animal only).

B. Bile sample preparation for GCMS

In 10 x 1.5 cm teflon capped centrifuge tubes, 2 ml aliquots of bile (pH 8.3) were centrifuged at 2000 rpm for 15 minutes, decanted and diluted to 4 ml with water and adjusted to pH 10 with a combination of 0.1M NaOH and a 0.12 sodium borate buffer. Nonconjugated metabolites were extracted with three, three ml aliquots of one of the following extraction solvents: 1. Distilled in glass grade ethyl acetate, 2. Distilled in glass grade chloroform containing 1% ethanol, 3. Distilled in glass grade methylene chloride, 4. Reagent grade benzene, 5. Freshly distilled ether. Samples were vortexed vigorously and centrifuged to separate phases. The organic phases were dried over K_2CO_3 ($CHCl_3$, CH_2Cl_2) or Na_2SO_4 (ethyl acetate, benzene and ether) and then evaporated to dryness in a 38^o water bath under a stream of dry nitrogen. Samples were stored at -5^o and reconstituted with 20 ul of reagent grade methanol or HPLC grade acetonitrile just prior to GCMS analysis. Samples were concentrated and rerun under column

overload conditions to detect minor metabolites.

The aqueous remainder containing conjugated metabolites was adjusted to pH 5 with 1 M acetic acid and lyophilized. The residue was reconstituted in 2 ml of 0.1 M, pH 5 sodium acetate buffer, then 0.2 ml of Glucurase^{R} was added. After a gentle mix, the sample was incubated for 18 hours at 38^{o}. The hydrolyzed recipavrin metabolites were then extracted as outlined above, using the same solvent chosen for the extraction of nonconjugated metabolites.

Ethyl acetate (distilled-in-glass grade) was adopted as the solvent of choice for metabolite characterization.

C. Derivatization Methods

i. Trimethylsilylation

The conjugated bile extract was transferred to a one ml reaction vial, evaporated free of methanol and diluted with 30 ul of silylation grade acetonitrile and 30 ul of BSTFA. After heating at 60^{o} for four hours, the sample was concentrated under a stream of nitrogen and analyzed by GCMS.

ii. Methylation

a. N- and O-methylation

The methanolic bile extract was diluted with 50 ul of methelute^{R}. The sample was concentrated under dry nitrogen and analyzed by GCMS.

b. O-Methylation

The methanolic bile extract was diluted to 0.5 ml with methanol. Diazomethane, prepared from Diazald^{R} was bubbled through the solution for one minute. The sample was concentrated under nitrogen and analyzed by GCMS.

6. CHEMICAL SYNTHESES

A. Flash chromatography

Using the method of Still *et al.*, (1978), a 15 x 1 cm bed of flash chromatography grade silica gel was packed dry over a 0.5 cm bed of fine glass beads. Eluent, proven by TLC to have sufficient polarity to move the desired component to an rf of 0.3-0.4 was forced through the column under pressure with nitrogen gas until no air bubbles remained in the packing. The samples (100 mg) were loaded dissolved in 0.5 ml of mobile phase and the column was run at 2 ml per minute. Two ml fractions were collected. The same procedure was followed using a 2.5 x 15 cm column loaded with up to 500 mg of sample and 5 ml fractions were collected.

B. Synthetic compounds related to methadone

The isolation and characterization of the major oxidation products of EDDP (1) were reported previously (Slatter, 1983, Abbott, Slatter and Kang, 1986).

i. General procedure for meta-chloroperbenzoic acid (MCPBA) oxidations of methadone (8) and EDDP (1).

Method 1: To a solution of 100 mg (2.6×10^{-4} mol) of ($\pm$) EDDP perchlorate in 5 ml of $CHCl_3$ at 0°, was added 100 mg (5.2×10^{-4} mol) of MCPBA in 5 ml of 0° $CHCl_3$. After standing at 0° for 12 hours the reaction mixture was filtered, washed twice with cold 1.5 M NaOH solution, twice with water, and dried over K_2CO_3 and evaporated. The yellow oil was analyzed by packed column GCMS (3% OV-17 on 100-120 mesh Gas Chrom Q, 150-280° at 4° per min) and found to contain EMDP (5) (tr=18.2 min.), EDDP (1) (tr=20.0 min), diketone (3) (tr=21.8 min.), DDP (4) (tr=24.2 min.), and the oxaziridine (2) (eluted as the formamide (6), tr=29.5 min.). The individual components were isolated in pure form as described previously (Slatter, 1983, Abbott, *et al.*, 1986).

High resolution mass spectrum of the oxaziridine (2), (source temp.=120°): M^+ 309 (1.5, $C_{20}H_{23}NO_2$); 222 (28, $C_{16}H_{16}N$); 56 (80, C_3H_6N), remaining ions arise from thermal degradation to the formamide, 73 (100, C_3H_6NO); 253 (60, $C_{17}H_{19}NO$); 207 ($C_{16}H_{15}$), etc.

Method 2: The above procedure was repeated at one tenth scale using one equivalent of EDDP free base (freshly extracted from pH 10 aqueous solution with chloroform) and 2 equivalents of MCPBA over suspended excess K_2CO_3 (50 mg).

Method 3: The above procedure was repeated at one tenth scale using one equivalent of methadone HCl and 2 equivalents of MCPBA.

ii. GCMS identification of 6-N-methylformamido-4,4-diphenyl-3-heptanone (Methadone tertiary formamide (49)).

In methadone oxidation mixtures and old nonconjugated bile extracts a compound with the mass spectrum summarized below was observed.

GCMS of 49: (Condition F, tr=14.17 min.) M^+ 323 (0); 86 (100); 58 (41); 87 (40); 207 (25); 105 (20); 129 (18); 267 (18); 208 (15); 72 (14); 30 (13); 165 (9).

iii. Synthesis of 2,3-dimethyl-5,5-diphenylcyclopent-2-enone (50)

The diketone (3) (4,4-diphenyl-2,5-heptanedione) was dissolved in EtOH and heated under reflux in an equal volume of 0.75 M ethanolic sodium hydroxide solution for three hours. The solution was diluted with 4 volumes of water and extracted with ether. The ether phase was washed twice with water dried over Na_2SO_4, and evaporated. The brown oil was flash chromatographed in 98:2 hexane:ethyl acetate. Only one new component was present. It reacted quickly with iodine vapour and gave mass spectral results in accord with a cyclopentenone structure. The clear oil was analyzed by UV, IR and NMR and found to contain only the title cyclopentenone and not the regioisomer 3-ethyl-4,4-diphenylcyclopent-2-enone (51).

GCMS of 50: (condition F, tr= 6.6 min.): M^+ 262 (100); 185 (80); 247 (52); 233 (52); 219 (22); 165 (22).

NMR (270 MHz): 1.78 (s, CH_3-C-C=O); 2.13 (s, $C\underline{H}_3$-C-CH_2); 3.32 (s, CH_2); 7.12-7.32 (m, C_6H_5). Singlets at 1.78 and 3.32 broadened by homoallylic coupling.

IR (film): 1697 (s, C=O str.); 1655 (s, C=C str.).

UV (CH_3CN): 316 (419, cyclopentenone R band); 240 (8,122, cyclopentenone K band); 204 (17,819, arom.); 256 (arom., buried).

C. Synthetic compounds related to recipavrin

Some synthetic methodology and spectra for important synthetic intermediates and precursors of the formamide metabolite (12) have been reported previously (Slatter, 1983) and are summarized here. Improvements and new spectral data are detailed.

i. 1,1-Diphenyl-3-butanone (14) (CAS registry 5409-60-9) (Slatter, 1983)

Diphenylbutanone was synthesized using the method of Burckhalter, *et al.*, (1951). B.p. 125° at 0.3 mm. M.p.= 46° (lit. 46°).

^{1}H NMR: (100 MHz): 2.08 (s, CH_3); 3.22 (d, CH_2); 4.63 (t, CH); 7.15-7.4 (m, Ph_2).(Slatter, 1983)

ii. Syn/anti 1,1-diphenyl-3-butanone oxime (19) (CAS registry 36317-57-4)(Slatter, 1983)

Using the method of Morgan and Beckett (1975), diphenylbutanone (14) was added to a 1.1 molar excess of

hydroxylamine hydrochloride in pH 8.5 methanol solution(pH adjusted with 2M NaOH). The solution was stirred overnight and diluted with water, extracted with $CHCl_3$, dried over Na_2SO_4 and distilled (170° at 0.15 mm) to afford a viscous yellow oil which NMR revealed to be a 2:1 ratio of anti and syn isomers. The oxime was also characterized by GCMS as syn and anti TMS ethers (52 a and b) and O-methyl ethers (16 a and b) following derivatization with BSTFA and TMAH respectively. The oxime methyl ether (16) was also prepared from diphenylbutanone (14) using O-methylhydroxylamine hydrochloride (MOX) reagent under similar conditions.

^{1}H NMR (19) (100 MHz): Anti isomer: 1.8 (s, CH_3); 2.97 (d, CH_2); 4.35 (t, CH); 7.3 (bs $(C_6H_5)_2$); 9.0-9.05 (s, NOH). Syn isomer: 1.57 (s, CH_3); 3.15 (d, CH_2); 4.4 (t, CH); 7.3 (bs $(C_6H_5)_2$); 9.0-9.5 (bs, NOH)(Slatter, 1983).

iii. N,N,α-trimethyl-γ-phenylbenzenepropanamine hydrochloride (Recipavrin^R (9)) (CAS 13957-55-6)(Slatter, 1983).

Using the method of May and Mossetig (1948), 2-dimethylamino-4,4-diphenylvaleronitrile (4 g, (7.46 mmol) was refluxed overnight in 10 ml diethylene glycol containing 4 g KOH. The dark solution was cooled, diluted with 40 ml H_2O extracted with ether, dried over Na_2SO_4, and filtered. Precipitation from ether by the dropwise addition of ether saturated with gaseous HCl afforded 3.66 g (88%) of beige crystals. Recrystallization twice from benzene afforded

white crystals m.p. 153° (lit. 151-155°) (CAS 22173-83-7).

IR (film, free base): 1600 (m); 1580 (w); 1500 (m); 1450 (m); 1272 (m, amine); 1138 (m);1035 (m); 1060 (m); 750 (s); 715 (s).

iv. N,N,α-trimethyl-γ-phenylbenzenepropanamine N-oxide (recipavrin N-oxide (53))

Using the method of Craig and Purushothaman (1970), 300 mg of recipavrin hydrochloride was extracted from 5 ml of pH 12 solution with chloroform. Recipavrin free base (262 mg (1.02 mmol) in chloroform was treated with 220 mg MCPBA (1.02 mmol) in chloroform at 0-5°. After stirring for three hours, the ice bath was removed and the solution was passed through a 3 x 2.5 cm column packed with 80-200 mesh alumina. The column was washed with chloroform and then the N-oxide was eluted with 25% methanol in chloroform. The solvent was evaporated and the chromatography was repeated.

GCMS (decomposes to Cope elimination products cis and trans 1,1-diphenyl-2-butene (54 and 55)): Cis isomer: tr=7.92 min. M^+ 208 (6); 167 (100); 165 (26); 152 (18); 115 (10); 208 (6); 193 (5); 128 (4). Trans isomer: tr=8.26 min. M^+ 208 (68); 115 (100); 193 (58); 178 (42); 91 (38); 178 (38); 130 (38); 165 (36).

NMR (N-oxide)(80 MHz, some recipavrin present in spectrum): 1.37 (d J=6.4 Hz, $C\underline{H}_3CH$); 3.03 (d or two singlets J=1.8 Hz, $^-ON^+(CH_3)_2$); 3.55-4.10 (m, $Ph_2C\underline{H}$);

3.0-3.5 (m, C<u>H</u>CH_3); 1.70-2.5 (m, CH_2); 7.05-7.45 (m, Ph_2).

v. *a*-Methyl-γ-phenylbenzenepropanamine (CAS registry 29869-77-0) (dinorrecipavrin (20)) (Slatter, 1983)

The primary amine was synthesized by acid hydrolysis of the secondary formamide (12) followed by extraction and distillation of the free base (clear liquid, 170° at 4 mm). The reductive hydroxylamination of diphenylbutanone (14) with ammonium acetate and sodium cyanoborohydride in methanol (Slatter, 1983) was abandoned. The hydrochloride salt was precipitated with gaseous HCL (m.p. 172°, Lit. 175° (Burckhalter *et al.*, 1951)).

^{1}H NMR (300 MHz): (free base): 1.08 (d, CH_3); 1.22 (s, NH_2); 1.75 (m, C<u>H</u>CH_3); 2.09 (m, CH_2); 4.08, (t, C<u>H</u>Ph_2); 7.22 (m, Ph_2).

vi. (±) N-hydroxy-*a*-methyl-γ-phenylbenzenepropanamine (primary hydroxylamine (22)) (Slatter, 1983)

The NMR and IR spectral results reported previously (Slatter, 1983) for the hydroxylamine (22) corresponded to the autoxidized product (56). An improved synthetic method is detailed below along with correct spectral results.

Using the method of Morgan and Beckett (1975), a stirred solution of 0.239 g (0.001 mol) of oxime (19) in 1.5 mL MeOH was added 63 mg (0.001 mol) sodium

cyanoborohydride. HCl (2M) was then added dropwise to maintain pH 3-4 for 15 min. until gas evolution slowed. The solution was stirred for three hours, then adjusted to pH 1.5-2.0. After gas evolution ceased, the MeOH was evaporated and the residue was dissolved in water, adjusted to pH 8 with 20% K_2CO_3, extracted with ether, dried over Na_2SO_4, and concentrated by flash evaporation at room temperature. The residue was dissolved in 40:60 hexane:EtOAc and flash chromatographed on a 10 x 1 cm silica gel column. After eluting the oxime (0-25 mL), the hydroxylamine was collected (25-70 mL). After evaporation the product (48 mg, 20%) was analyzed or used in subsequent reactions immediately, since it was rapidly autoxidized. The product gave black spots when visualized with Tollen's reagent after TLC separation. Most of the hydroxylamine decomposed during GCMS analysis, however a small portion survived the GC sector to give a satisfactory mass spectrum. The major decomposition product was the primary amine. O-TMS (57) and methyl derivatives were prepared for GCMS analysis. The permethyl derivative (58) was identical to the O-methylated secondary hydroxylamine.

GCMS: (underivatized (22)): tr=15.02 min.: M^+, 241 (9); 60 (100); 44 (73); 165 (64); 167 (55); 208 (32); 152 (27); 91 (18). (TMS derivative): tr= 15.5 min.: M^+ 313 (8); 132 (100); 44 (52); 116 (50); 75 (30); 167 (30); 118 (20); 91 (10).

NMR (400 MHz): 1.09 (d, CH_3); 1.9-2.0 (m, CH_aH_b); 2.35-2.45 (m, CH_aH_b); 2.83-2.93 (m, $CHCH_3$); 4.01-4.09

(t-dd, C<u>H</u>Ph_2); 5.3-5.7 (br.s, N<u>HOH</u>); 7.1-7.3 (m, arom.).

<u>IR</u>: (film): 3470-3100 (br. s, NHOH str.); 3080-2830 (s, several bands); 2000-1700 (w, overtones); 1598 (m, arom.); 1570 (w-m, arom.); 1490 (s); 1445 (s); 1365 (m); 1150 (w-m); 1095 (m); 1062 (m); 1025 (m-s); 1005 (br. m); 905 (w); 880 (w); 780 (w-m) 745, 737, (s); 700 (s).

vii. Spectral characterization of the hydroxylamine autoxidation product (56).

<u>NMR</u> (300 MHz): 1.09 (d, CH_3); 1.88-2.0 (m, C<u>H_a</u>H_b); 2.32-2.44 (m, CH_a<u>H_b</u>); 2.8-2.92 (m, C<u>H</u>CH_3); 3.98-4.07 (t-dd, C<u>H</u>Ph_2); 7.1-7.3 (m, arom.). Spectrum is identical to the hydroxylamine without the NHOH resonance.

<u>IR</u>: (nujol) 3250 (sharp m); 3000-2800 (s, nujol); 1598 (w-m, arom.); 1570 (w, arom.); 1495-1450 (s, nujol); 1430 (m); 1370 (m, nujol); 1150 (w-m); 1110 (w); 1065 (m); 1020 (m-s); 935 (m); 915 (w-m); 850 (w); 835 (w-m); 780 (w-m); 750 (s); 738 (s); 702 (s).

viii. ($\pm$) N,α-dimethyl-γ-phenylbenzenepropanamine (norrecipavrin (15)) (CAS 29869-78-1)(Slatter, 1983)

Norrecipavrin was synthesized by reductive amination of diphenylbutanone (14) with methylamine and sodium cyanoborohydride in acidic MeOH using the method of Morgan and Beckett (1975) (Slatter, 1983). The hydrochloride (CAS 61721-58-2) was precipitated from ether and recrystallized once from ethyl acetate and acetonitrile. The mono trimethylsilyl

derivative was also synthesized and characterized by GCMS. Norrecipavrin was also obtained by acid hydrolysis of the tertiary formamide (26).

ix. (±) N-hydroxy-N,α-dimethyl-γ-phenylbenzenepropanamine (secondary hydroxylamine (17))(Slatter, 1983)

To 0.5 mL of an aqueous solution of 0.42 g (0.005 mol) N-methylhydroxylamine hydrochloride was added 1.0 g (0.004 mol) diphenylbutanone (14) in 25 mL MeOH. After adjusting to pH 6, 0.35 g of sodium cyanoborohydride was added and pH 5-6 was maintained by the dropwise addition of 5% HCl, until the pH remained constant. After stirring for an additional hour, the pH was lowered to one with 6N HCl, gas evolution was allowed to subside and the solution was diluted with 10 mL H_2O, washed with ether, adjusted to pH 8 with 5% KOH, saturated with NaCl, extracted with ether, dried and evaporated to afford 0.24 g (50%) of a clear oil. Most of the hydroxylamine decomposed during GCMS analysis to the corresponding secondary amine. A small portion survived the GC to give a satisfactory mass spectrum. O-TMS (18) and O-methyl (57) derivatives were prepared for GCMS analysis.

GCMS: (underivatized (17)): tr=15.2 min.: M^+ 255 (6); 74 (100); 58 (55); 167 (45); 165 (20); 152 (10); 115 (8); 222 (8). (TMS derivative (18): tr= 15.2 min.: M^+ 327 (4); 146 (100); 58 (28); 167 (15); 132 (5); 73 (5); 165 (5); 208 (3). (O-methyl derivative (58)): tr= 13.5 min. M^+ 269 (3); 88 (100); 58 (34); 167 (16); 165 (12); 152 (6); 115 (4); 77(4).

^{1}H NMR (100 MHz): 1.06, (d, CH_3CH);4.1 (t, $CHPh_2$); 2.0 (m, CH_3CH); 2.4-2.8 (m (buried), CH_2); 2.56 (s, CH_3N); 7.1-7.4 (m, Ph_2); 9.7 (bs, OH).(Slatter, 1983).

IR (nujol mull): 3200 (broad m, NH,OH str.); 1950 (w); 1590 (m); 1580 (m); 1490 (s); 1450 (s); 1370 (s); 1360(m); 1220, 1190, 1160, 1130 (m); 930 (w); 910 (w); 800 (m); 780, 750, 730 (m-s); 700 (s).(Slatter, 1983)

x. (±) *a*-Methyl-(N-methylene)-γ-phenylbenzenepropanamine N-oxide (methylene nitrone (24))(Slatter, 1983)

Using the method of Coutts *et al.*, (1978), to benzene in a Dean and Starks apparatus was added 720 mg of the primary hydroxylamine (22) in 10 ml benzene. The solution was refluxed for two hours and then flash evaporated. The residue was flash chromatographed on a 2.5 x 10 cm column using $CHCl_3$: EtOH (99.25:0.75) to elute the yellow forerun. Then the eluent was changed to $CHCl_3$:MeOH:EtOH (98.25:1.0:0.75) to elute the white band. This fraction was rechromatographed in the same solvent to yield 285 mg (38%) of a clear oil. GCMS: decomposes to varying degrees in the GC inlet (see discussion).

GCMS: tr, nitrone (24)= 17.8 min. Mass spectrum: M^+ 253 (2); 165 (100); 56 (66); 91 (58); 167 (57); 115, (47); 152 (46); 193 (41); 208 (40); 73 (38); 130 (36); 179 (30); 178 (26); 236 (20); 222 (10).

^{1}H NMR (400 MHz): 1.42 (d CH_3); 2.23 (dd, CH_aH_bCH); 2.74 (dd, CH_aH_bCH); 3.79 (m, $CHCH_3$); 5.98 (d J=8Hz,

CH_aH_b=N); 6.41 (d, CH_aH_b=N); 7.1-7.4 (m, arom.)(Slatter, 1983).

^{13}C NMR: (Broad Band decoupled, splitting from SFORD): 19.92 (q, CH_3); 39.41 (t, CH_2CH); 47.74 (d, $CHPh_2$); 69.03 (d, $CHCH_3$); 122.29 (t, N=CH_2); 126.51-128.90 (8 doublets, arom.); 143.05, 144.16 (s, arom. C-C). (Slatter, 1983).

IR (film): 3395 (w-m, br., assoc. H_2O), 3020, 2970, 2920 (w-m); 1600 (m); 1566 (m-s, nitrone); 1490 (m-s); 1450 (m-s); 1300 (m); 1065 (s, nitrone); 740, 730, (m-s, arom.); 702 (s, arom.)(Slatter, 1983).

xi. Cis/trans N-(*a*-methyl-γ-phenylbenzenepropylidene) methylamine-N-oxide (methyl nitrone (44))

Using the method of Coutts *et al.*, (1978), N-methylhydroxylamine hydrochloride (3.72 g, 44.6 mmol) was dissolved in 5 ml H_2O and adjusted to pH 8 with 3M NaOH. Diphenylbutanone (14) (2g, 8.9 mmol) in 150 ml benzene was added. The solution was refluxed overnight in a Dean and Starks apparatus. The benzene solution was filtered and flash evaporated. Flash chromatography of 1 g (20%) of the amber residue on a 2.5x10 cm column in 96:3:1 $CHCl_3$:MeOH:EtOH was done and the low rf component was collected. Chromatography was repeated affording 240 mg (21%) of pure nitrone, which forms a white waxy solid when pumped under high vacuum. NMR revealed a 4.3:10 ratio of cis and trans isomers.

GCMS: tr=20.37 min.: M^+ 253 (6); 56 (100); 165 (82); 91 (70); 73 (50); 193 (50) 208 (47); 57 (45). Mass Spectrum (Direct insertion probe): M^+ 253 (9); 56 (84); 165 (22); 167 (15); 57 (15); 237 (13); 152 (13); 222 (5).

^{1}H NMR (300 MHz): Trans isomer (Major): 1.66 (s, CH_3CH); 3.27 (d J=6 Hz, CH_2); 3.59 (d, J=0.7 Hz, N-CH_3); 4.66 (t, J=6Hz, $CHPh_2$); 7.12-7.24 (m, 2H, arom.); 7.24-7.45 (m, 8H, arom.). Cis (minor) isomer: 2.04 (d, J=0.75 Hz, CH_3CH); 3.12 (d, J=5.6 Hz, CH_2); 3.28 (buried, NCH_3); 4.2 (t, J=5.8 Hz, $CHPh_2$); 7.12-7.45 (m, arom.).

^{13}C NMR (100 MHz): Trans isomer: 19.48 (CH_3CH); 38.81 (CH_2); 45.80 (NCH_3); 47.85 ($CHPh_2$); 126.31 (para, arom.); 127.65, 128.32 (ortho, meta, arom.); 142.27 (arom. C-C); 143.65 (C=N). Cis isomer: 18.39 (CH_3CH); 40.73 (CH_2); 46.60 (NCH_3); 48.55 ($CHPh_2$); 126.89 (para, arom.); 127.51, 128.68 (ortho, meta, arom.); 142.27 (arom C-C); 145.27 (C=N).

IR (nujol mull): 3000 (s, nujol); 2000-1800 (w, arom. overtones); 1660 (w); 1592 (m, arom.); 1560 (w); 1495 (m) 1470 (nujol); 1375 (nujol); 1335 (w-m); 1290 (w); 1245 (m-s); 1220 (m-s); 1160 (m-s); 1120 (w); 1040 (m); 1030 (m); 1120 (m); 935 (w-m); 920 (w-m); 790 (w-m); 740 (s); 702 (s); 692 (s).

xii. (±) α-Methyl-(N-methylene)-γ-phenylbenzenepropanamine (polymer) (methylene imine (23) as triazane (41))(Slatter, 1983)

A methanolic solution of dinorrecipavrin was treated overnight with a 1.3 molar excess of 38% aqueous formaldehyde over molecular sieve. After filtration and evaporation, GCMS revealed complete conversion to a compound that chromatographed as the monomer.

GCMS (packed column): tr= 2.16 min. M^+ (monomer (23)) 237 (4); 57 (100); 222 (18); 56 (16); 91 (9); 167 (8); 58 (8); 165 (8); 152 (5).(Slatter, 1983)

NMR (100 MHz): arylaliphatic resonances: 1.17 (d, J=6.4 Hz, CH_3, 26mm=9protons, distorted); 2.0-2.5 (m, $C\underline{H}_a\underline{H}_bCHCH_3$, 19mm=6 protons); 2.75-3.1 (m, $CH_2C\underline{H}CH_3$, 9mm=3 protons); 4.0-4.2 (m, $CHPh_2$, 9mm=3 protons); 7.1-7.4 (97mm=30 protons); triazane and related resonances: 3.22 (s, 28mm=9 protons); 3.33 (s, 7mm=2 protons); 3.45 (s, 3mm=1 proton); 3.5 (br. s, 3mm= 1 proton); 4.28 (s, 20mm=6 protons);4.48 (d, 6mm= 2 protons); 4.62 (s, 3mm=1 proton) (Slatter, 1983).

IR (film): 3095 (m-s); 3065, 3025, (m-s arom.); 2965 (aliph CH str.); 2935 (CH_3); 1598, 1583 (m-s, arom); 1490 (s), 1448 (s) (arom.); 1378 (br. m); 1260 (w); 1235 (w); 1150 (s); 1068, 1030, 1018, 1000 (br, m); 965 (w-m); 910 (br. w-m); 780 (w); 850 (m); 738 (m-s); 704 (s, arom.).(Slatter, 1983).

xiii. (±) 2-(4',4'-Diphenyl-but-2'yl) oxaziridine (25)(Slatter, 1983)

Following the modified method of Krimm (1958), 750 mg (2.88 mmol) of dinorrecipavrin HCl (20) in 10 ml H_2O was cooled to 0°. Aqueous formaldehyde (38%) (0.5 ml, 6.33 mmol) was added, after stirring for half an hour. To the stirred solution was added 1 g (5 mmol) MCPBA (85%) in 20 ml $CHCl_3$. After 2 hours , 10 ml of 1.1 M $CaCO_3$ was added dropwise. The chloroform phase was separated, washed three times with 1 M NaOH to hydrolyze the intermediate perester, then twice with water, dried over K_2CO_3 and evaporated at room temperature. Flash chromatography in 9:1 pet. ether (30-60°): EtOAc afforded 164 mg (22%) of two diastereomeric oxaziridines which appeared as black spots when TLC plates were developed with Dragendorf's reagent. The chromatography was repeated and the major diastereomer was isolated in pure form for NMR analysis. Samples decomposed on standing to the isomeric secondary formamide (12).

GCMS: Identical to the secondary formamide (12). (packed column): tr=5.5 min. M^+ 253 (25); 73 (100); 208 (42); 167 (40); 165 (28); 193 (27); 130 (37); 181 (23); 72 (23); 115 (18); 58 (12); 44 (10). A peak collected off a GC column in an ice cooled glass tube was eluted with $CDCl_3$ and found by NMR to be identical to the secondary formamide (12) synthesized by refluxing the primary amine (20) in ethyl formate. Mass Spectrum (direct inlet): identical to sec formamide (12)(Slatter, 1983).

^{1}H NMR: Major diastereomer: (400 MHz): 1.2 (d, CH_3); 1.86 (m, $C\underline{H}CH_3$); 2.21 (dd, $C\underline{H}_aH_bCH$); 2.34 (dd, $CH_a\underline{H}_bCH$); 3.28 (d, J=10 Hz, oxaz. $C\underline{H}_aH_b$); 3.82 (d, J=10 Hz, oxaz. $CH_a\underline{H}_b$); 4.01 (t, $C\underline{H}Ph_2$); 7.1-7.4 (Ph_2). Minor diastereomer: (100 MHz): 1.12, (d, $C\underline{H}_3CH$); 1.85 (m, $C\underline{H}CH_3$); 2.24 (dd, $C\underline{H}_aH_bCH$); 2.64 (dd, $CH_a\underline{H}_bCH$); 3.46 (d, J=10 Hz, oxaz. $C\underline{H}_aH_b$); 3.95 (d, J=10 Hz, oxaz. $CH_a\underline{H}_b$); 4.25 (t, $C\underline{H}Ph_2$); 7.1-7.4 (m, Ph_2).(Slatter, 1983).

^{13}C NMR: Major diastereomer: (100 MHz): (ppm, Broad Band decoupled, splitting from SFORD): 19.73 (q, CH_3); 40.12 (t, $\underline{C}H_2CH$); 48.23 (d, $CHPh_2$); 65.09 (d, $\underline{C}HCH_3$); 71.97 (t or dd, oxaz. CH_2); 126.18-128.94 (d, Ph_2); 144.12, 144.26 (arom. C_5H_5-$\underline{C}$-CH) (Slatter, 1983).

IR (film): 3100-2900 (m, series of bands) 1600 (m, arom.); 1585 (w, arom.); 1520 (s); 1480 (s); 1390 (m); 1250 (m-s, oxaz.); 1170 (w-m); 1120 (w-m); 1065 (w-m); 1035 (w-m, oxaz.); 970 (w-m); 935 (w-m, oxaz.); 770 (m-s); 700 (s).(Slatter, 1983)

xiv. (±) N-(1-methyl-3,3-diphenylpropyl) formamide (12) (Slatter, 1983)

Because of extensive reference to (±) N-(1-methyl-3,3-diphenylpropyl) formamide (12) in this thesis, for convenience, this compound will be referred to as the secondary formamide.

The secondary formamide (12) was synthesized by refluxing dinorrecipavrin free base in excess ethyl formate for several days using the method of Moffat *et al.*, (1962)(Slatter, 1983). The presence of suspended K_2CO_3 shortened the reaction time. Recrystallization from benzene/petroleum ether (60-80°) afforded a quantitative yield of white rods (m.p. 102-103°). NMR revealed a mixture of two rotamers.

The secondary formamide (12) was also obtained using the method of LeBelle *et al.*, (1973) for the Leuckart-Wallach reaction of diphenylbutanone (14) in refluxing formamide. After four hours, dilution with water, extraction with chloroform and evaporation afforded an 80% yield of the secondary formamide which was separated from the less polar byproduct, 4-(2,2-diphenylethyl) pyrimidine (59) by flash chromatography in hexane : EtOAc (4:1) or by fractional distillation (170-180° at 0.3mm) (pyrimidine) and 200-205° at 0.3mm (secondary formamide (12)). The secondary formamide (12) solidifies into a waxy mass in the receiving flask. A TMS derivative (60) was formed slowly at 80°.

GCMS (12): M^+ 253 (44); 73 (100); 167 (84); 165 (66); 208 (60); 44 (50); 193 (42); 181 (38).

GCMS (TMS derivative (60)): M^+ 325 (6); 145 (100); 73 (70); 165 (42); 167 (40); 221 (36); 152 (24); 130 (23).

^{1}H NMR (400 MHz): Trans rotamer: 1.19 (d, J=6.7 Hz, CH_3); 2.0-2.1 (m, $C\underline{H}_aCH_b$); 2.25-2.35 (m, J_{ab}= 14 Hz, $CH_aC\underline{H}_b$,); 4.02 (t, distorted, Ar_2CH and m, CH-N (buried)); 5.16 (s, broad, NH); 7.1-7.35 (arom.); 8.02-8.08 (s, HC=O). Cis rotamer: 1.24 (d, J=6.8 Hz, CH_3); 2.1-2.2 (m or ddd, $C\underline{H}_aCH_b$-CH_x, J_{ab}=12.8 Hz, J_{ax}=9 Hz); 2.25-2.35 (m, buried, $CH_aC\underline{H}_b$); 3.3-3.4 (m, CH-N); 4.04 (t, buried, Ar_2CH); 5.35 (s, broad, NH); 7.1-7.3 (arom.); 7.82 (d, broad, J=12 Hz, HC=O,).

^{13}C NMR (100 MHz, Shift from BB splitting from SFORD): Trans rotamer: 21.07 (q, CH_3); 42.71 (t, $\underline{C}H_2$-CH); 46.40 (d, $\underline{C}H$-CH_3); 48.22 (d, Ar_2-$\underline{C}H$); 126.19-128.89 (d, arom. $\underline{C}H$); 144.43 (s, arom. C-R, weak); 160.24 (d, H$\underline{C}$=O). Cis rotamer: 23.08 (q, CH_3); 43.17 (t, $\underline{C}H_2$-CH); 46.40 (overlap, d, CH_2-$\underline{C}H$); 47.86 (Ar$\underline{C}$H); 126.19-128.89 (d, arom.CH); 144.15 (s, arom. C-R); 163.76 (d, H$\underline{C}$=O).

IR (film): 3350-3120 (broad m-s, NH str.); 3065 (m), 3030 (m) (arom. CH); 2970 (m, aliph. CH str.); 2960 (m-s, CH_3); 2830 (m, assym. CH_3); 1950 (w), 1885 (w), 1810 (w) (arom. overtones); 1670 (m-s, cis C=O str.); 1652 (s, trans C=O str.); 1602 (m), 1585 (w-m) (arom. C-C skel. str.); 1548 (m-s), 1540, (w-m shoulder, amide II); 1495 (m), 1450 (m) (arom.); 1385 (m-s, C-N str. amide); 1245 (w-m, broad, amide III); 1140 (w, CH in plane bend); 1062 (w-m), 1030 (w-m) (arom.); 1015 (w-m); 783-760 (w-m out of plane NH wag); 780 (w-m); 745 (m-s), 735 (m), 700 (s) (arom. CH bend).

IR ($CHCl_3$ solution): 3439 (m, sharp, trans NH)); 3397 (cis NH); 1697 (s, trans C=O); 1685 (s, cis C=O); 1550-1510 (m, broad, $CHCl_3$ overlap), 1400 (m, broad) (C-N str. amide).

xv.Characterization of the Leuckart specific byproduct 4-(2,2-diphenylethyl) pyrimidine (59)

GCMS: M^+ 260 (44); 167 (100); 165 (45); 169 (32); 152 (25); 183 (20).

^{1}H NMR (270 MHz): 3.51 (d, J=8Hz, CH_2); 4.62 (t, J=8Hz, $Ar_2\underline{C}H$); 6.93 (d, J=5 Hz, HC_5 pyrimidine); 7.2 (arom.); 8.49 (d, J=4Hz, HC_6 pyrimidine); 9.16 (s, HC_2 pyrimidine).

IR (nujol mull): 1582 (m-s), 1577 (m) (arom.); 1550 (w-m, C=N str.); 1495 (m, arom.); 1312 (w); 1159 (w); 1080 (w); 1032 (w, arom.); 992 (w); 959 (w), 910 (w) (arom.); 752 (m), 739 (m), 702 (s) (arom.).

xvi. ($\pm$) N-(1-methyl-3,3-diphenylpropyl) formohydroxamic acid (61)

Using the method of Fishbein, *et al.*, (1968), to 250 mg (1.04 mmol) of freshly synthesized primary hydroxylamine (22) in 2.5 ml EtOH was added dropwise a solution of 50 mg (2.1 mmol) sodium metal in 2.5 ml EtOH sufficient to make the solution mildly alkaline. Then 84 ul (77 mg, 1.04 mmol) ethyl formate was dissolved in 1 ml of EtOH and added to the reaction mixture. The mixture was cooled and the remaining sodium ethoxide was added.

After 30 minutes the solution was allowed to come to room temperature and stirred for two additional hours. A white precipitate formed. The solution was brought to pH 4 with dilute HCl and flash evaporated. The residue was dissolved in a two phase mixture of EtOAc and water. The organic phase was separated and washed with water. The polar product was purified free of oxime and diphenylbutanone by flash chromatography in 2:1 hexane:EtOAc. The product was an orange red oil. The product was derivatized with TMAH for GCMS analysis.

GCMS (packed column, tr=5 min.): M^+ 283 (5); 72 (100); 208 (85); 42 (74); 167 (43); 193 (38); 130 (35); 165 (30); 74 (24); 91 (21); 115 (18); 181 (15); 252 (3); 239 (1).

IR (film): 3600-3000 (s br., OH); 3000-2850 (s)200-1800 (arom. overtones); 1665 (s, C=O); 1600 (m); 1585 (w); 1490 (m-s); 1448 (m-s); 1390 (m); 1170 (m); 1110 (w); 1075 (w); 1045 (w); 1025 (m); 1000 (w); 985 (w); 910 (w); 863 (m-s); 840 (w); 782 (w-m); 748, 735 (s); 700 (s); 680 (m).

xvii. (±) N-methyl-N-(1-methyl-3,3-diphenylpropyl) formamide (26)

Because of extensive reference to (±) N-methyl-N-(1-methyl-3,3-diphenylpropyl) formamide in this thesis, this compound will be referred to as the tertiary formamide (26).

The tertiary formamide was synthesized by the Leuckart-Wallach reaction of diphenylbutanone (14, 300 mg) in excess (3 mL) refluxing N-methylformamide. After 24 hours the dark brown solution was diluted with water and extracted with three 25 mL aliquots of chloroform. The chloroform extract was backwashed with water, dried over Na_2SO_4 and evaporated to afford a brown liquid that was analyzed by GCMS and found to be 90% tertiary formamide. The crude product was flash chromatographed twice on a 1x15 cm column using 20% EtOAc in hexane. After evaporation, 150 mg (42%) of a pale yellow oil was analyzed by NMR and IR spectroscopy and then used for metabolism experiments. Larger quantities of the formamide could also be purified by distillation (b.p. 173° at 0.04mm) yielding a viscous yellow oil which precipitated a pale yellow solid (m.p. 85-88°) from MeOH solution when stored at -5°. ^{1}H NMR revealed a 1.2:10 ratio of cis and trans rotamers (LaPlanche and Rogers, 1964). The tertiary formamide was also obtained by on column methylation of the secondary formamide with TMAH (Slatter, 1983).

^{1}H NMR (300 MHz): Trans rotamer: 1.23 (d, J=7 Hz, $C\underline{H}_3$-CH); 2.25 (t, CH_2); 2.79 (s, NCH_3); 3.37-3.50 (m, $C\underline{H}$-CH_3); 3.72-3.82 (t, $C\underline{H}Ph_2$); 7.12-7.37 (m, Ph); 7.72 (s, CHO). Cis rotamer: 1.13 (d, J=6.5 Hz, $C\underline{H}_3$-CH); 2.25 (t (buried), CH_2); 2.62 (s, NCH_3); 3,37-3.5 (m (buried), $C\underline{H}CH_3$); 3.81-3.89 (t, $C\underline{H}Ph_2$); 7.12-7.37 (m, Ph); 7.99 (s, CHO).

^{13}C NMR (75 MHz): trans rotamer: 19.48 ($\underline{C}H_3CH$); 24.17 (N$\underline{C}H_3$); 39.22 (CH_2); 47.75 ($Ph_2\underline{C}H$); 51.57 ($CH_3\underline{C}H$); 126.31-128.91 (arom. CH); 142.65, 144.16, (arom.$\underline{C}$-C); 162.91 ($\underline{C}$HO). Cis rotamer: 17.9 ($\underline{C}H_3CH$); 29.6 (N$\underline{C}H_3$); 39.22 ($\underline{C}H_2$ (buried)); 45.8 ($Ph_2\underline{C}H$); 48.2 ($CH_3\underline{C}H$); 126.31-128.91 (arom. $\underline{C}$H (buried)); 142.16, 144.16 (arom $\underline{C}$-C (buried)); 162.87 ($\underline{C}$HO)

IR (film): 3057, 3045 (w, arom. CH); 2967 (m, aliph. CH); 2929, 2867 (m,CH_3); 1666 (s, C=O str.); 1595 (m), 1585 (w)(C-C skel. str.); 1490 (w-m, CH_3-N); 1446, 1425, 1403 (w-m, N-CH_3, amide); 1322 (w-m), 1303 (w), 1248 (w-m), 1200 (w),1185 (w), 1155 (m); 1088, 1030 (m-s, arom. CH bend); 1060 (w-m); 920, 855, 850 (w); 790, 755 (w-m), 742 (w-m), 708 (m-s) (arom. CH bend).

xviii. (±) N-hydroxymethyl-N-(1-methyl-3,3-diphenylpropyl) formamide (carbinolamide (47))

Using the method of Nair and Francis (1980), the secondary formamide (500 mg, .02 mol) was dissolved in 5 mL MeOH and added to a stirring methanolic solution of 324 uL (0.04 mol) of a 38% aqueous solution of formaldehyde and 2 g of suspended K_2CO_3. The suspension was stirred for 24 hours at room temperature, filtered through a double thickness of filter paper and evaporated under reduced pressure. A portion of the gelatinous precipitate was dissolved in BSTFA, heated overnight at 60° and analyzed by GCMS. The carbinolamide TMS derivative (62) was present as a minor componment (4%) along with the N-TMS derivative of the starting material.

GCMS (TMS derivative (62)): tr=22.92: M^+ 355 (3); 85 (100); 167 (97); 265 (76); 165 (70); 144 (64); 73 (60); 103 (48).

xix.(±) N-(1-methyl-3,3-diphenylpropyl) acetamide (63)

The acetamide was synthesized from dinorrecipavrin (20) and acetic anhydride (1 eq. each) in dry benzene containing two equivalents of dry pyridine. The mixture was heated in a reactivial^R overnight at 60°.

GCMS: tr=19.16; M^+ 267 (12); 87 (100); 167 (14); 44 (61); 72 (16); 167 (22); 167 (14); 86 (13); 208 (10).

^{1}H NMR (80 MHz): Trans rotamer: 1.2 (d, $C\underline{H}_3$-CH); 1.8 (s, CH_3C=O); 2.05-2.4 (ddd, CH_2); 4.0 (m, $C\underline{H}$-CH_3); 4.0 (dd, buried, $C\underline{H}Ph_2$); 5.2 (bs, NH); 7.1-7.4 (m, Ph). Cis rotamer: 1.4 (d, $C\underline{H}_3$-CH); 2.0 (s, CH_3C=O); 2.05-2.4 (ddd, CH_2); 3.8 (m, $C\underline{H}$-CH_3); 4.0 (dd, buried, $C\underline{H}Ph_2$); 5.2 (bs, NH); 7.1-7.4 (m, Ph).

IR (film): 3480 (bm, NH str.); 1642 (s, C=O str.); 1550 (m); 1255 (w).

xx. (±) N-(1-methyl-3,3-diphenylpropyl) ethanimine (64)

The ethanimine was synthesized by condensation of acetaldehyde and dinorrecipavrin in ether solution. The product was characterized by GCMS only.

GCMS: tr=11.41: M^+ 251 (8); 71 (100); 165 (46); 236 (43); 167 (30); 70 (26); 152 (24); 105 (23).

xxi. (±) 3-Carbylamino-1,1-diphenylbutane (recipavrin isocyanide (65))

A mixture of 730 mg (13 mmol) of powdered KOH in 1.8 ml benzene was warmed to reflux with stirring in an apparatus protected with a $CaCl_2$ guard tube. Gradually, 500 mg (2.2 mmol) of dinorrecipavrin free base in 0.5 ml of chloroform was added. The heat was reduced and the flask was cooled with an ice bath if the reflux became too vigorous. After 30 minutes reflux the suspension was filtered and flash evaporated. The residue was flash chromatographed in 20% EtOAc in hexane, affording 47mg (9%) of the isocyanide.

GCMS (packed column, tr =2.9 min., possible isomerization to the nitrile): M^+ 235 (0.5); 167 (100); 132 (88); 117 (53); 165 (39) 152 (24) 193 (20); 166 (17); 168 (16).

NMR (300 MHz): 1.48 (d, CH_3); 2.27 (m, CH_2); 3.40 (m, $C\underline{H}CH_3$); 4.10-4.25 (m, $CHPh_2$); 7.25 (Ph_2).

IR (film): 3050 (m); 2150 (s, isocyanide); 1960, 1900, 1820, 1740, 1670 (w, arom overtones); 1600 (m); 1585 (m); 1500 (s); 1450 (s); 1370 (m); 1350 (m); 1270 (w); 1220 (w); 1190 (w); 1140 (m); 1100 (m); 1065 (m); 1040 (m); 1020 (m); 925 (w); 850 (w); 755 (s); 745 (s); 710 (s).

xxii. (±) N-(1-methyl-3,3-diphenylpropyl) carbamic acid methyl ester (dinorrecipavrin methylcarbamate (66))

To 75 mg (0.33 mmol) dinorrecipavrin free base in 10 ml benzene was added 38.4 ul (47 mg, 0.5 mmol) methyl chloroformate. After reflux overnight, the solvent was evaporated and the residue flash chromatographed.

GCMS: (packed column, tr=4.5 min.): M^+ 283 (0.6); 167 (100); 102 (69); 208 (67); 165 (47); 130 (43); 193 (40); 168 (31); 152 (28).

NMR (400 MHz): 1.05 (d J=7 Hz, $C\underline{H}_3CH$); 1.9-2.1 (m (sharp), $CH\underline{H}_a\underline{H}_b$); 2.13-2.3 (bm, $CH\underline{H}_a\underline{H}_b$); 3.54 (s, OCH_3); 3.75-3.9 (bm, $C\underline{H}CH_3$); 3.9-4.0 (t, $Ph_2C\underline{H}$); 4.35-4.45 (bs, NH); 7.05-7.24 (m, Ph_2).

IR (film): 3400 (w-m, br., NH); 3320 (m,br., NH); 3100-2850 (m); 1740-1700 (s); 1600 (w); 1540 (m-s); 1390 (m-s); 1450 (m-s); 1360 (w-m, br.) 1280 (w); 1250 (m); 1190 (w-m); 1110 (w); 1090 (w-m); 1060 (m); 910 (w); 780 (w-m); 750, 740 (m); 700 (s).

xxiii.($\pm$) N-(1-methyl-3,3-diphenylpropyl) carbamic acid ethyl ester (dinorrecipavrin ethylcarbamate (67))

To 24 mg (0.11 mmol) dinorrecipavrin free base in 10 ml benzene was added 15.3 ul (17.3 mg, 0.16 mmol) ethyl chloroformate. After reflux overnight, the solvent was evaporated and the residue flash chromatographed to give 19 mg (60 %) of the carbamate.

GCMS (packed column, tr=4.9 min.): M^+ 297 (1); 167 (100); 208 (52); 130 (50); 165 (48); 44 (40); 31 (39); 193 (36); 152

(30); 116 (28); 115 (28); 179 (20).

NMR (400 MHz): 1.15 (d J=6 Hz, $C\underline{H}_3CH$); 1.18-1.3 (t, $C\underline{H}_3CH_2$); 2.05-2.15 (m (sharp), $CH_a\underline{H}_b$); 2.2-2.36 (bm, $CH_a\underline{H}_b$); 3.55-3.7 (bm, $C\underline{H}CH_3$); 4.0-4.14 (t (buried), $Ph_2C\underline{H}$); 4.0-4.14 (q (overlap), $C\underline{H}_2CH_3$); 4.3-4.4 (bm, NH); 7.1-7.3 (m, Ph_2).

IR (film): 3400 (w-m, br., NH); 3320 (m,br., NH); 3100-2850 (m); 1740-1700 (s); 1600 (w); 1540 (m-s); 1390 (m-s); 1450 (m-s); 1380 (w-m) 1330 (m); 1250 (m-s); 1210 (w-m); 1100 (m-s); 1060 (m-s); 1040 (m); 780 (w-m); 750, 740 (m); 700 (s).

xxiv. ($\pm$) N-methyl-N-(1-methyl-3,3-diphenylpropyl) carbamic acid methyl ester (norrecipavrin methyl carbamate, (68))

Using the method of Kapnang and Charles (1983), 66 mg of recipavrin free base (0.26 mmol) in dry benzene was added 22 ul (0.28 mmol) methyl chloroformate. After reflux overnight, the solution was evaporated and flash chromatographed in 85:15 hexane:EtOAc and the eluent evaporated to afford 62 mg (76 %) of the carbamate.

GCMS (packed column tr=4.6): M^+ 297 (<1); 116 (100); 208 (34); 59 (22); 167 (15); 130 (12); 193 (10).

IR (film, weak sample): 3400 (w-m, br., H_2O); 3100-2850 (m); 1710 (s); 1600 (w): 1490 (m); 1460 (m-s); 1390 (w); 1360 (w-m, br.); 1260 (w); 1190 (w-m); 1150 (w-m); 1060 (w); 1030 (w); 750, 740 (m); 700 (s).

xxv. (±) N-methyl-N-(1-methyl-3,3-diphenylpropyl) carbamic acid ethyl ester (norrecipavrin ethyl carbamate (69))

Using the method of Kapnang and Charles (1983), 66 mg of recipavrin free base (0.26 mmol) in dry benzene was added 26 ul (0.28 mmol) ethyl chloroformate. After reflux overnight, the solution was evaporated and flash chromatographed in 85:15 hexane:EtOAc and evaporated.

GCMS (packed column tr= 4.8 min.); M^+ 311 (<1); 130 (100); 58 (79); 208 (44); 29 (28); 102 (22); 86 (18); 167 (17); 193 (12).

NMR (400 MHz): 1.10 (d, J=7Hz, $C\underline{H}_3CH$); 1.07 (t, $C\underline{H}_3CH_2$); 2.05-2.4 (m, $CH_a\underline{H}_b$); 2.78 (s, NCH_3); 3.78-3.90 (m, $C\underline{H}CH_3$); 3.92-4.30 (t, (buried), $Ph_2C\underline{H}$); 3.92-4.30 (q, (buried), $C\underline{H}_2CH_3$); 7.04-7.25 (m, Ph_2).

IR (film): 3100-2850 (m); 1740-1700 (s); 1600 (w); 1585 (w); 1495 (m-s); 1450 (m-s); 1410 (m-s); 1370 (m) 1330 (s); 1200 (m); 1150 (m-s); 1110 (w); 1062 (w); 1032 (w); 910 (w); 770, 750, 740 (m); 700 (s).

D. Synthetic Compounds Related to Promethazine

Reaction vessels were protected from light during all procedures. Solvents were dried as described previously. Glassware was oven dried prior to use. Solid starting materials were dried in vacuo prior to use.

i. 10-(2-Propynyl) phenothiazine (70)

The method of Clement and Beckett (1981a) was used. A 56% yield of beige crystals was obtained. NMR, IR, and GCMS results were as reported in the literature.

ii. 10-(2-Propanone) phenothiazine (71)

The method of Clement and Beckett (1981a) was used. Mercuric sulfate was prepared by the method of Newman (1960). Four g (46%) of product were recovered. NMR, IR, and GCMS results were as reported in the literature.

iii. 10-(2-formamidopropyl) phenothiazine (72)

10-(2-Propanone) phenothiazine (2 g, 7.8 mmol) was refluxed in 20 ml formamide for 4 hours in the dark. The solution was cooled, diluted with water and extracted with chloroform. The chloroform was dried over K_2CO_3 filtered and evaporated. The residue was dissolved and flash chromatographed on a 2.5 x 10 cm column in chloroform. The phenothiazine formamide (72) was eluted last (rf=0.18 in chloroform). Chromatography was repeated. After a short $CHCl_3$ prerun to elute a pyrimidine byproduct (73), the product was eluted with $CHCl_3$/MeOH 98/2. Evaporation afforded 600 mg (27%) of yellow crystals. NMR revealed a 4:1 ratio of cis and trans rotamers.

GCMS: (tr= 9.34): M^+ 284 (28); 212 (100); 180 (46); 213 (16); 178 (8); 198 (7); 181 (7); 152 (4); 286 (3); 77 (2). 30 eV GCMS and 70 eV direct probe mass spectrum were

identical. On column derivatization with TMAH gave a peak with mass spectrum and retention time identical to the tertiary formamide (74) described below.

^{1}H NMR: (400 MHz): Major rotamer: 1.27 (d, J=6.4 Hz, CH_3); 3.74-3.84 (dd, $C\underline{H}_a\underline{H}_b$); 4.08-4.17 (dd, $CH_a\underline{H}_b$);4.39-4.51 (m (sextuplet), $C\underline{H}CH_3$); 5.50-5.70 (bs, NH); 8.10 (s, CHO); 6.92-7.00 (m, arom. C_1, C_9); 7.00-7.08 (m, arom C_3, C_7) 7.14-7.24 (m, arom. C_2, C_4, C_6, C_8); Minor rotamer: 1.32 (d, J=6.4 Hz, CH_3); 3.78-3.84 (dd, buried, $C\underline{H}_a\underline{H}_b$); 3,90-4.00 (dd, overlap, $CH_a\underline{H}_b$); 3.90-4.00 (m, overlap, $C\underline{H}CH_3$); 5.55-5.70 (bs, buried, NH); 7.87 and 7.90 (d, J=12 Hz, CHO); 6.81-6.87 (d, J=8Hz, arom. C_1, C_9); 6.92-7.00 (m, arom. C_3, C_7); 7.14-7.24 (m, arom. C_2, C_4, C_6, C_8); H_2O at 1.64 ppm.

^{13}C NMR: (BB shift (20 MHz with splitting from SFORD (100 MHz), 111 mg sample): Trans rotamer: 18.1 (q, CH_3); 42.7 (d, $\underline{C}HCH_3$); 51.7 (t, CH_2); 161.04 (d, CHO); 138.52 (s weak, arom. C-N); 145.78 (s, weak, arom.C-S). Cis rotamer: 19.89 (q, CH_3); 45.25 (d, $\underline{C}HCH_3$); 53.75 (t, CH_2); 163.81 (d, CHO); 145.21 (s, weak, arom. C-N); 145.35 (s, weak, arom.C-S). Aromatic resonances for cis and trans rotamers 115.81; 116.26; 116.75; 122.05; 122.56; 123.04; 123.53; 126.28; 126.52; 126.79; 127.10; 127.54; 127.73; 128.03; 128.22.

IR: ($CHCL_3$ solution, 0.5mm cell): 3432 (w-m, sharp, NH trans); 3400 (w, shoulder, NH cis); 2930 (w-m); 2870 (w-m, $CHCl_3$); 1692 (s, C=O trans); 1681 (s, C=O cis);

1594 (m, $CHCl_3$); 1575 (m, $CHCl_3$); 1389 (m);1345 (m); 1130 (m-s, $CHCl_3$); 1108 (w); 1090 (w); 1051 (w); 1039 (m, sharp).

IR (film, Perkin Elmer FTIR): 3389 (w-m, sharp, NH); 3272 (m,broad, NH); 3060, 2975, 2929, 2869 (w-m); 1664 (s, 2 peaks, C=O); 1592 (m), 1571 (m), 1538 (m, broad), 1486 (m), 1459 (s), 1383 (m-s), 1341 (m-s), 1307 (m-s), 1286 (m), 1254 (m-s), 1225 (m-s), 1163 (w), 1132 (m), 1108 (w), 1051 (w), 1039 (m), 909 (m), 855 (w, broad), 752 (s), 730 (m-s), 696 (m).

iv. Leuckart specific byproduct, 4-(1-(10-phenothiazinyl) methyl) pyrimidine (73)

GCMS (packed column, 200-280° at 8° per minute, tr=9.6 min.) M^+ 291 (11); 198 (100); 39 (44); 154 (18); 199 (17); 45 (16); 69 (12).

v. 10-(2-Formamidopropyl) phenothiazine N_{10}-oxide (75)

Using the method of Clement and Beckett (1981b) for the synthesis of promethazine N-oxide, to 250 mg of 10-(2-formamidopropyl) phenothiazine (72) in 1.5 ml MeOH was added 1.25 ml of 30% H_2O_2. After 30 minutes the excess H_2O_2 was destroyed with 100 mg MnO_2. The solution was filtered, diluted with water and extracted with chloroform. The organic phase was dried over Na_2SO_4, filtered and evaporated, to afford 140 mg (53%) of a yellow waxy solid (mp less than 60°) that decomposed to the formamide during GCMS analysis. NMR and IR revealed only one rotamer.

The polar product was flash chromatographed in 97.5/2.5 $CHCl_3$/MeOH.

GCMS: Decomposes and is desorbed slowly as the secondary formamide (72).

Direct insertion probe mass spectrum: M^+ 300 (3); 212 (100); 180 (46); 198 (28); 213 (16); 284 (12); 200 (12); 152 (8); 179 (8); 229 (4); 77 (2).

^{1}H NMR: 1.1 (d, J=7.2 Hz, CH_3); 4.2-4.34 (m, $C\underline{H}CH_3$); 4.59-4.645 (dd, $CH_a\underline{H}_b$); 6.6 and 6.62 (bd, J=6Hz, NH); 7.66 and 7.71 (d, J=8.5 Hz, cis CHO); 7.19-7.3 (m, arom. C_3, C_7); 7.5-7.65 (m, arom C_3, C_7, C_2, C_8) 7.83-7.9 (m, arom. C_1, C_9); aromatic assignments tentative.

^{13}C NMR (100 MHz): 18.58 (CH_3); 46.00 ($\underline{C}HCH_3$); 50.82 (CH_2); 161.40 (CHO); 140.52 (weak, arom. C-N); 140.56 (weak, arom.C-S). Arom. CH resonances: 118.18; 118.60; 122.41; 122.51; 122.73; 127.58; 129.82; 132.54; 132.61; 132.67.

IR: ($CHCl_3$ solution 0.5 mm): 3438 (w, sharp, NH); 3284 (w-m, broad); 3204 (w, shoulder); 2944, 2874 (w-m, aliph.); 1682 (s, C=O); 1601 (m); 1590, 1580 (m); 1386 (w-m); 1368 (m); 1360 (m, shoulder); 1327 (m); 1127 (w); 1100 (w); 1068 (w); 1050 (w); 1040 (w); 1010 (s, N-O str.).

IR (film, Perkin Elmer FTIR): 3700-3200 (m, broad); 3350-3100 (m-s); 3035 (m, broad); 2972 (m); 2929 (m); 2873 (m); 1670 (s, one peak); 1585 (s); 1538 (m, broad); 1486 (m); 1461 (s); 1381 (m-s); 1361 (m-s); 1315 (w);

1250 (s); 1174 (w-m); 1149 (w-m); 1130 (w-m); 1098 (m); 1066 (w-m); 1047 (m-s); 1009 (s); 920 (m); 851 (m); 755 (s); 730 (s); 671 (m).

vi. 10-(N-methyl-2-formamidopropyl) phenothiazine (74)

10-(2-Propanone) phenothiazine (71) (2 g, 7.8 mmol) was refluxed in 20 ml N-methylformamide for 5 hours in the dark. The solution was cooled, poured onto 75 ml ice water and the beige precipitate was extracted with chloroform. The chloroform was dried over K_2CO_3 filtered and evaporated to afford 1.7 g of crude product. The residue was dissolved and flash chromatographed on a 2.5 x 10 cm column. with a chloroform prerun and product elution with $CHCL_3$/MeOH 95/5. The phenothiazine N-methyl formamide was eluted last. Chromatography was repeated. NMR revealed a 2:1 ratio of cis and trans rotamers.

GCMS (packed column, condition F, tr=10.5 min.): M^+ 298 (12); 212 (100); 180 (47); 100 (28); 213 (21); 58 (12); 30 (12); 179 (10); 214 (8); 198 (8);152 (6); 299 (4).

^{1}H NMR: (400 MHz): Major rotamer: 1.27-1.32 (d J=7.2 Hz, CH_3); 2.65-2.69 (s, NCH_3); 3.90-3.96 (dd, $C\underline{H}_a\underline{H}_b$); 3.82-3.99 (m (sextuplet) buried, $C\underline{H}CH_3$); 6.92-6.96 (m, arom. 2 protons); 7.11-7.21 (m, arom. 6 protons); 7.72 (s, CHO). Minor rotamer: 1.22-1.27 (d J= 7.2Hz, CH_3); 2.76-2.80 (s, NCH_3); 4.02-4.12 (m, $C\underline{H}_a\underline{H}_b$); 4.73-4.85 (m (sextuplet), $C\underline{H}CH_3$); 6.81-6.88 (d J=8 Hz, arom.); 6.92-6.96 (d J=8 Hz, arom.); 6.96-7.01 (d J=8 Hz, arom.);

7.11-7.21 (m, arom.); 8.01 (s, CHO). Overlapping cis and trans aromatic resonances make aromatic assignments uncertain.

IR (film): 3065 (w-m); 2970 (m); 2940 (m); 2860 (m); 1670 (s); 1592 (m-s); 1572 (m-s); 1485 (m-s); 1465 (s, broad); 1408 (m-s); 1380 (m); 1338 (m-s); 1285 (m-s); 1252 (m-s); 1218 (m-s); 1160 (w); 1130 (m); 1105 (m); 1078 (m); 1038 (m); 930 (w); 860 (w); 835 (w); 750 (s); 665 (m).

vii. 10-(N,N-dimethyl-N-oxo-2-aminopropyl) phenothiazine (promethazine N-oxide (76), Clement and Beckett, 1981b)

Using the method of Craig and Purushothaman (1970), a 1.1 molar excess of MCPBA in ice chilled chloroform was added to a cooled and stirred solution of promethazine HCl in chloroform. After stirring for three hours the solution was passed through a bed of alkaline alumina (100-200 mesh, 20 times the combined weight of the starting materials). Promethazine was washed out with $CHCl_3$, then the N-oxide was eluted with 1/1 $CHCl_3$/MeOH. GCMS revealed the Cope elimination product 10-(2-propenyl)phenothiazine. Two minor long retention time products had mass spectral characteristics of the Cope elimination product with an additional high mass ion m/z 255 in each, suggesting that the aromatic N-oxide and sulfoxide analogues of promethazine N-oxide were also formed to a minor extent. The product was purified by flash chromatography in 90/10 $CHCl_3$/MeOH.

Direct inlet mass spectrum: (decomposes to the Cope elimination product (77)): M^+ 239 (100); 237 (44); 198 (39); 223 (29); 240 (20); 224 (13).

^{1}H NMR (80 MHz): 1.38 (d J=6 Hz, $C\underline{H}_3CH$); 3.17 (d J=10Hz, $O^-N^+(C\underline{H}_3)_2$); 3.55-3.87 (m, $CH_3C\underline{H}$); 4.15-4.53 (dd, $C\underline{H}_aH_b$); 5.45-5.62 and 5.64-5.77 (doublets J=4Hz each, $CH_a\underline{H}_b$); 7.12-8.00 (arom.); H_2O at 2.25-2.62 ppm.

IR (film, Perkin Elmer FTIR): 3700-3100 (s, broad, H_2O); 3070, 3030, 2982, 2960 (m-s); 2361, 2229 (w-m); 1666 (s); 1586 (s); 1485 (s); 1459 (s); 1364 (m); 1342 (m-s); 1301 (w); 1254 (s); 1172 (w); 1151 (w); 1099 (m-s); 1022 (s); 923 (w); 869 (w); 757 (s); 704 (w-m).

viii. Characterization of promethazine free base (10)

An injectable formulation of 25 mg of promethazine HCl (Phenergan^R) was adjusted to pH 10 and extracted with chloroform, dried over K_2CO_3, evaporated and characterized by GCMS, NMR and IR for comparison to compounds 72, 74, 75 and 76.

GCMS (packed column, 200-280°at 8° per minute, tr=6.25 min): M^+ 284 (0.2); 72 (100); 42 (6); 44 (5); 73 (5); 56 (4); 199 (2); 198 (2); 167 (1).

NMR (80 MHz): 1.06 (d, J=6.4 Hz, $C\underline{H}_3CH$); 2.35 (s, $N(C\underline{H}_3)_2$); 2.87 -3.12 (m, $CH_3C\underline{H}$); 3.5-3.83 (dd, $C\underline{H}_aH_b$); 3.5-3.84 and 3.9-4.22 (dd, $CH_a\underline{H}_b$); 6.75-7.3 (arom.).

IR: (film): 3050 (m); 2965, 2940, 2860, 2790, 2770 (m-s); 2000-1770 (w, arom. overtones); 1592, 1572 (s, sharp); 1500-1450 (vs, broad); 1360 (m-s); 1335 (m-s); 1305 (m); 1285 (s); 1250 (s); 1235 (s); 1182 (m); 1159 (m); 1129 (m-s); 1103 (m-s); 1038 (s); 972 (w-m); 927 (w-m); 872, 850 (w-m); 750 (vs); 725 (m).

E.Synthetic compounds related to amphetamine

i. (±) α-Methyl-(N-methylene)-β-phenylbenzeneethanamine (78)

The method of Reynolds and Cossar (1971) was used. To a flask containing 150 ul of 3N NaOH and 2 ml benzene was added a solution of 275 mg (2.7 mmol) of amphetamine sulfate in 1.5 ml H_2O. Then 266 ul (3.3 mmol) of 37% aqueous formaldehyde was added slowly over gentle heat. The benzene layer was separated, dried and evaporated, affording 201 mg of crude product, which was flash chromatographed to afford a cloudy liquid with a nutty smell. NMR revealed a 3:2:5 mixture of 1,3,5-hexahydrotriazene (79), 1,3,5-hexahydrooxadiazene (80), and 1,3,5-hexahydrodioxazene (81). The presence of excess formaldehyde favors 1,3,5-hexahydrodioxazene formation.

GCMS (packed column): tr=2.3 min.: M^+ (monomer (78)) 147 (0.1); 56 (100); 91 (10); 65 (5); 57 (4); 51 (3); 132 (3); 77 (2); 54 (2).

^{1}H NMR (80 MHz): Common resonances: 2.25-2.75 (CH_aH_b); 2.9-3.5 (m, CH_aH_b and CH); 7.0-7.4 (m, 88 mm, arom.): Triazane component: 1.02 (br. d, J=6Hz, 27mm=9 protons, CH_3); 3.75 (s, 17mm=6 protons,NCH_2N); When the spectrum is run at 60°, resonances associated with the triazane disappear and are replaced by a pair of singlets at 2.77 (NCH_2N, 6 protons) and 2.85 (CH_3, 9 protons). The other two components are unaffected by the higher temperature. Oxadiazane component: 1.22 (d, J=6Hz, 12mm=6 protons, CH_3); 3.95 (s, 4mm=2 protons, NCH_2N); 4.55 (s, 8mm=4 protons, NCH_2O). Dioxazane component: 1.25 (d, J=6 Hz, 15mm=3 protons, CH_3); 4.78 (s, 20mm=4 protons, NCH_2O); 5.22 (s, 10mm=2 protons, OCH_2O).

IR (film): 3010 (m-s); 2942 (s); 2900 (s); 2841 (m-s); 2820-2760 (m, CH_2N); 2000-1800 (w, arom . overtones); 1607 (m, arom.); 1585 (w, arom.); 1495 (m-s, arom.); 1452 (s, arom.); 1402-1335 (m-s, aliph.); 1290 (w); 1233-1080 (multiple bands, s, t-amine); 1185 (s); 1150 (s); 1108 (s); 1080 (s); 1040 (s, arom.); 1000 (s, arom.); 958 (m); 920 (s, ether); 800 (w); 744 (s, arom.); 700 (s, arom.).

ii. (±) 2-(3'-phenylprop-2'-yl) oxaziridine (82)

To 300 mg (1.6 mmol) amphetamine sulfate in 2 ml H_2O was added 240 ul (3 mmol) of 38% aqueous formaldehyde. Then 575 mg (3.3 mmol) of 85% metachloroperbenzoic acid in 5 ml $CHCl_3$ was added and the 2 phase mixture was stirred for 2 hours. Solid K_2CO_3 was added until gas evolution ceased. The solution was filtered, the chloroform phase separated, dried over K_2CO_3,

evaporated and flash chromatographed in 97:3 hexane: EtOAc to afford 17 mg (6%) of a 1.23:1 ratio of N,C-diastereomeric oxaziridines. The GCMS results were identical to N-formylamphetamine (83).

^{1}H NMR (400 MHz): (major diastereomer): 1.06 (d, J=6.4 Hz, CH_3); 2.08-2.21 (m, $CH_3C\underline{H}$); 2.74-2.93 (dd, Jab=4.6 Hz, $CH_{\underline{a}}CH_b$); 3.11-3.2 (dd, $CH_aC\underline{H}_b$); 3.55-3.75 (d, Jab=10Hz, $C\underline{H}_aH_b$ oxaz.ring); 3.9-4.1 (d, Jab=10 Hz, $CH_a\underline{H}_b$ oxaz. ring); 7.25 (C_6H_5). (Minor diastereomer): 1.35 (d, J=6.4 Hz, CH_3); 2.1-2.2 (m, $CH_3C\underline{H}$); 2.74-2.85 (dd, $C\underline{H}_aH_b$); 2.85-2.95 (dd, buried, $CH_aC\underline{H}_b$); 3.12-3.26 (d, Jab=10 Hz, $C\underline{H}_aH_b$ oxaz. ring); 3.6-3.8 (d, Jab=10 Hz, $CH_a\underline{H}_b$ oxaz. ring); 7.25 (m, C_6H_5).

iii. (±) N-(1-methyl-2-phenylethyl) formamide (83) (CAS registry 42044-69-9)

Using the method of Moffat *et al.*, (1962), amphetamine free base was refluxed over K_2CO_3 in ethyl formate for 2 days. The solution was filtered and evaporated. The amber liquid product was purified by flash chromatography in 20:80 EtOAc:petroleum ether (30-60°). NMR revealed a 3:1 ratio of trans and cis rotamers.

GCMS (packed column, condition G): tr=3.1 min.: M^+=163 (0), 72 (100); 118 (61); 44(46); 91 (22); 117 (14); 65 (10); 119 (68).

GCMS (TMS derivative, (84))(packed column, condition G): tr=1.6 min. M^+-15, 220 (5); 144 (70); 72 (30); 75 (32); 91 (30); 118 (20).

^{1}H NMR (80 MHz) Trans rotamer: 1.15 (d, J=6.5 Hz CH_3); 2.63-2.91 (dd, CH_2); 4.06-4.7 (m, $CHCH_3$); 5.01-5.87 (br s, NH); 7.0-7.5 (C_6H_5); 8.1 (s, CHO). Cis rotamer: 1.27, (d, CH_3); 2.63-2.91 (dd (buried), CH_2); 3.5-4.0 (m, $CHCH_3$); 5-6 (br. s (buried), NH); 7.0-7.5 (m, C_6H_5); 7.9 (d, J=12 Hz, CHO).(lit. values: 1.13 (d); 2.77 (m); 7.17 (s).

7. EXPERIMENTS TO DETERMINE THE SOURCE OF FORMAMIDE METABOLITES OF METHADONE AND RECIPAVRIN

A. Solvent related artifact control experiments for methadone metabolites

To preclude the possibility of phosgene or peroxide related generation of formamides in the conjugated fraction of bile from methadone dosed rats, the metabolite extraction method of Horning and Mitchell (1984) was used. Extracts were reconstituted with 50 ul of 5 mg/ml (250 ug) diphenylbutanone stock solution just prior to analysis of 1 ul by GCMS. Ether and chloroform extractions were performed with and without pretreatment of the solvent with type 4A molecular sieve (Burfield, 1982) and distillation just before use. This procedure removes organic peroxides from ether and phosgene from chloroform. The area of the m/z 167 ion peak common to diphenylbutanone (14) and the secondary formamide (12)

was integrated to estimate the relative amount of secondary formamide (12) present, expressed as % of diphenylbutanone.

Following the standard extraction protocol, the following alkali/solvent pairs were employed for the basification and extraction of nonconjugated and conjugated metabolites: A. 1 M $NaOH/CHCl_3$, Sample workup A was repeated with the final sample dissolution for GCMS in EtOH rather than MeOH; B. 1 M $NaOH/CH_2Cl_2$; C. 2 g solid NH_4CO_3/EtOAc; D. 2 g solid NH_4CO_3/diethyl ether; E. 2 g solid NH_4CO_3/benzene; F. 1 M NaOH/EtOAc, without prior extraction of nonconjugated metabolites (all metabolites extracted following β-glucuronidase hydrolysis); G. 1 M $NaOH/CHCl_3$, without prior extraction of nonconjugated metabolites. Lastly, bile was stored exposed to light and air for a period of one week to determine whether formamides could be detected in the nonconjugated fraction.

B. Effect of added synthetic secondary formamide on peak shape and retention time of the recipavrin formamide metabolite (12)

The conjugated fraction extracted from 5 ml recipavrin dosed rat bile was concentrated and 1 ul aliquots were spiked with aliquots of a 30 ug/ml stock solution of the synthetic secondary formamide (12) according to the following table. One uL aliquots of each sample were analyzed by GCMS without correction for volume differences.

Table 1. Table showing volumes of bile extract and synthetic formamide (12) used in the dilution experiment.

Sample No.	bile extr.(uL)	formamide soln. (uL)	formamide inj. (ng)	bile extract inj. (uL)
1	1	0	0	1
2	0	1	30	0
3	1	1	15	0.5
4	1	10	27.3	0.09

The relative amount of the formamide was compared to that of the recipavrin phenol metabolite, by comparing the peak heights of the molecular ions (253 and 269 respectively) and the peak heights of ions corresponding to the diarylmethyl cation (167 and 183 respectively). The concentration effects on retention time and peak shape of secondary formamide (12) derived ions were also estimated.

C. Experiments to establish that the secondary formamide metabolite (12) of recipavrin arises from a glucuronide precursor

i. Sulfatase hydrolysis of the recipavrin conjugated metabolites

Bile (1.5 ml) from a rat dosed with recipavrin D_3 hydrochloride was extracted free of nonconjugated metabolites by the standard method. The aqueous phase was adjusted to pH 5 with 4 M HCl and lyophilized. The residue was taken up in 3 ml pH 5, 0.1 M sodium acetate buffer and then hydrolyzed

overnight at 38° with sulfatase (0.5 mg of 21 units per mg, dissolved in 2 ml sodium acetate buffer, β-glucuronidase activity= 2.8 Fishman units per mg, Sigma product S-9754, Lot 40F-9550). The conjugated metabolites were then extracted by the standard method and analyzed by GCMS. A parallel incubation was performed using β-glucuronidase.

ii. Control incubation of recipavrin conjugated fraction without β-glucuronidase enzyme.

Bile (1.5 ml) from a rat dosed with recipavrin hydrochloride was extracted free of nonconjugated metabolites under standard conditions. The aqueous phase was adjusted to pH 5 with 4 M HCl and lyophilized. The residue was taken up in 3 ml pH 5, 0.1 M sodium acetate buffer and then held overnight at 38° without enzyme treatment. The bile sample was then extracted under standard conditions and analyzed by GCMS. A parallel incubation was performed using β-glucuronidase.

D. Free radical oxidation of recipavrin and norrecipavrin as a source of the secondary formamide (12)

i. Incubation of recipavrin (9) with blank bile under simulated workup conditions

a. Blank bile (5 ml) was spiked with 1 ml of a 1 mg/ml aqueous solution of recipavrin hydrobromide, adjusted to pH 10 with 1 M sodium hydroxide, allowed to stand overnight, adjusted to pH 5, treated with β-glucuronidase

for 36 hours at 38°, extracted and analyzed by GCMS by standard procedures.

b. Bile from a recipavrin-D_3 dosed rat was spiked with 10 mg of recipavrin-D_0 and stored at room temperature for 7 days prior to extraction at pH 5, pH 10 and pH 12.

ii. Incubation of norrecipavrin with blank bile under simulated workup conditions

A 2 ml aliquot of control bile was spiked with 50 ug of norrecipavrin HCl in 50 ul propylene glycol and 1.12 ug N-ethyl recipavrin in 0.5 ml H_2O, and carried through standard isolation procedures for nonconjugated metabolites. The extract was examined for the presence of the secondary formamide.

E. Effect of extraction pH on the observation of the recipavrin secondary formamide metabolite (12).

Bile from recipavrin dosed rats was worked up by the standard protocol. The conjugated fraction was extracted sequentially at pH 7, pH 10 and pH 12. Each organic extract was dried evaporated,reconstituted to a constant volume and analyzed by GCMS.

F. Effect of immediate sample preparation and storage on the secondary formamide metabolite of recipavrin

Bile from a recipavrin dosed rat was collected directly into an opaque tube containing a solution of 0.5 ml of glucurase and 1 ml of sodium acetate buffer. At the

end of 24 hours of bile collection, bile was adjusted to pH 5, ten ml was split into 2 five ml aliquots. One aliquot sat for an additional hour at 38°, was adjusted to pH 9.8 with NaOH (1 M) and borate buffer (pH 10), extracted with 3 five ml aliquots of EtOAc, dried, evaporated and immediately analyzed by GCMS. The remaining aliquot was adjusted to pH 5 and stored for two weeks at -5°, thawed, treated with Glucurase^{R} for 36 hours at 38°, adjusted to pH 10, extracted with EtOAc, dried, evaporated and analyzed by GCMS.

G. Solvent related artifact control experiments for recipavrin metabolites.

i. Comparison of chloroform and EtOAc extraction solvents on the recovery of the formamide metabolite of recipavrin.

Bile from recipavrin dosed rats was isolated using either distilled in glass grade chloroform (stabilized with 1% EtOH) or distilled in glass grade EtOAc. The amount of secondary formamide (12) present was estimated relative to other metabolites.

H. Solid phase extraction (SPE) of recipavrin metabolites

A Baker-10 SPE manifold was used with a water aspirator vacuum. Cartridges were available in 1, 3 and 6 ml sizes. Cartridges were loaded until adsorbed bile pigments were visible to the bottom of the cartridge packing.

i. Cartridge conditioning and sample elution (J.T. Baker Ltd., 1982)

Baker 10 SPE cartridges were conditioned as follows:

a. Reversed phase octadecyl (RPC_{18}) cartridges.

To extract non polar solutes by partition from aqueous solution, RPC_{18} cartridges were washed with 2 column volumes of MeOH, then two column volumes of water or buffer, then the buffered aqueous sample was applied to the cartridge. The sorbent was not allowed to run dry. The column was washed with two column volumes of water or buffer, then allowed to air dry for 3 minutes, just prior to sample elution. A sample equivalent to 0.5 ml bile per ml of SPE adsorbent was applied and eluted using successive aliquots of 1 column volume of increasing proportions of organic solvent in water.

b. Forward phase silica gel ($FPSiO_2$) cartridges.

To extract moderately polar solutes by adsorption from organic solution, $FPSiO_2$ cartridges were washed with 2 column volumes of the solvent that the solute was to be applied in. The column was not allowed to run dry prior to sample application. The sample (equivalent to 0.5 ml bile per ml of SPE adsorbent) was applied, the column was washed with two column volumes of the dissolution solvent, then air dried for three minutes. The samples were then eluted with successive one column volume aliquots of solvents of gradually increasing polarity.

ii. Amberlite XAD-2 column preparation

XAD-2 beads were packed dry into a 1 x 15 cm glass column then washed with EtOH and then with water. The aqueous sample (10 ml of 50% aqueous bile or urine solution adjusted to pH 8-9) was applied, and allowed to percolate slowly through the column bed at 2 ml per minute. The column was washed with water until the eluent was almost colorless then the free and conjugated metabolites were eluted with EtOH until the eluent was almost free of yellow coloration. The samples were concentrated under nitrogen and subjected to further SPE steps.

iii. Preliminary solid phase extraction experiments

a. Elution of synthetic reference compounds from RPC_{18} columns

Aqueous solutions (300 ug/ml) of the synthetic standards diphenylbutanone (14) (a non polar neutral metabolite), dinorrecipavrin free base (20) (a medium polarity basic metabolite) and the secondary formamide (12)(a medium polarity neutral metabolite and target compound) were prepared and one ml of each solution was applied to separate 1 ml RPC_{18} columns. The samples were eluted with successive one column volumes of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% aqueous MeOH. The aliquots were monitored for the solutes by UV absorbance at 254 nm, using a matching concentration of aqueous MeOH as the reference solution.

b. RPC_{18} test elutions using UV and TLC detection of eluates

Solutions of d-glucuronic acid (5 mg/ml), methadone HCl (1 mg/ml of free base), and phenolphthalein glucuronide (5 mg/ml, turns pink in alkaline solution) were adjusted to pH 8 with 0.01 M NaOH and one ml of each solution was applied to separate RPC_{18} columns. Aliquots of bile (0.5 ml) from control and recipavrin dosed rats were treated similarly. The columns were washed with 2 column volumes of water and then eluted with successive one column volume aliquots of 20, 40, 60, 80, and 100% aqueous EtOH. Each eluate was spotted on 3 separate UV fluorescent silica gel TLC plates. Each plate was checked for adequate sample application with 254 nm light and then developed by spraying with one of the following reagents: Dragendorf's reagent for basic nitrogen compounds, naphthoresorcinol reagent plus heat (70^{o} for 15 minutes) for glucuronides, and 50 % sulfuric acid in 1:1 aqueous EtOH followed by heating at 100^{o} to detect all carbon compounds.

iv. Fractionation of bile components by SPE methods

a. Attempted fractionation of biliary recipavrin metabolites using RPC_{18} columns.

Bile (2 ml) from a recipavrin dosed rat was passed through a RPC_{18} column. The column was washed with one column volume of water, then with successive one column volume aliquots of MeOH, isopropanol, EtOH, hexane, 0.05

M sodium acetate in 50% aqueous MeOH, 0.05 M sodium borate in 50 % aqueous MeOH, and 0.05% acetic acid in 50% aqueous MeOH. Each aliquot was blown free of organic solvent under nitrogen, adjusted to pH 5 and treated with 0.25 ml β-glucuronidase at 38° overnight. The solutions were extracted at pH 5, then adjusted to pH 12 and re-extracted.

b. Preliminary cleanup and deionization of bile using a XAD-2 column

Bile from a recipavrin dosed rat (5 ml) was diluted with 5 ml H_2O and passed through a XAD-2 column. The aqueous wash was discarded since glucuronidase hydrolysis and extraction failed to reveal significant amounts of recipavrin metabolites. The EtOH eluent was further purified by further SPE procedures.

c. Separation of all metabolites from extremely polar bile constituents

The eluent (equivalent to 1 ml bile) was passed through a 3 ml SiO_2 SPE column and followed by a one column volume wash with successive aliquots of EtOH, 60% aqueous EtOH, and then H_20. The eluents were spotted on a TLC plate and visualized with naphthoresorcinol and heat to locate glucuronides.

The eluent (equivalent to 1 ml bile) was passed through a 3 ml SiO_2 SPE column and followed by a one column volume wash with successive aliquots of EtOH and 60% aqueous EtOH.

The EtOH in each eluate was evaporated under nitrogen and the residue or aqueous remainder was diluted with 200 ul of 0.1 M pH 5 sodium acetate buffer and 100 ul of glucurase^{R} was added. After an overnight incubation at 38^{o}, the samples were diluted with 0.7 ml of water, adjusted to above pH 9 with 25 ul 1.0 M NaOH, and applied to a RPC_{18} column. The column was washed with successive aliquots of alkalinized water, 40% aqueous EtOH, and 100% EtOH. Samples were concentrated under nitrogen. The concentrated EtOH sample was analyzed by GCMS. The aqueous samples were extracted with 3 four ml aliquots of EtOAc, dried over Na_2SO_4, evaporated, reconstituted in EtOH and analyzed by GCMS.

d. Separation of nonconjugated metabolites on $FPSiO_2$ cartridges

1. To separate the nonconjugated metabolites from the crude XAD-2 EtOH eluate on a silica gel column, a benzene/ EtOH (92/8) solvent system was developed for synthetic samples of the recipavrin secondary formamide (12) and the recipavrin N-methyl nitrone. Both test compounds are more polar than any of the nonconjugated metabolites, but presumably are not as polar as the glucuronide conjugates (TLC rf nitrone (44) =0.18, rf secondary formamide (12) = 0.44). This eluent was used to selectively elute the nonconjugated fraction in the following procedure.

The XAD-2 EtOH eluate equivalent to 1 ml bile and containing 250ug methadone free base as an internal standard was evaporated to dryness under nitrogen, then dissolved with 0.8 ml EtOH and diluted slowly with 9.2 ml benzene. This fraction was passed through a 6 ml $FPSiO_2$ cartridge. The cartridge was washed with 5 column volumes of 92/8 benzene/EtOH to elute the nonconjugated metabolites. Conjugated metabolites were eluted with 5 column volumes of EtOH. The nonconjugated fraction was concentrated under nitrogen and analyzed by GCMS.

I. Attempts to increase secondary formamide (12) production by the addition of formic acid, formaldehyde, or formaldehyde and hydrogen peroxide during sample preparation.

Bile from a recipavrin dosed rat was treated as shown in the following table.

Table 2. Table showing volumes of reagents added to bile from recipavrin dosed rats.

Reagent added	TREATMENT					
	A Control	B 90% H_2CO_2	C 37% H_2CO	D 30% H_2O_2	E 30% H_2O_2	E 37% H_2CO
1. Bile	2 ml	2 ml	2 ml	2 ml	2 ml	
2. Water	180 ul	160 ul	120 ul	60 ul	60 ul	
3. Treatment reagent	None	20 ul	60 ul	120ul	120ul	60ul
4. Internal standard*	1 ml	1 ml	1 ml	1 ml	1 ml	
5. MeOH	2 ml	2 ml	2 ml	2 ml	2ml	

* Internal standard 2.24 ug/ml N-ethylrecipavrin

Each sample was mixed well, centrifuged at 2500 rpm for 30 min, decanted free of precipitated cholesterol, adjusted to pH 10 $\pm$ 0.1 with 1M sodium hydroxide and 1 ml of pH 10 0.12 M sodium borate buffer, and extracted with three 4 ml aliquots of EtOAc. The organic phase containing the nonconjugated fraction was separated, backwashed with 1 ml water, dried over sodium sulfate, evaporated to dryness, reconstituted in 50 ul MeOH and 1 ul was analyzed by GCMS under standard operating conditions with a helium back pressure of 8 psi.

The aqueous phase was diluted with the backwash from the nonconjugated fraction, adjusted to pH 5 with 4 M HCl and 0.1 M acetic acid, frozen in liquid nitrogen, lyophilized, diluted with 3 ml pH 5, 0.1 M sodium acetate buffer, and incubated with 0.5 ml glucurase (gas evolved from H_2O_2 samples), incubated at 38° for 4 hours, treated with an additional 0.2 ml glucurase, and held overnight at $38^{\ominus}$, centrifuged at 2500 rpm for half an hour, decanted from sediment, spiked with 1 ml of 1.8 ug/ml terodiline hydrochloride, adjusted to pH 10 $\pm$ 0.1. Each sample was then retreated with the same volume of the same treatment reagent used in the nonconjugated fraction. The pH was checked and readjusted to 10 if necessary, and the samples were extracted with 4, three ml aliquots of EtOAc. The organic phase was separated, dried over sodium sulfate, evaporated, reconstituted in 50 ul MeOH and 1 ul was injected into the GCMS and run under standard operating conditions with a helium back pressure of 8 psi.

J. Attempts to decrease secondary formamide (12) production with the use of antioxidants and a formaldehyde complexing agent

l-Ascorbic acid (0.1 M) was prepared in MeOH. Butylated hydroxy toluene (BHT, 0.1 M) was prepared in MeOH. Dimedone (5,5-dimethyl-1,3-cyclohexanedione, 0.1 M) was prepared in MeOH.

Samples of bile (1 ml) from a recipavrin dosed rat were diluted with 1.5 ml water and 1 ml pH 10 sodium borate buffer (0.12 M), resulting in a final pH of 9.5-9.6. Each sample was extracted free of nonconjugated metabolites with three 3 ml aliquots of EtOAc. The aqueous phase was then spiked with 0.5 ml of MeOH (control samples), or 0.5 ml of BHT or ascorbic acid solution and allowed to stand overnight in the refrigerator. Samples were adjusted to pH 5 with sodium hydroxide, frozen in liquid nitrogen, lyophilized, reconstituted in 3 ml sodium acetate buffer (0.1 M, pH 5), incubated overnight with 0.2 ml glucurase at 38^{o}, adjusted to pH 10 with sodium hydroxide and 1 ml pH 10 borate buffer, and the samples were extracted with three 3 ml aliquots of EtOAc. The organic phase was separated, dried over sodium sulfate, evaporated, reconstituted in 0.5 ml of a 4.5 ug/ml methanolic solution of N-ethylrecipavrin hydrochloride. The samples were evaporated to dryness reconstituted in 10 ul MeOH and 1 ul was injected into the GCMS and run under standard operating conditions with a helium back pressure of 8 psi.

The amount of secondary formamide (12) was estimated by the ratio of integrated areas of the m/z 167 peak of the secondary formamide (12) (tr=21.00 min.) to that of the internal standard N-ethylrecipavrin (85)(tr=16.13 min.). This value was then expressed as a percent of secondary formamide (12) in the treated sample relative to the control.

K. Effect of the antioxidant BHT, formic acid and formaldehyde on dinorrecipavrin metabolite profiles

Following β-glucuronidase hydrolysis, the conjugated fraction aliquots of bile from dinorrecipavrin dosed rats was treated with 1 ml of methanolic 0.1 M butylated hydroxytoluene, or 1 ml of 0.1 M aqueous formic acid, or 1 ml of 0.1 M aqueous formaldehyde. Samples were alkalinized and the standard workup protocol was continued. Metabolite profiles were compared by visual inspection of the m/z 167 mass chromatograms and checked for the presence of the secondary formamide.

L. Decomposition of recipavrin N-oxide (53) under simulated workup conditions

Recipavrin N-oxide was purified twice on an alumina column. A stock solution of 17.3 mg/ml in chloroform was prepared for decomposition experiments.

Aliquots of 50 ul (865 ug) of recipavrin N-oxide were added to tubes containing 2 ml of one of the following solutions: 1. Water; 2. Sodium acetate buffer,

0.1 M, pH 5; 3. Sodium acetate buffer 0.1 M, pH 5 plus 250 ul glucurase; 4. Sodium borate, 0.12 M, pH 10; 5. 3 M Sodium hydroxide; 6. Ferric chloride, 5 mg/ml; 7. Ferric chloride, 5 mg/ ml plus 300 ul of 30% hydrogen peroxide.

The tubes were mixed well and incubated overnight at 40°, allowed to stand exposed to light for 24 hours at room temperature, then adjusted to pH 11 with 3 M NaOH, extracted with 4 two ml aliquots of EtOAc, dried over sodium sulfate, evaporated to dryness and reconstituted with 20 ul MeOH for GCMS analysis of 1 ul aliquots. The GCMS was operated under standard conditions with a helium back pressure of 15 psi.

Degradation products were identified by their mass spectra when possible. Total ion current peak areas of the degradation products were compared to determine the effects of sample treatments on the amount of each degradation product. The control sample was a 50 ul aliquot of the N-oxide stock solution evaporated to dryness and reconstituted in 20 ul MeOH for GCMS analysis of a 1 ul aliquot.

M. GCMS analysis and decomposition of the methylene nitrone (24)

The methylene nitrone was synthesized and purified as described earlier. Two stock solutions (10 and 100 ug/ul) were prepared in EtOH for experiments to determine the effects of sample loading, chromatographic conditions and the presence of bile constituents on the thermal degradation of the nitrone in the GC inlet.

Injection port liners were classified as either clean (boiled in nitric acid prior to use), dirty (previously used for at least ten bile sample analyses and visibly lined with brown material) or silanized (a new injection port liner treated with 5% DMCS in toluene overnight and washed to neutrality with MeOH).

GCMS analysis of each stock solution was performed on the GCMS equipped with either the clean or dirty injection port liner. On a separate occasion the nitrone was analyzed by GCMS on the silanized system. A last experiment involved the addition of 11 mg of nitrone to 5 ml of control rat bile followed by extraction with EtOAc from pH 9.5 solution, followed by drying, evaporation, and reconstitution of the organic phase to 110 ul. One ul (approximately 100 ug) was analyzed by GCMS.

III. RESULTS AND DISCUSSION

The first objective of the thesis was to conclude the work on the methadone formamide (6) with work on pH effects on the oxidation of EDDP (1) and the mechanism of the oxidation. This initial section concludes with details of the effect of workup conditions on the isolation of the formamide (6). Subsequent sections describe the use of recipavrin (9) as a model compound in experiments concerning the source of formamide metabolites of tertiary arylaliphatic amines.

1. FINAL EXPERIMENTS CONCERNING THE SYNTHESIS AND POSSIBLE SOURCES OF THE METHADONE FORMAMIDE METABOLITE

A. Mechanisms for the peroxidation of EDDP to an oxaziridine, diketone and related compounds.

The peroxidation of the major methadone metabolite (±) 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate (EDDP, 1) with m-chloroperbenzoic acid (MCPBA) afforded diastereomeric 2-(4',4'-diphenylheptan-5'-one-2'-yl) oxaziridine (2), 4,4-diphenyl-2,5-heptanedione (3), the known compounds 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP, 5) and 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (DDP, 4) and other minor byproducts (Figure 1)(Slatter, 1983, Abbott, Slatter and Kang, 1986). The oxaziridine was labile, and decomposed upon standing, when refluxed in m-xylene, and in the GC inlet to the isomeric formamide, 6-formamido-4,4-diphenyl-3-heptanone (6). The GCMS characteristics of the formamide were identical

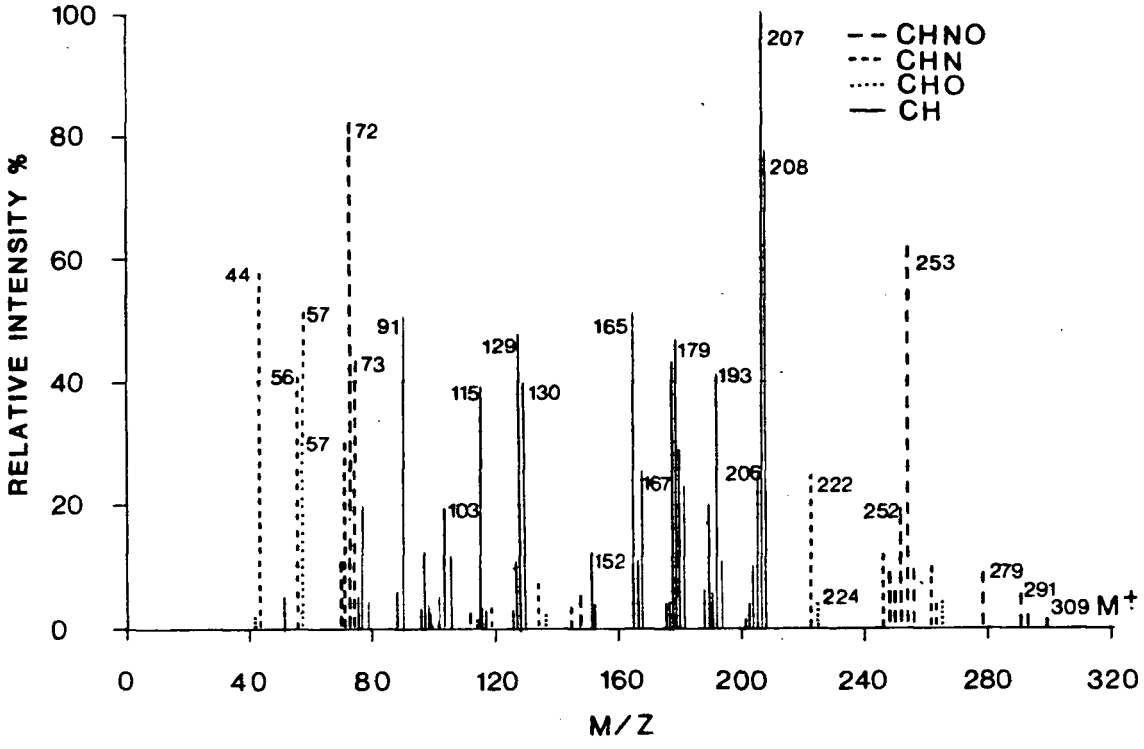
to a new metabolite of methadone isolated by solvent extraction of β-glucuronidase-hydrolyzed rat bile (Abbott, Slatter, Burton and Kang, 1985). Several experiments were done to refine the mechanism proposed for this oxidation.

i. High resolution mass spectrum of the methadone oxaziridine (2)

Figure 13 shows a composite high resolution mass spectrum of the methadone oxaziridine that was recorded at the lowest possible source temperature (120°). As expected from GCMS results, thermal decomposition to the formamide dominates the mass spectrum (figure 1b). The presence of the ion m/z 56 is the only indication that decomposition at this temperature is not spontaneous. This ion ($C_3H_6N^+$) corresponds to the desoxygenated iminium cation $CH_3-CH=N^+=CH_2$, an alpha cleavage product which is also common to the isomeric nitrone functional group in amphetamine related compounds (Coutts *et al.*, 1978).

ii. MCPBA oxidation of methadone HCl.

When the tertiary amine, methadone HCl (8) was oxidized with MCPBA, an additional minor oxidation product with a mass spectrum in accord with the the tertiary formamide structure (49) was obtained (figure 14). This is in accord with the results obtained for other aliphatic formamides generated by the peroxidation of N-methylamines (Sayigh and Ulrich, 1963).



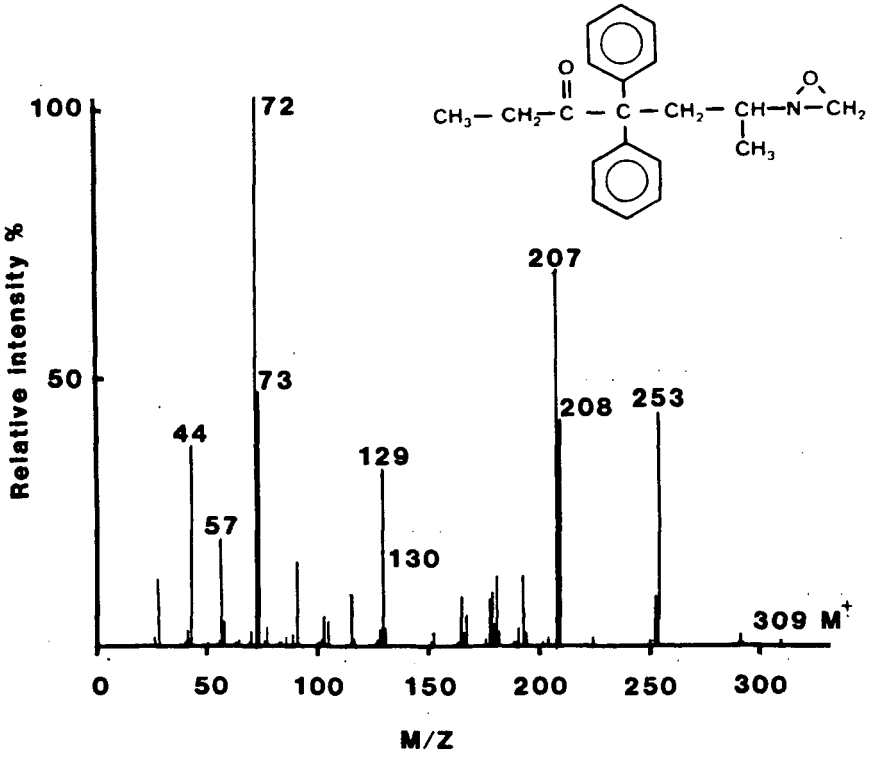


Figure 13. (top) Composite of high resolution mass spectral results for the methadone oxaziridine (2) (source temperature 120°). (bottom) Mass spectrum of the methadone formamide (6) arising from GCMS analysis of the methadone oxaziridine.

This suggested that if post enzymatic peroxidative generation of the secondary formamide from secondary amines were occurring then it was also likely that tertiary formamides would arise from tertiary amines, especially since tertiary amines are more readily oxidized than secondary amines (Beckwith, *et al.*, 1983). This was born out when bile from methadone dosed rats was left exposed to air for one week at room temperature and then examined by GCMS and found to contain the tertiary formamide in the nonconjugated fraction (appendix). This observation of post enzymatic oxidation required that future sample preparations should be treated so as to avoid possible artifactual generation of formamides in bile by post enzymatic oxidation.

iii. Peroxidation of EDDP free base in the presence of suspended K_2CO_3

The formamide isolated as a methadone metabolite had been isolated under alkaline conditions, while the oxidation of EDDP to the oxaziridine (2) was done under acidic conditions. The oxidation conditions were modified to see if EDDP produced oxaziridines by peroxidation under alkaline conditions.

Oxidation of the EDDP free base in the presence of suspended K_2CO_3 did not afford the oxaziridine. The major products were EMDP (5) and DDP (4). The diketone (3) was cyclized to only one of two possible regioisomeric cyclopentenones, 2,3-dimethyl-5,5-diphenylcyclopent-2-enone (50).

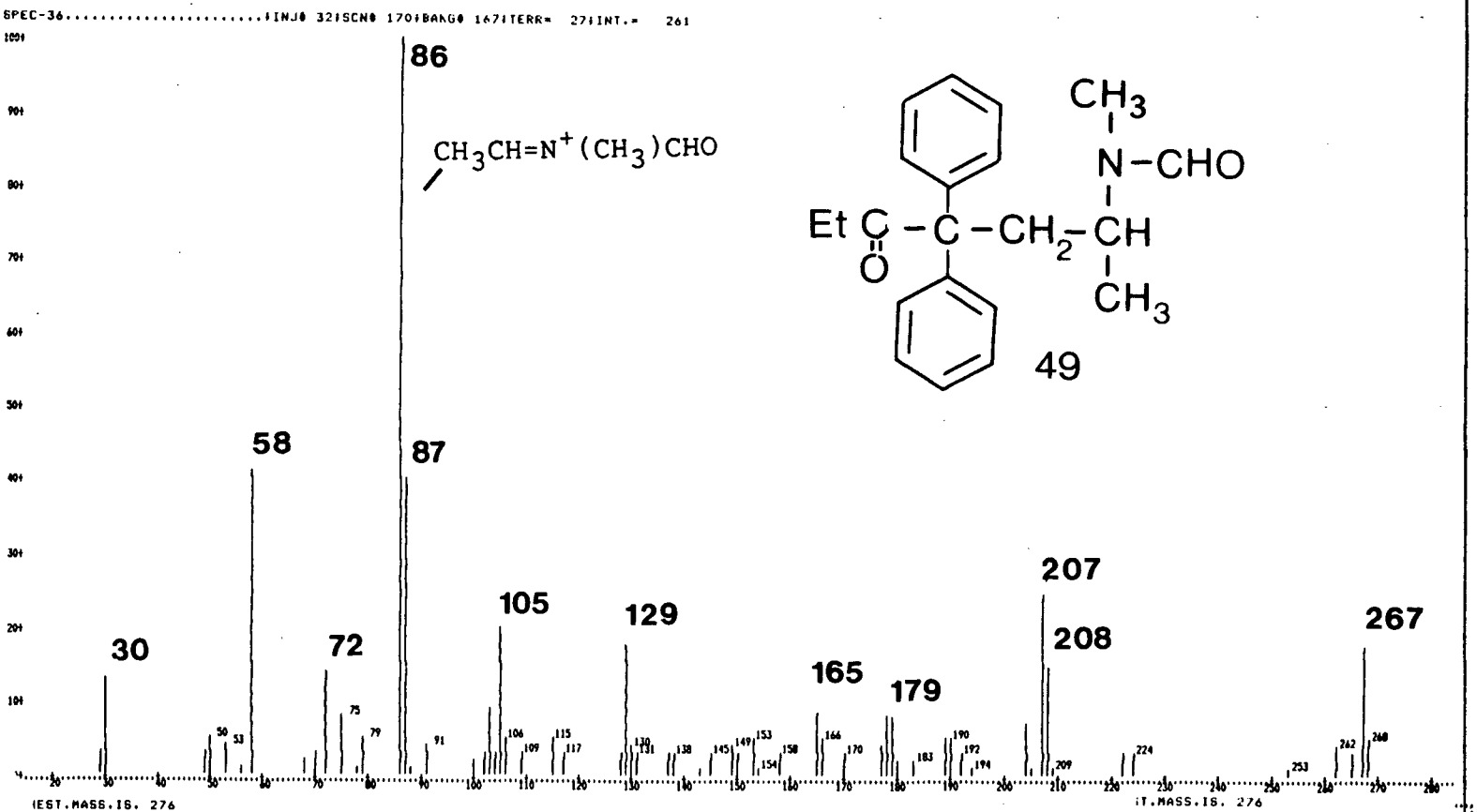
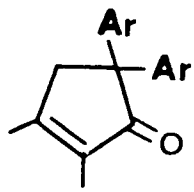
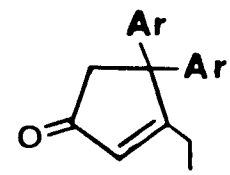


Figure 14. Mass spectrum of the tertiary formamide (49) formed by MCPBA oxidation of methadone HCl. M^+ 323 absent.

To confirm which of two possible regioisomeric cyclopentenones was produced by this aldol condensation, the diketone was refluxed briefly in 0.37 M ethanolic sodium hydroxide. The cyclopentenone product was detected on the TLC plate by its rapid reaction with iodine vapour. Only one regioisomer was obtained (figure 15), with NMR (figure 16), IR (appendix) and UV spectra in accord with the structure 2,3-dimethyl-5,5-diphenylcyclopent-2-enone (50) and not 3-ethyl-4,4-diphenylcyclopent-2-enone (51). NMR revealed singlets at 1.78, 2.13 and 3.32 ppm for $C\underline{H}_3CC{=}O$, $C\underline{H}_3CCH_2$, and $C\underline{H}_2$ respectively (figure 16). Homoallylic coupling broadened the resonances at 1.78 and 3.32 ppm. The UV spectrum had cyclopentenone K and R bands at 240 and 316 nm respectively. The calculated values for the cyclopentenone system enone pi-pi* transition (K band) with α and β substituent is 236 nm versus 224 nm for the single beta substituent of the other regioisomer. The R band n-pi* is a weak band between 310 and 330 nm and was not diagnostic (Silverstein *et al.*, 1981).



50



51

Figure 15. Possible regioisomeric cyclopentenones (50 and 51) expected from the aldol condensation of the diketone.

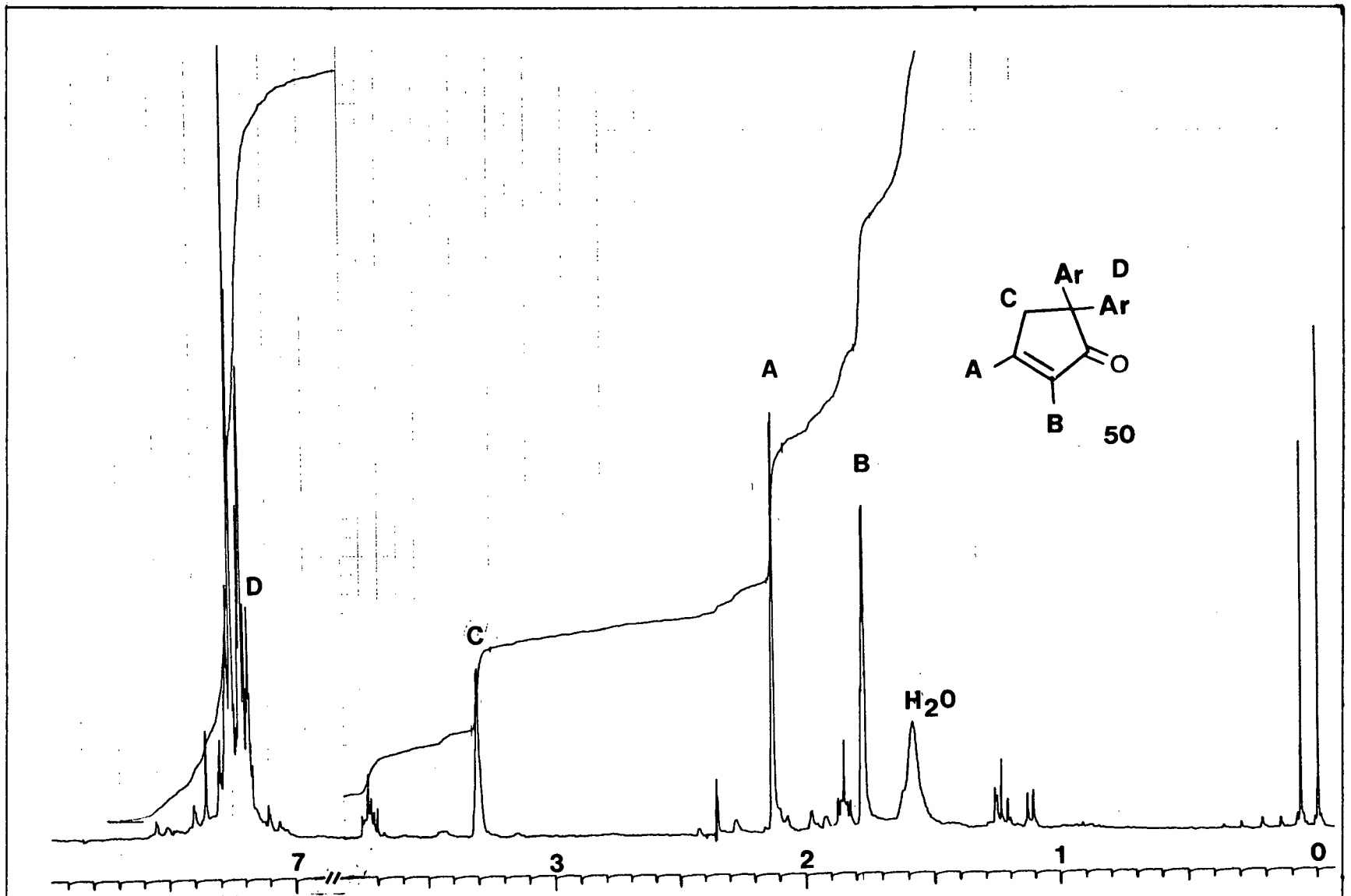


Figure 16. NMR spectrum of the cyclopentenone aldol product 2,3-dimethyl-5,5-diphenylcyclopent-2-enone (50).

iv. Mechanism for the peroxidation of EDDP

It has been established by Milliet and co-workers (1981, 1974a, 1974b) that peroxidation of N-methylpyrrolidine derivatives of the alkaloid conanine can afford open chain keto-oxaziridines, dicarbonyl compounds, cyclic oxaziridinium salts, lactams, pyrrolines and related compounds. The similarity between the arrays of oxidation products described for conanine and those of EDDP prompted the application of mechanisms described by Milliet *et al*. (1981, 1974a, 1974b) to the EDDP oxidation.

The peracid oxidation of imines is thought to proceed by a two step Bayer-Villiger type mechanism with rate determining oxidant addition to the C=N bond in the first step (Ogata and Sawaki, 1973). This mechanism, applied to EDDP is shown in figure 17. Ring closure with loss of chlorobenzoic acid follows in the second step to give an intermediate of type a.

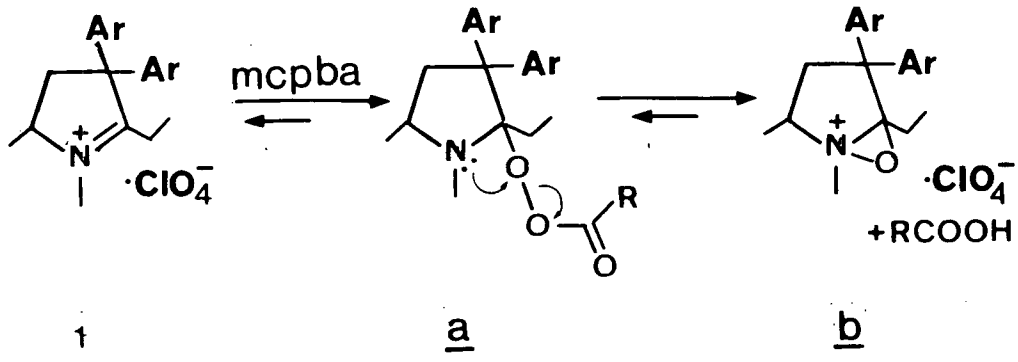


Figure 17. Bayer-Villiger MCPBA addition to $C=N^+$ bonds in the first step of the peroxidation of EDDP (1).

Milliet and co-workers (1974a, 1974b, 1981) have incorporated intermediate a into their mechanisms of peroxidation of N-methylpyrrolinium salts and free bases. Their mechanisms, modified to EDDP are shown in figure 18. They have proposed that elimination of the ring proton alpha to nitrogen in a (scheme 1) accounts for keto-imine c, thermolabile C-disubstituted keto-oxaziridine b and diketone (3).

We speculate that elimination of the alpha proton on the N-methyl carbon of a in a similar manner (figure 18, scheme 2) accounts for EMDP, in accord with the results of Milliet *et al.* (1981), as well as the methylene oxaziridine. The conanine analogues of the keto-imines c and d were observed by Milliet *et al.* (1981) following peroxidations with one equivalent of oxidant. Keto-imines were not observed in this study, however they are the conventional imine precursors for peroxidation to oxaziridines b and 2 respectively. The imine d is presumably unstable and could form a 1,3,5-triazane or related polymers (Emmons, 1957, Farrar, 1968), themselves amenable to peroxidation.

The initial addition of MCPBA to pyrrolidine salts is a reversible reaction, unaffected by oxidant concentration (Milliet, *et al.*, 1981). This could account for our observation of unreacted EDDP under a variety of reaction conditions, including large excesses of MCPBA.

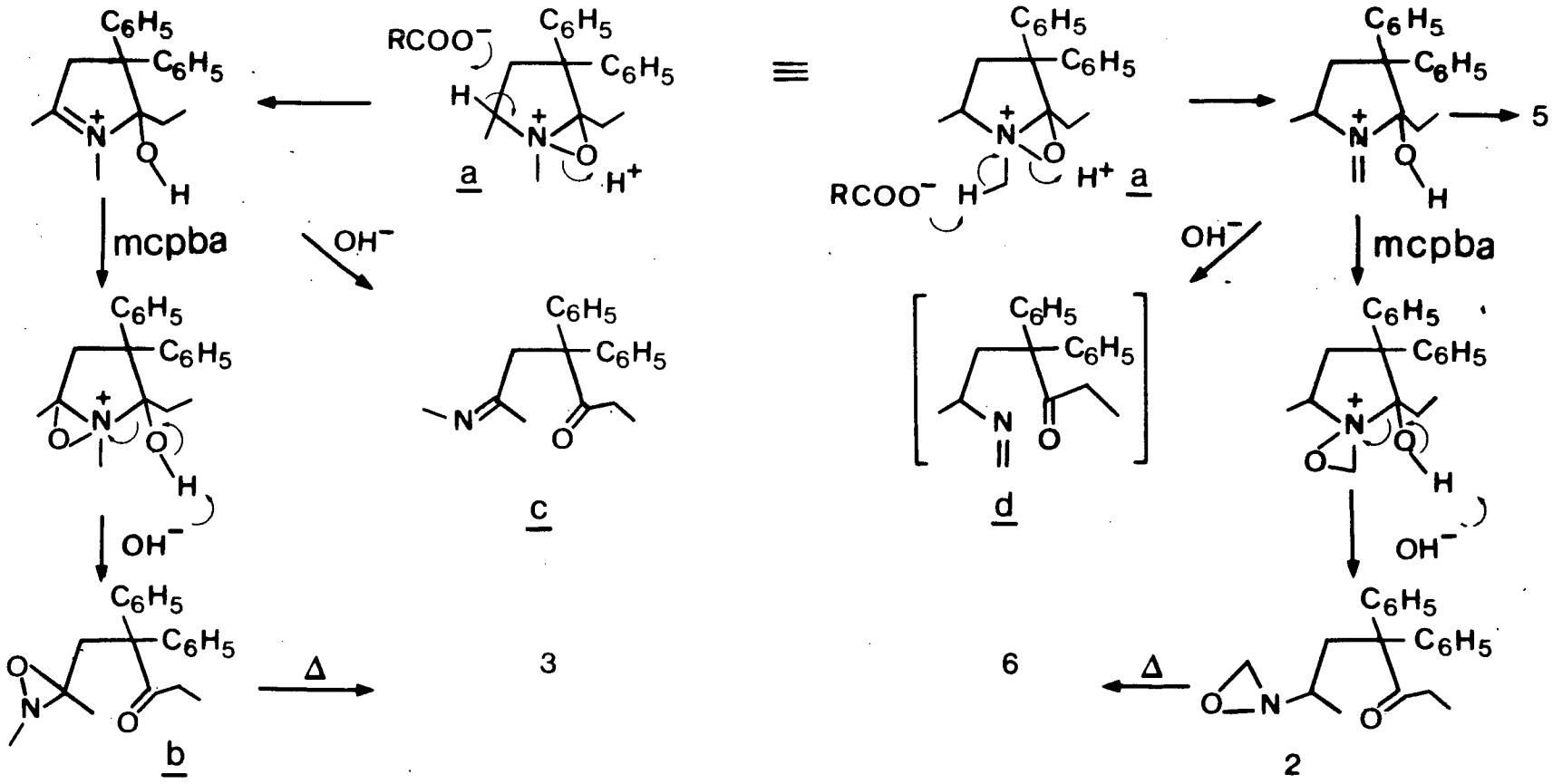


Figure 18. Possible mechanisms for the formation of the oxidation products of EDDP that were observed by GCMS.

The other product of the peroxidation of EDDP, the diphenylpyrrolidone DDP (4) could arise from the enamine tautomer of EDDP by known mechanisms (Milliet, *et al.*, 1981, Back, *et al.*, 1977) (figure 19), although the endocyclic double bond position is preferred in acid solution (Hassan and Casy, 1970).

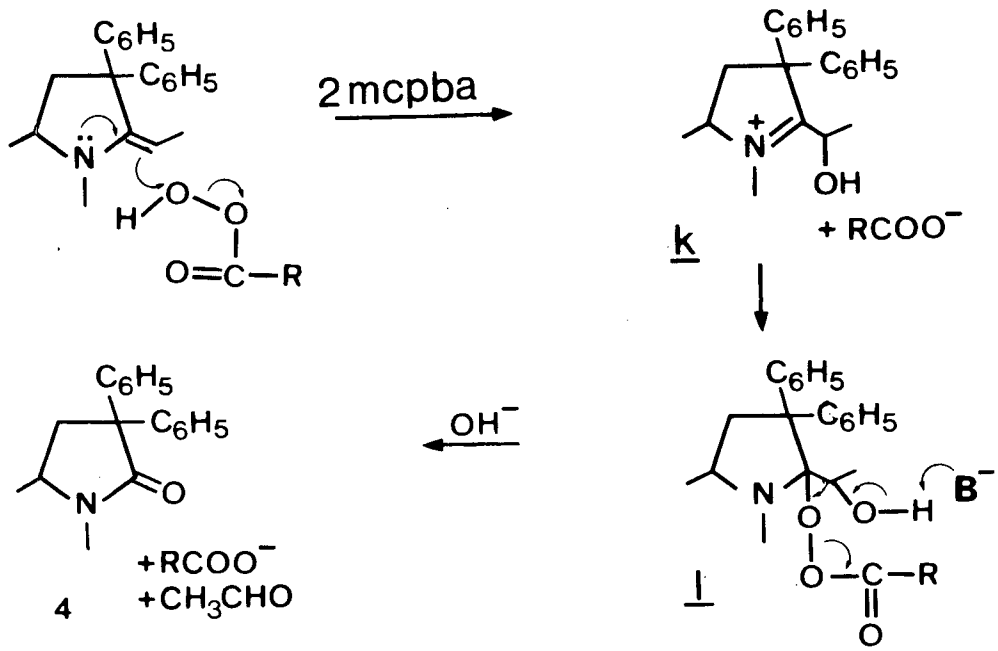


Figure 19. Mechanism for the formation of DDP (4) from oxidation of the enamine tautomer of EDDP.

B. Solvent control experiments in the isolation of the methadone formamide metabolite

To preclude the possibility of phosgene, peroxide and aldehyde related generation of formamides in the conjugated fraction of rat bile, the metabolite extraction method of Horning and Mitchell (1984) was used. Ether and chloroform extractions were performed with and without prior treatment with type 4A molecular sieve (Burfield, 1982, Burfield and Smithers, 1982) and distillation just before use. Molecular sieves remove organic peroxides from ether and phosgene related impurities from chloroform.

Bile extracts were reconstituted with diphenylbutanone (14, $Ph_2CH-CH_2-C(=O)CH_3$) added as an internal standard just prior to analysis by GCMS. The area of the m/z 167 ion peak (Ph_2CH^+) which is common to diphenylbutanone and the secondary formamide (6) was integrated to estimate the relative amount of the formamide present.

Following the standard extraction protocol, or the method employing solid ammonium carbonate for alkalinization (Horning and Mitchell, 1984), solvent/alkali pairs were employed for the extraction and basification of nonconjugated and conjugated metabolites. The chloroform extracted sample was also reconstituted with ethanol in place of methanol to preclude any contribution of formate or formaldehyde contaminants in methanol to the amount of the methadone formamide (6). Results of these sample isolation procedures are summarized in table 3.

Table 3. Recovery of the methadone formamide metabolite (6) using various sample isolation procedures.

Bile fraction[1]	Solvent/alkali/solvent used in workup[2]	formamide peak abund.[3]	%DPB[4]
A. Conj	$CHCl_3$/NaOH/MeOH	12000	1.12
A. Conj	$CHCl_3$[5]/NaOH/MeOH	13307	2.14
A. Conj	$CHCl_3$/NaOH/EtOH	13000	-
B. Conj	CH_2Cl_2/NaOH/MeOH	13500	2.72
C. Conj	EtOAc/NH_4CO_3/MeOH	12000	1.12
D. Conj	Ether/NH_4CO_3/MeOH	1600	0.23
D. Conj	Ether[5]/NH_4CO_3/MeOH	2000	0.08
E. Conj	C_6H_6/NH_4CO_3/MeOH	12000	0.75
F. All	EtOAc/NH_4CO_3/MeOH	14000	1.62
G. All	$CHCl_3$/NaOH/MeOH	-	2.32

[1] conj= conjugated fraction, all= conjugated and nonconjugated metabolites extracted together immediately after β-glucuronidase hydrolysis. [2]. solvent=extraction solvent, /alkali= 1M NaOH or solid NH_4CO_3 used to basify aqueous bile, /solvent= MeOH containing diphenylbutanone internal standard, or ethanol (EtOH) used to dissolve bile extract for GCMS. [3]. Formamide m/z 167 ion abundance from the integrated areas of the m/z 167 ion chromatogram. [4]. Formamide m/z 167 ion abundance from the integrated areas of the m/z 167 ion chromatogram expressed as percent of the m/z 167 ion abundance of the standard diphenylbutanone (DPB, 5ug per injection). [5]. Solvent received no pretreatment with molecular sieve.

The results in table 3 show that regardless of solvent or purification method, detectable amounts of the formamide metabolite were always observed. Halogenated solvents did result in higher recoveries, especially when the solvent was not

treated with molecular sieve prior to use. This indicates that a possible role of phosgene or formyl chloride in formamide generation cannot be ruled out. The formylation of basic compounds by these solvent impurities is a well known pitfall in drug metabolism methodology (Stillwell *et al.*, 1978, Cone *et al.*, 1981). However it is difficult to envisage dinormethadone, the requisite substrate in this formylation reaction, as a component of the conjugated fraction. It is generally accepted that any basic desalkyl metabolite of methadone spontaneously cyclizes to the corresponding pyrrolidine (EDDP and EMDP for normethadone and dinormethadone respectively)(Pohland, *et al.*, 1971). The phosgene mediated formylation of EMDP (5) and EDDP (1) was not investigated because neither EDDP or EMDP were components of the conjugated bile fraction.

The recovery of the formamide metabolite was lowest with diethyl ether, and was not altered significantly by pretreatment to remove organic peroxides. The lower recovery may be related to the solubility of the metabolite in ether. This speculation is based on the requirements for more polar chloroform/alcohol or ethyl acetate based solvent systems to dissolve and elute recipavrin derived formamides or nitrones for chromatography on silica gel.

Exchanging redistilled ethanol for methanol as the solvent used to dissolve the extract for injection, had no effect on the abundance of the formamide in the chloroform

extracted bile. This precluded the possibility that formate or formaldehyde in methanol was involved in the production of the formamide metabolite from an unidentified precursor.

C. Conclusions regarding the source of the methadone formamide metabolite

Peroxidation of EDDP (1) and methadone (8) has pointed to two possible precursors of the formamide metabolite of methadone, namely, the oxaziridine (2) and tertiary N-methylformamide (49). It was not possible to synthesize hydroxylamine and nitrone precursors due to cyclization of intermediates (Slatter, 1983).

Solvent control experiments have shown that solvents can contribute, but are not the sole source of the formamide metabolite of methadone. This could be due to chemical reaction with unidentified precursors or by different extraction efficiencies.

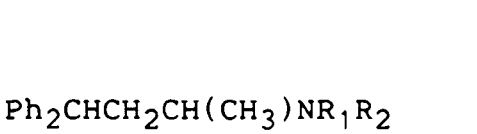
2. SYNTHESIS AND CHARACTERIZATION OF SYNTHETIC COMPOUNDS RELATED TO RECIPAVRIN

The synthesis of potential intermediates in the generation of a formamide metabolite of recipavrin was undertaken to search for these intermediates by GCMS and LCMS in bile extracts. The lability of potential intermediates under sample isolation conditions could also be determined. The synthesis and characterization of the secondary hydroxylamine (17), methylene nitrone (24) and oxaziridine (25) were reported previously (Slatter, 1983).

Spectral results for all compounds are summarized in the experimental section. New spectra not included in the text are presented in the appendix, indexed by compound name in the same order as the experimental section.

A. Synthesis and Characterization of the amines

The amines recipavrin (9), norrecipavrin (15) and dinorrecipavrin (20) are all known compounds and gave satisfactory NMR, IR, and GCMS analyses (Slatter, 1983). Norrecipavrin and dinorrecipavrin both formed TMS derivatives with BSTFA. Norrecipavrin was methylated with TMAH to give recipavrin.

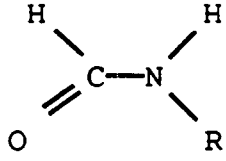


	9	$R_1=R_2=CH_3$
$Ph_2CHCH_2CH(CH_3)NR_1R_2$	15	$R_1=H$, $R_2=CH_3$
	20	$R_1=R_2=H$

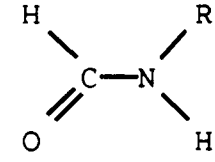
B. Synthesis and Characterization of the amides

The secondary formamide (12, $Ph_2CHCH_2CH(CH_3)N(H)CHO$) was obtained in good yield by refluxing dinorrecipavrin (20) with ethyl formate, or by Leuckart reaction of diphenylbutanone (14) and formamide using the method of Moffat, *et al*. (1962), and Moore (1949) respectively.

The proton NMR spectrum of the secondary formamide showed that two rotamers were present in a ratio of 2:1 (Figure 20). When the spectrum was run in DMSO-D_6 at 25°, the proportion of cis isomer decreased to 2.1:10 relative to the trans rotamer. The ratio decreased further to 1.6:10 when the spectrum was rerun in DMSO-D_6 at 90° C. The position of the NH and, to a lesser extent, the CHO resonances varied between samples. The rotational isomerism of the formamide functional group arising from amide resonance-mediated restricted rotation about the C-N bond has been well documented spectroscopically (LaPlanche and Rogers, 1964, Hallam and Jones, 1970) and is discussed further in the section on the promethazine formamides. The planar cis and trans orientations described by LaPlanche and Rogers are shown below. Cis and trans nomenclature is assigned based on the relation between the NH and C=O groups that are involved in hydrogen bonding. The trans rotamer predominates. In the ^{13}C NMR spectrum the rotamers were also apparent (Figure 21). The infrared spectrum had distinct cis and trans C=O stretches (Figure 22). The secondary formamide reacted slowly with BSTFA at 80° to form a mono TMS derivative (60) (Figure 23).



Trans (major)



Cis (minor)

A major byproduct of the Leuckart reaction was 4-(2,2-diphenylethyl) pyrimidine (59) (Figure 24). Formamide and pyrimidine byproducts of the Leuckart reaction of phenyl-2-propanone are important in the forensic investigation of clandestine amphetamine laboratories (Kram and Kruegel, 1977, Frank, 1983)

The tertiary formamide (26, $Ph_2CHCH_2CH(CH_3)N(CH_3)CHO$) was synthesized by Leuckart reaction of diphenylbutanone (14) and N-methyl formamide and by methylation of the secondary formamide (12) with TMAH. In the tertiary formamide, proton and ^{13}C NMR results showed that the trans rotamer predominates in $CDCl_3$ solution at ambient temperature (11.4% cis)(figure 25, 26). NMR results were in accord with those reported for the structurally similar formamide derivative of methamphetamine (Lebelle *et al.* 1973). Cis and trans C=O stretches were not resolved in the infrared spectrum (figure 27).

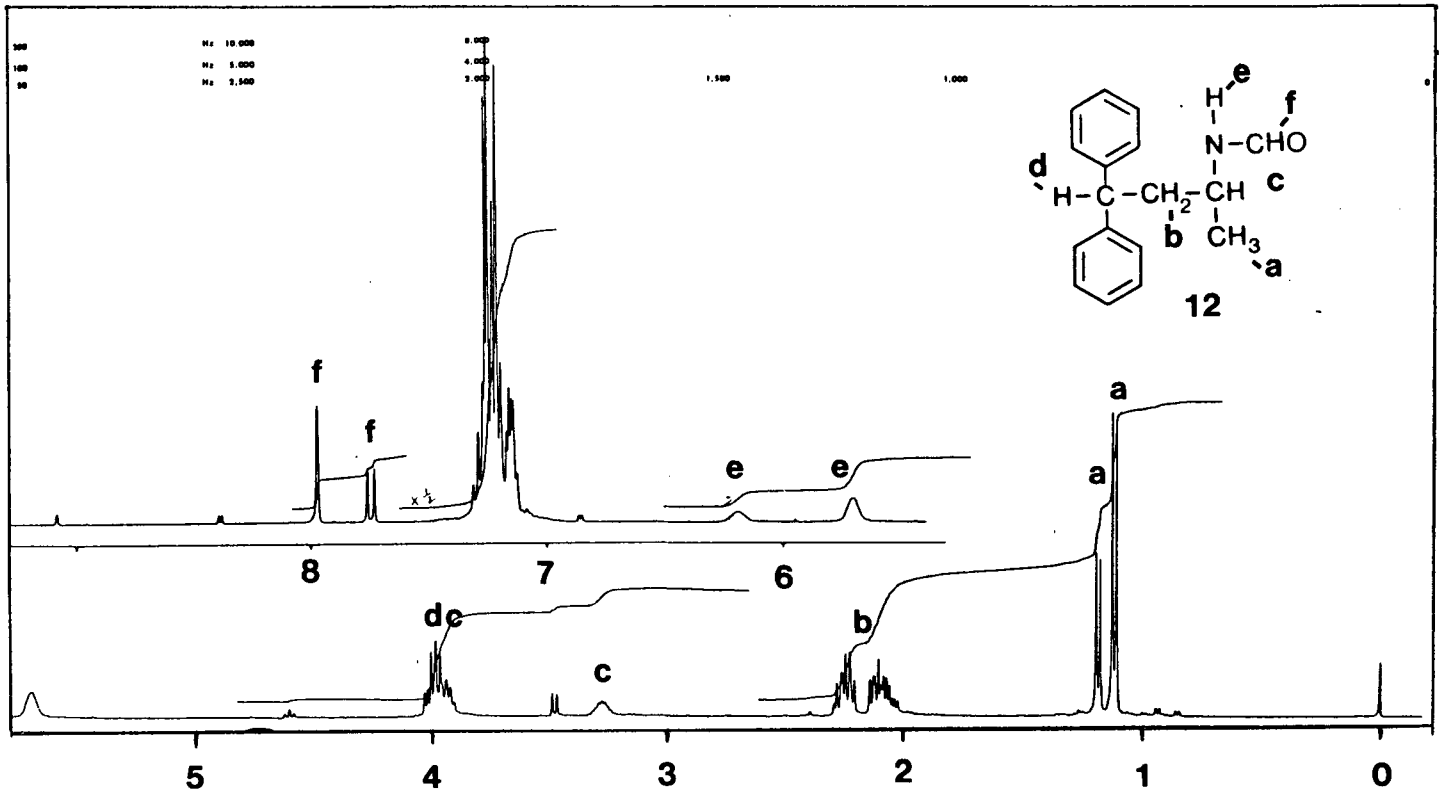


Figure 20. 400 MHz ^{1}H NMR spectrum of the secondary formamide (12).

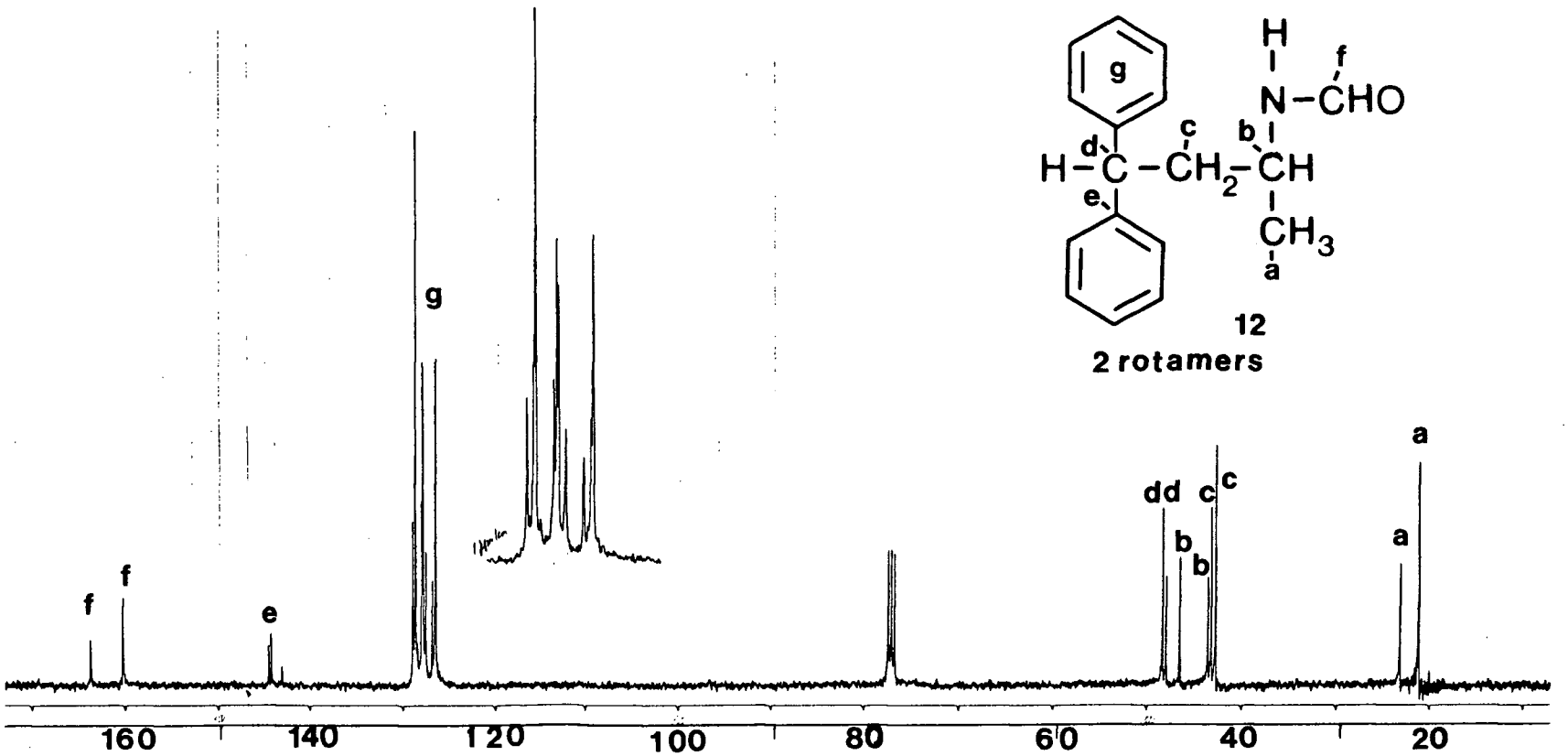


Figure 21. 75 MHz broad band decoupled ^{13}C NMR spectrum of the secondary formamide (12).

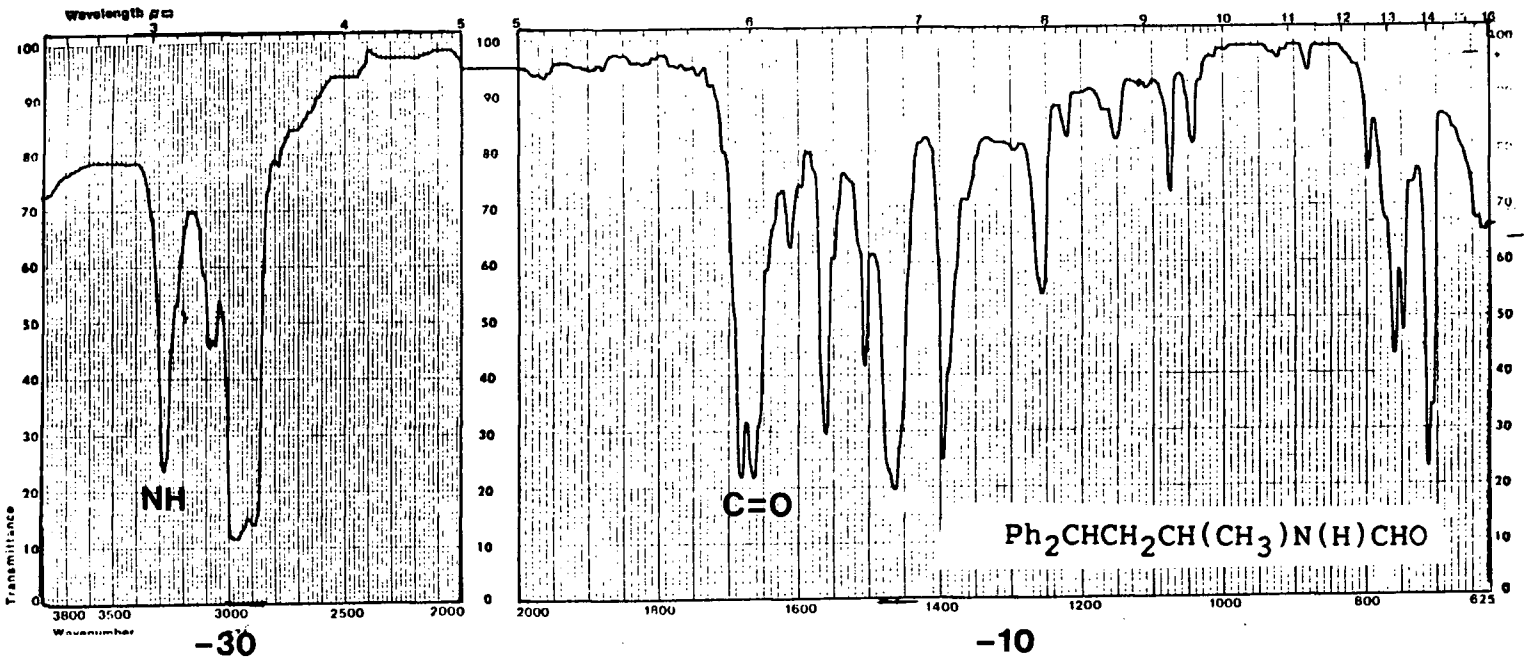


Figure 22. Infrared spectrum of a nujol mull of the secondary formamide. NH stretches 3260 (trans), 3200 (shoulder, cis). C=O stretches 1670 (trans), 1650 (cis).

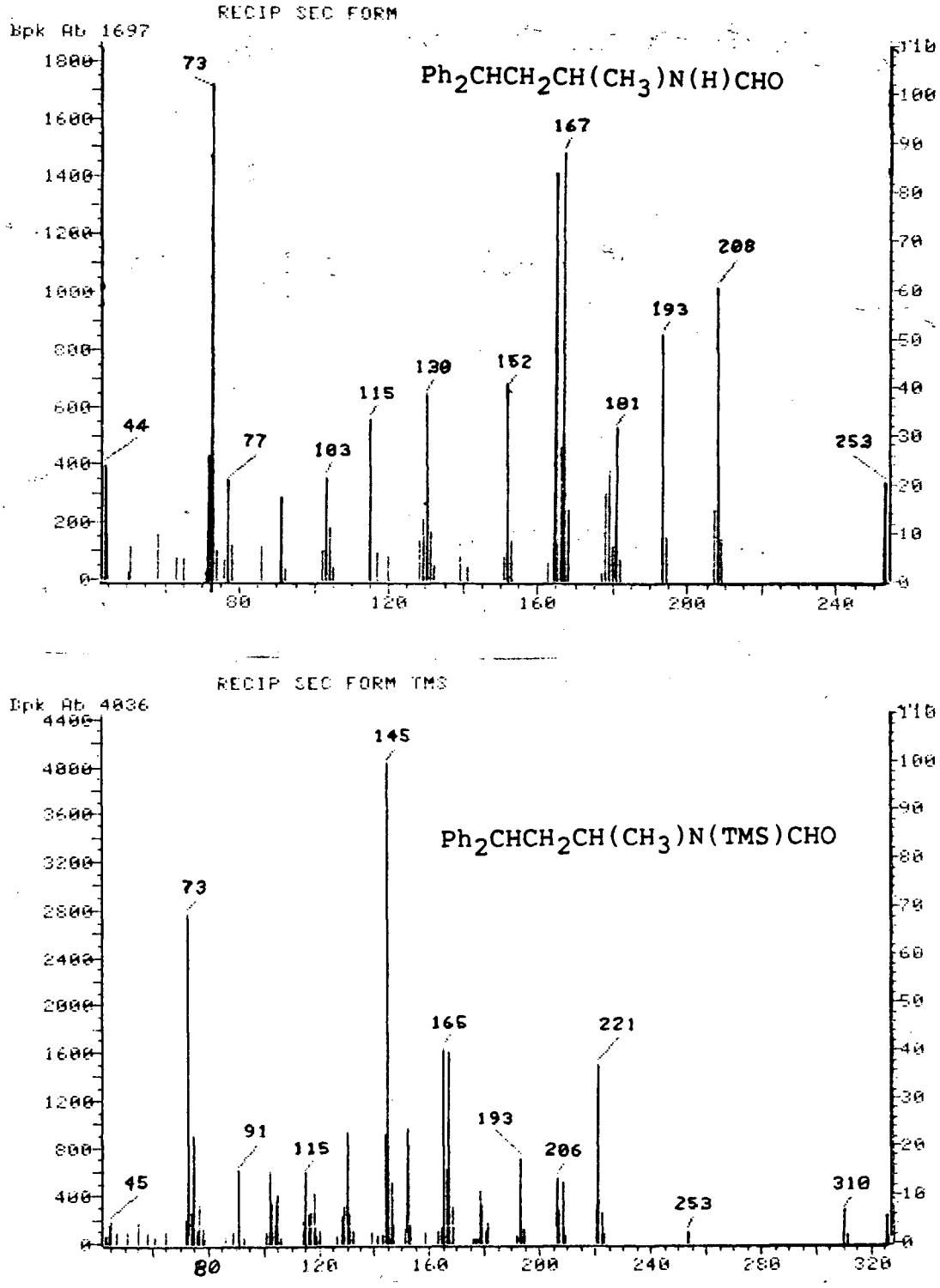


Figure 23. (top) Mass spectrum of the secondary formamide (12)(M^+ 253), (bottom) Mass spectrum of the secondary formamide mono TMS derivative (60)(M^+ 325).

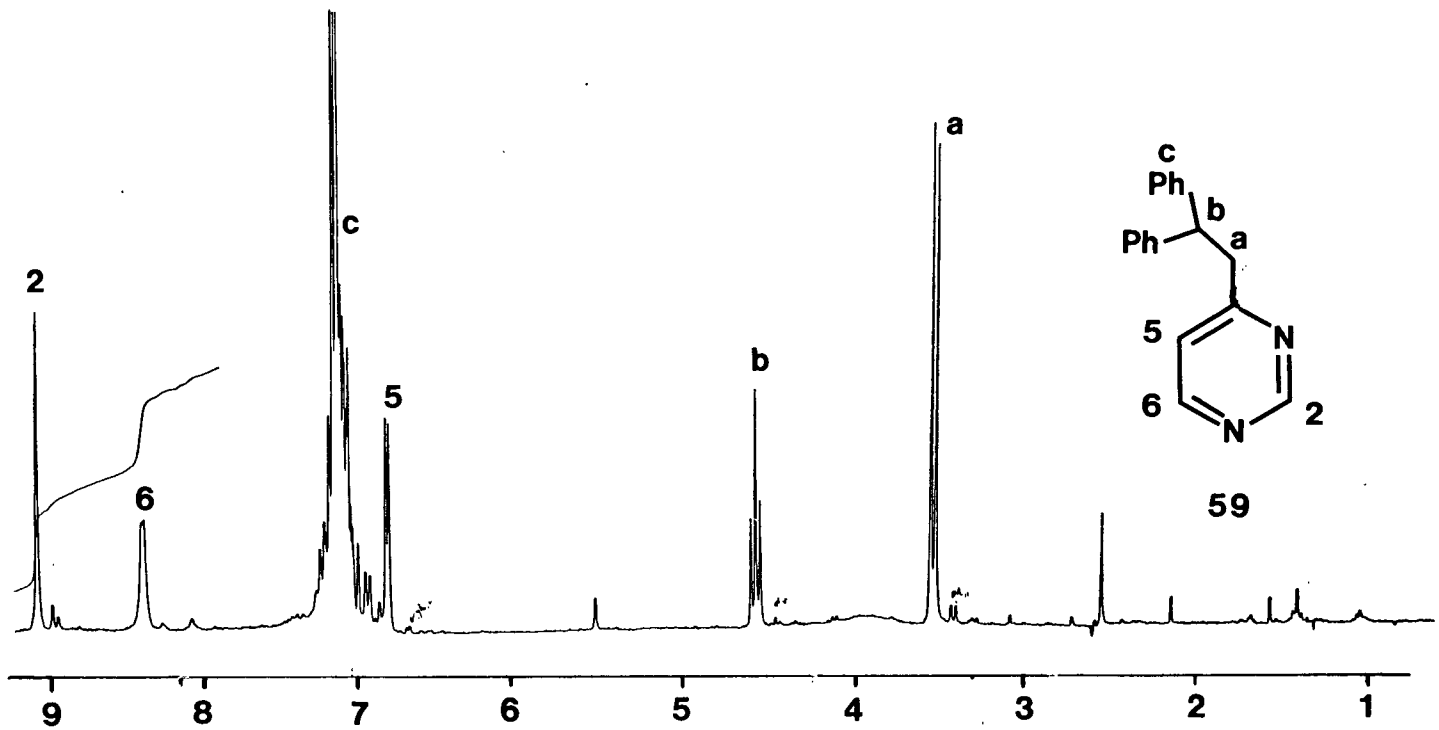


Figure 24. 270 MHz NMR spectrum of the pyrimidine byproduct (59) of formamide synthesis.

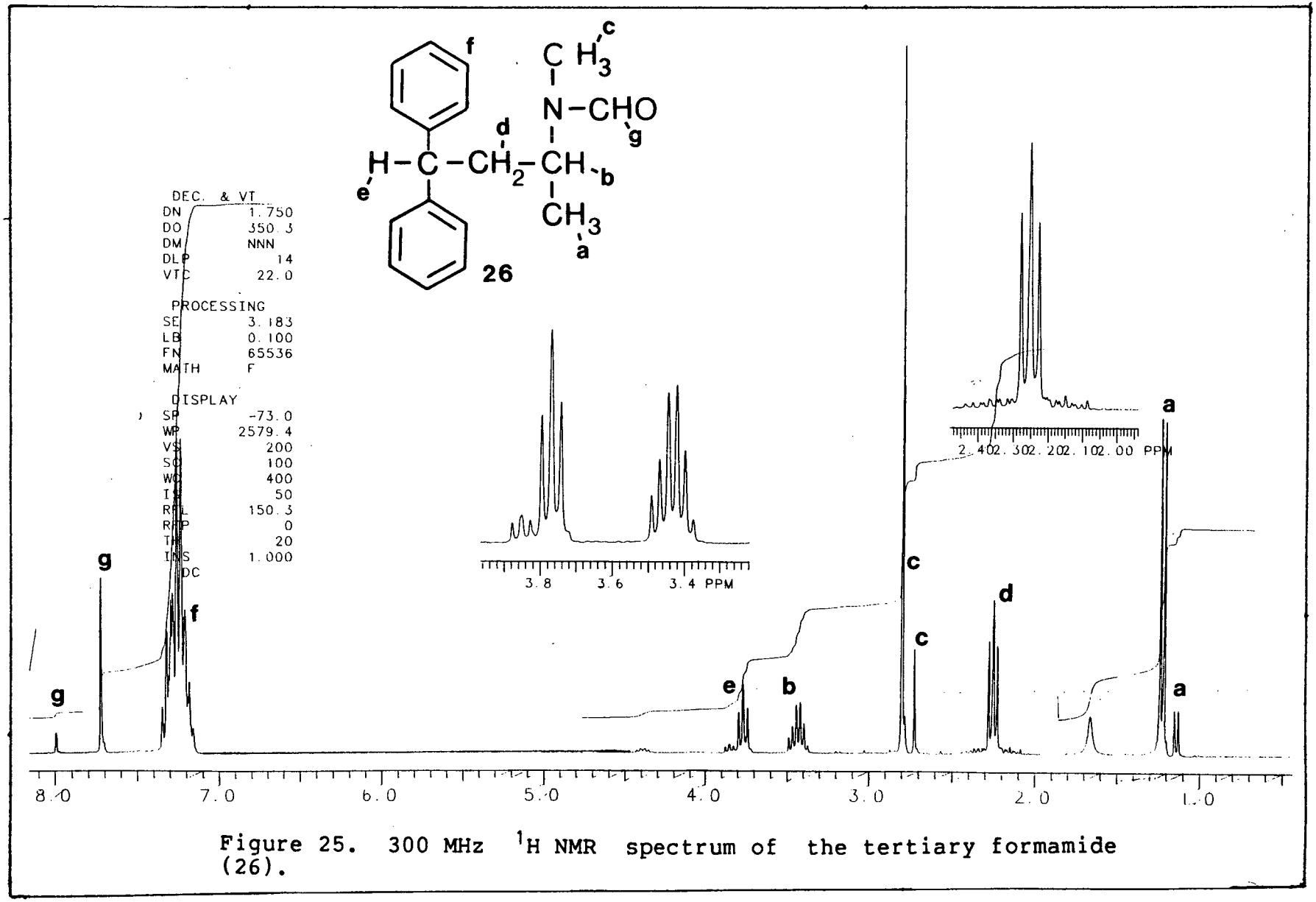


Figure 25. 300 MHz ^{1}H NMR spectrum of the tertiary formamide (26).

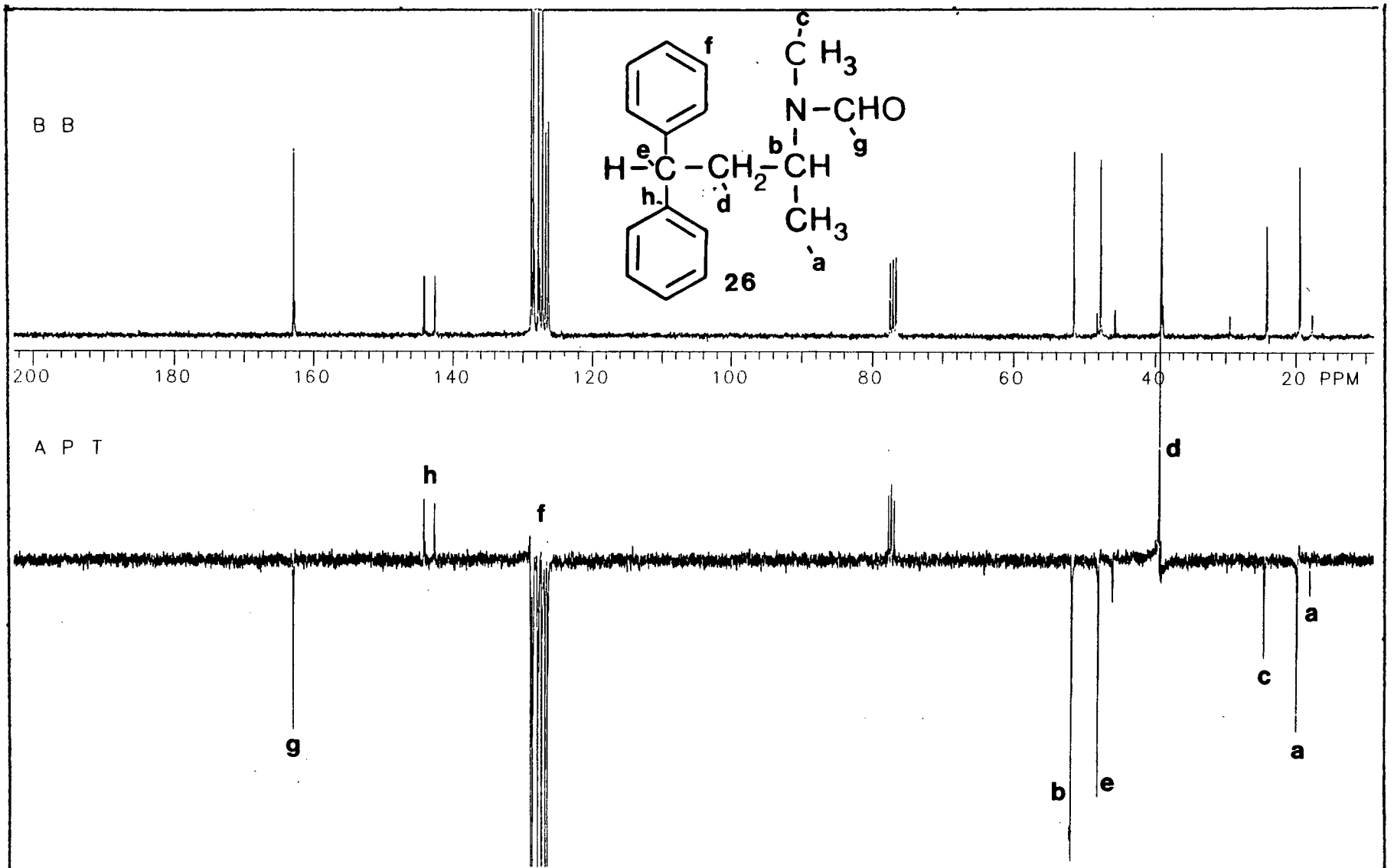


Figure 26. 75 MHz ^{13}C NMR spectrum of the tertiary formamide (26). Top: broad band decoupled. Bottom: Attached proton test (CH and CH_3 project down, C and CH_2 project up).

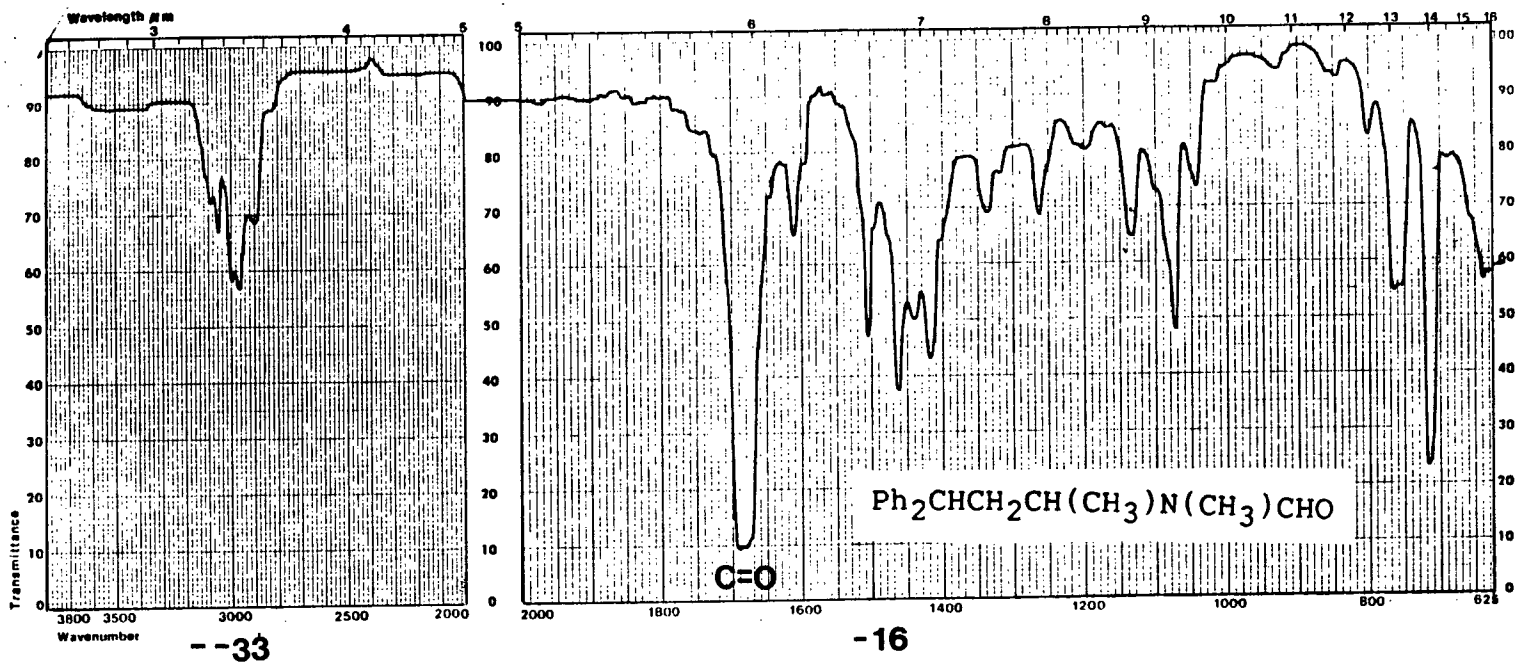
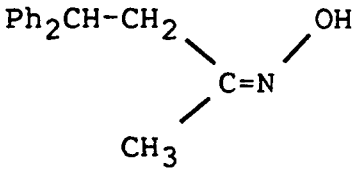


Figure 27. Infrared spectrum of a thin film of the tertiary formamide (26). C=O stretch at 1666 cm^{-1}.

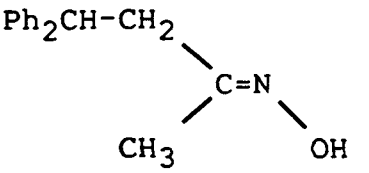
The secondary acetamide (63, $Ph_2CHCH_2CH(CH_3)NHC(=O)CH_3$), a potential metabolite of recipavrin was synthesized by acetylation of dinorrecipavrin (20) with acetic anhydride. The mass spectra of the tertiary formamide (26) and the isomeric secondary acetamide are similar, their mass spectra differing only in the presence of ions at m/z 44 (acetamide) or m/z 58 (formamide). Both ions are related to the base peak at m/z 87 by loss of the acyl fragment.

C. Synthesis and Characterization of oximes, hydroxylamines and their oxidation products

The oxime (19, ($Ph_2CHCH_2C(CH_3)=N-OH$)) was synthesized by condensation of diphenylbutanone (14) and hydroxylamine hydrochloride (Slatter, 1983). GCMS and NMR analysis revealed both syn (Z) and anti (E) geometric isomers. The predominant anti (E) isomer elutes at longer retention time. The ratio of geometric isomers is a function of the bulk of each alpha-C substituent, with the largest C=N substituent trans to the N-OH bond in the anti (major) isomer (Gorrod and Christou, 1986). Mass spectra of the underivatized oximes were similar although the syn isomer had more intense M^+-17 and arylaliphatic fragments but did not have a detectable molecular ion. The oxime formed a TMS ether derivative (52) with BSTFA and was O-methylated with TMAH to give the oxime ether 16 ($Ph_2CHCH_2C(CH_3)=NOCH_3$).



Syn (Z) oxime 19



Anti (E) oxime 19

The primary hydroxylamine (22, $Ph_2CHCH_2CH(CH_3)N(H)OH$) was obtained by reduction of the oxime (19) with sodium cyanoborohydride. Satisfactory NMR results for the free base were only obtained with freshly prepared samples (figure 28). The NMR results were similar to those of N-hydroxyamphetamine (Beckett *et al*. 1975). The product autoxidized within hours to the azoxy dimer ($R-N=N^+(O^-)-R$). This was evident by the loss of the strong NH and OH stretch in the infrared spectrum (figure 29) and by the disappearance of the NHOH resonance in the NMR spectrum. As a consequence of the facile autooxidation, freshly prepared hydroxylamine was used in the synthesis of the methylene nitrone (24). The secondary hydroxylamine (17, $Ph_2CH-CH_2-CH(CH_3)-N(CH_3)OH$) was oxidized to the methylene nitrone (24, $R-N^+(O^-)=CH_2$) by yellow mercuric oxide, in mildly alkaline solution and in control bile under simulated workup conditions (Slatter, 1983).

The hydroxylamines both formed O-TMS derivatives with BSTFA. Methylation with TMAH afforded N-methoxy-N,α-dimethyl-γ-phenylbenzenepropanamine (58) from both the primary and secondary hydroxylamines. When freshly silanized GC accessories were used, small amounts of the intact hydroxylamines survived the GC sector to give satisfactory mass spectra with base peaks

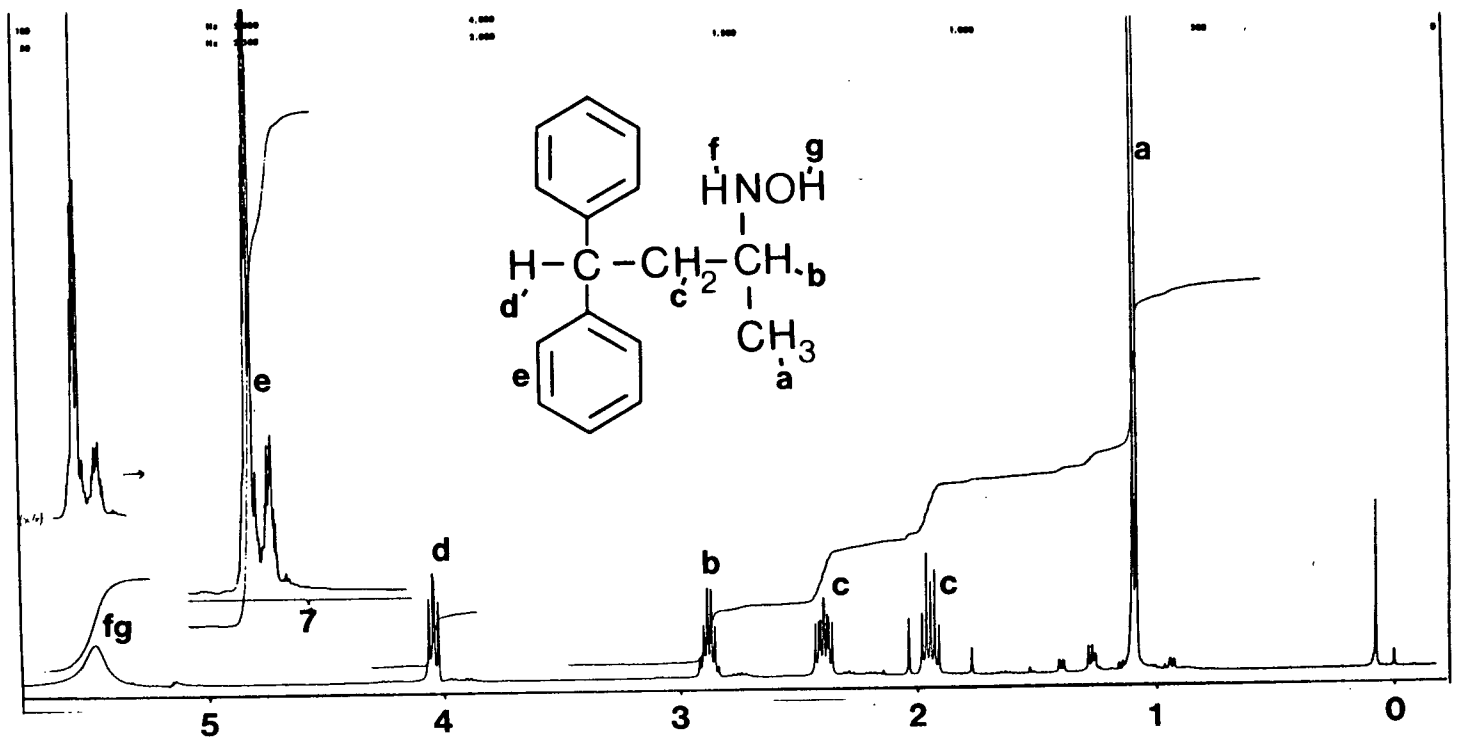


Figure 28. 300 MHz NMR spectrum of the primary hydroxylamine (22).

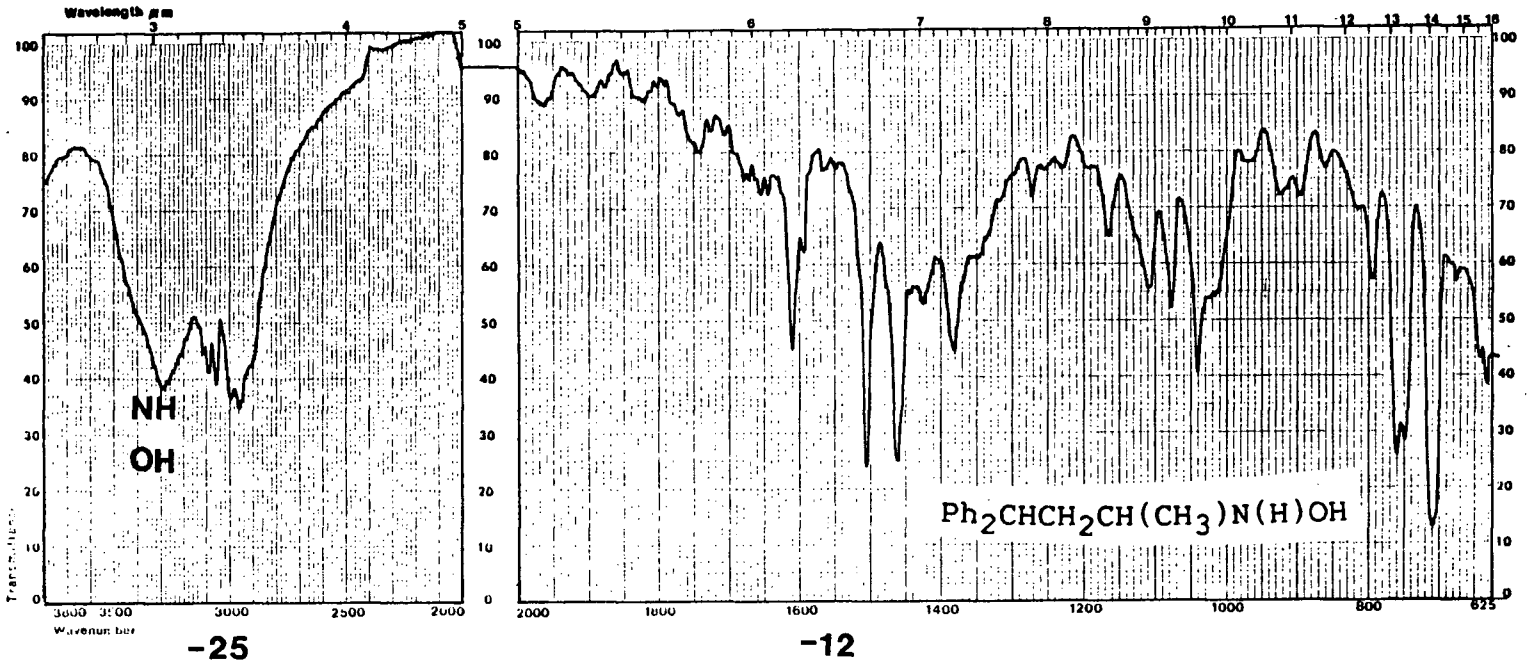


Figure 29. Infrared spectrum of a thin film of the primary hydroxylamine (22). NH and OH stretch between 3600 and 3100 cm^{-1}.

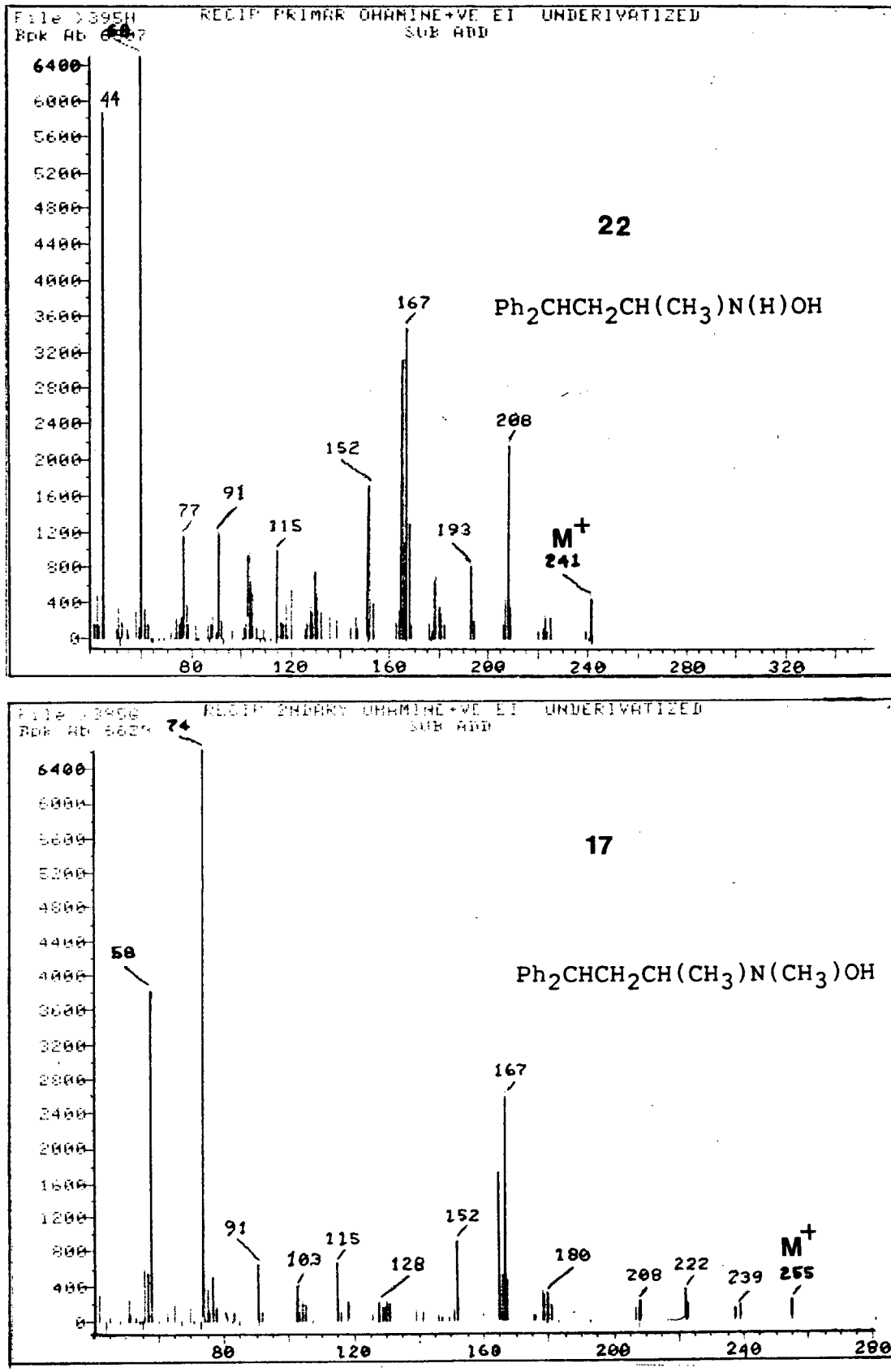


Figure 30. Mass spectra of the underivatized primary (22, top) and secondary (17, bottom) hydroxylamines.

arising by cleavage of the C-C bond adjacent to nitrogen (Figure 30). The major degradation products were the respective amines. This mass spectral fragmentation and chemical instability has been seen in the amphetamine hydroxylamines (Beckett *et al.* 1973, Beckett and Achari, 1977).

D. Synthesis and Characterization of the recipavrin N-oxide

The N-oxide (53, $Ph_2CHCH_2CH(CH_3)-N^+(O^-)(CH_3)_2$) decomposed to cis and trans 1,1-diphenylbut-2-ene Cope elimination products (54 and 55, $Ph_2CHCH=CHCH_3$) and other minor byproducts when analyzed by GCMS. The tertiary formamide (26) was present as a minor byproduct of the oxidation.

E. Synthesis and Characterization of the Nitrones

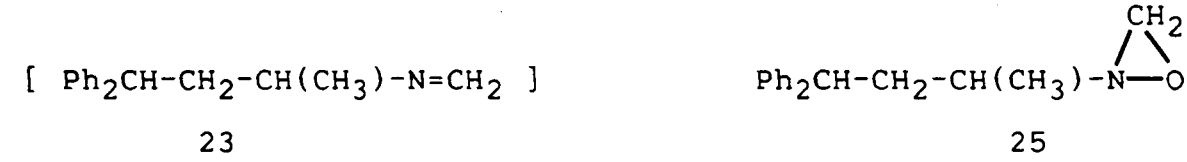
The methylene nitrone (24, $Ph_2CHCH_2CH(CH_3)-N^+(O^-)=CH_2$) was obtained from the condensation of the primary hydroxylamine (22) and formaldehyde (Slatter, 1983). Spectral results of 24 were comparable to that of a similar amphetamine nitrone (Coutts *et al.* 1978). The methylene nitrone is possibly a recipavrin metabolite which could degrade thermally or chemically to the isomeric secondary formamide (12) (see nitrone degradation studies). Because this nitrone is a possible precursor of the secondary formamide, the complete synthesis and characterization reported previously (Slatter, 1983) are included in the experimental section.

The isomeric N-methyl nitrone (44, $Ph_2CHCH_2C(CH_3)=N^+(O^-)CH_3$) was obtained from condensation of N-methylhydroxylamine

and diphenylbutanone (14). The N-methyl nitrone was isolated as a mixture of cis (Z) and trans (E) isomers, which co-eluted under standard GCMS conditions. The geometric isomers (43% cis) were apparent in the NMR spectra (figure 31,32).

F. Synthesis and Characterization of the methylene imine (23) (polymer) and oxaziridine (25)

The oxaziridine (25) has been characterized in detail (Slatter, 1983). Because the oxaziridine is a possible precursor to the formamide, the complete synthesis and spectral results were included in the experimental section.



[$Ph_2CH-CH_2-CH(CH_3)-N=CH_2$] $Ph_2CH-CH_2-CH(CH_3)-N(CH_2)-O$

23 25

The oxaziridine was synthesized by a modified method of Krimm (1958). The synthesis involves the in situ peroxidation of an unstable methylene imine (23) derived from the condensation of dinorrecipavrin (20) and formaldehyde.

The condensation products of primary amines and formaldehyde are 1,3,5-triazacyclohexanes (triazanes), 1,3-dioxa-5-aza-cyclohexanes (dioxazanes) and 1-oxa-3,5-diazacyclohexanes (oxadiazanes) (Baker, *et al.*, 1978). Analytical results, particularly molecular weight determinations, of the condensation products are often confusing (Farrar, 1968). The polymers dissociate in the

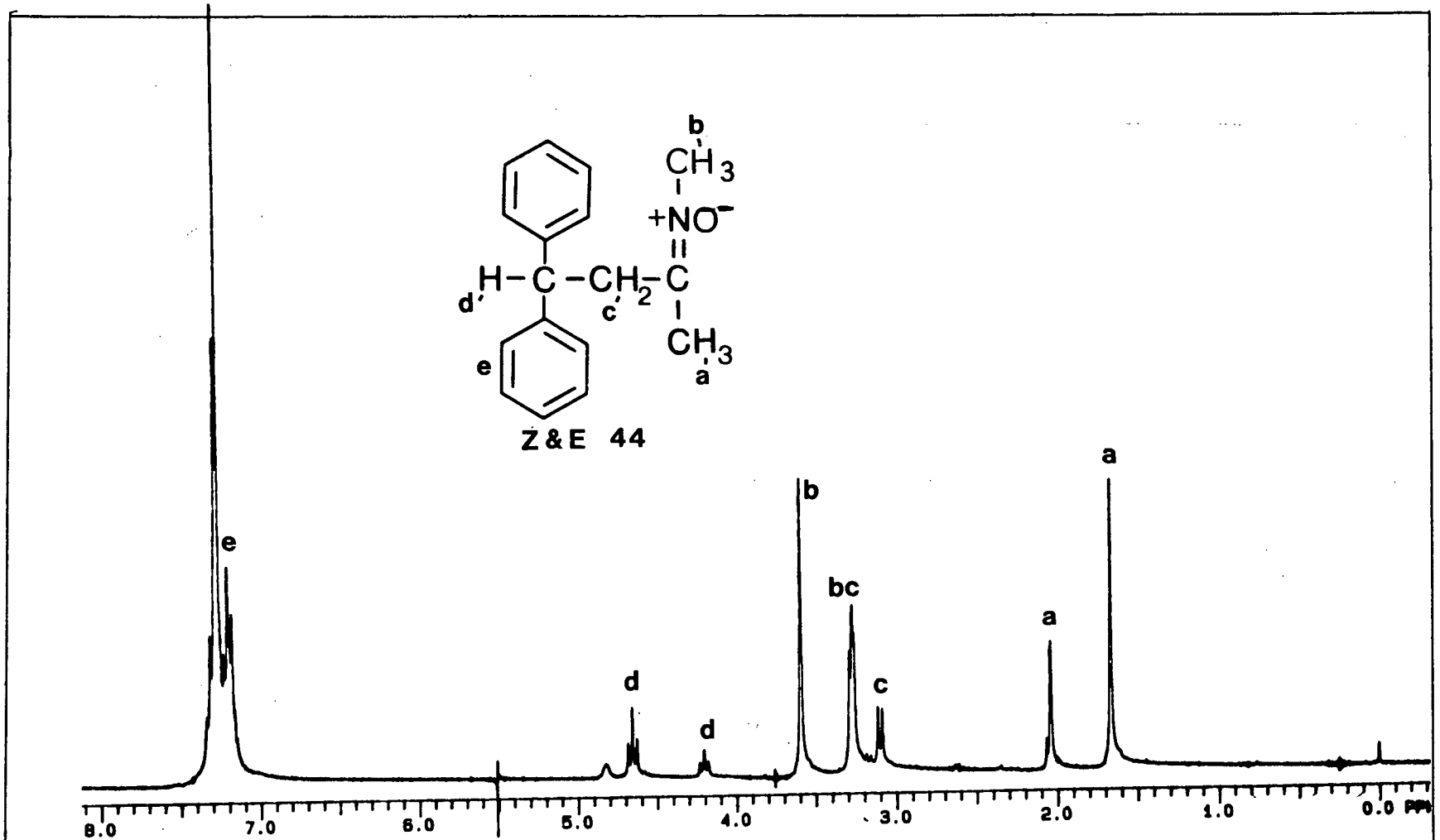


Figure 31. 300 MHz ^{1}H NMR spectrum of the N-methyl nitrone (44).

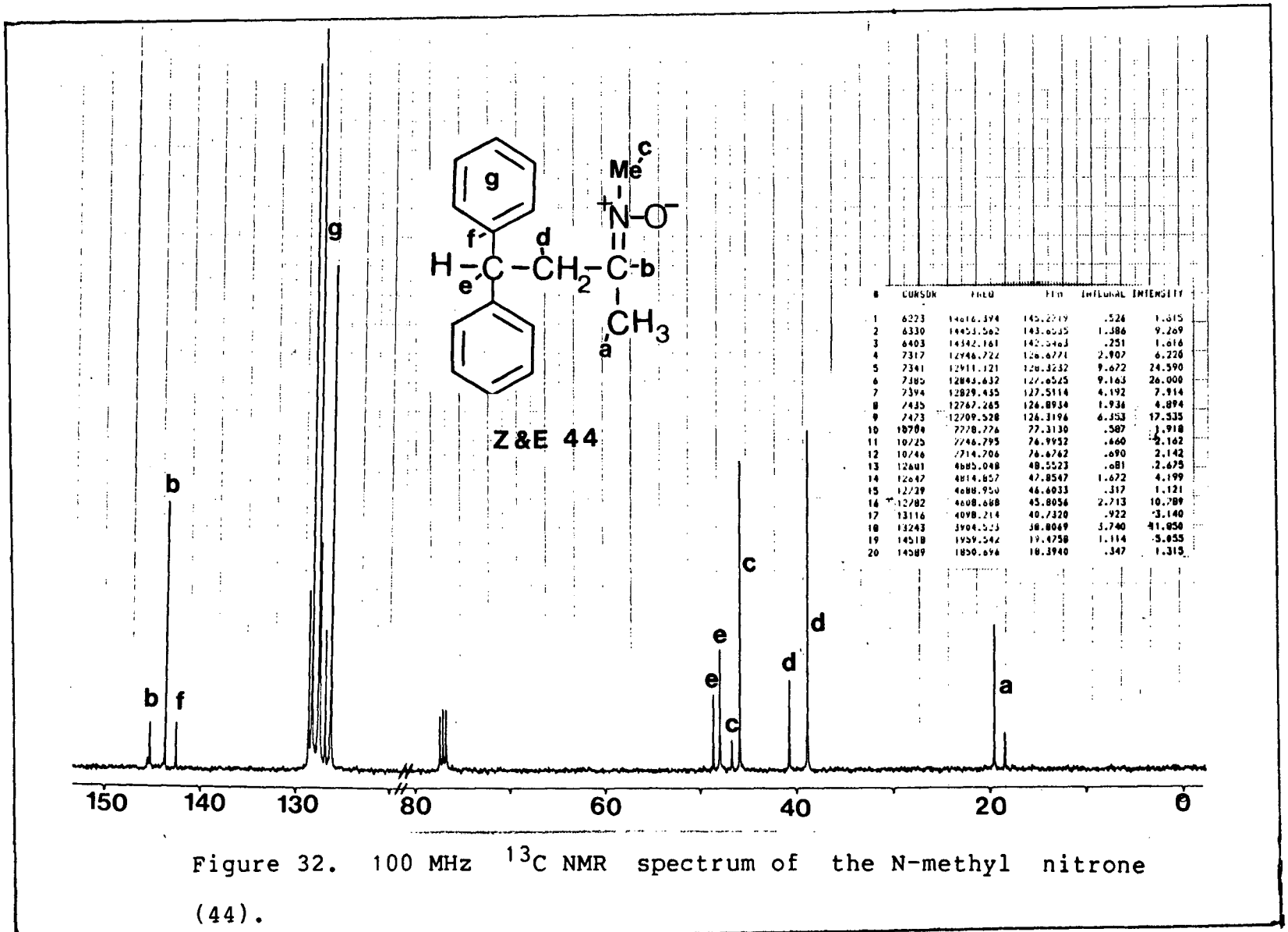


Figure 32. 100 MHz ^{13}C NMR spectrum of the N-methyl nitrone (44).

vapour phase to give a single peak by GC. Some degree of dissociation and disproportionation in solution can result in increasing amounts of the triazane plus formaldehyde (Baker, *et al.*, 1978). Ring NMR resonances occur as singlets between 3.1 and 3.6 ppm (Jones, *et al.*, 1970) consistent with rapid ring reversal and nitrogen inversion (Bushweller, *et al.*, 1974).

Emmons (1957) first described the in situ peroxidation of the triazane compounds to monomeric oxaziridines and postulated that the acidic reaction conditions apparently depolymerize the intermediate trimer, leaving the imine available for peroxidation. Reynolds and Cossar (1971) have shown that HCl salts of hexahydrotriazanes are equivalent to the methylene imine in reactivity towards nucleophiles in Mannich reactions.

Two equivalents of MCPBA were required for the in situ oxidation of the triazane (41). A combination of $CaCO_3$ and NaOH for alkalinization and perester hydrolysis respectively gave the best results. Much lower yields resulted from the use of K_2CO_3 in place of $CaCO_3$.

The N,C-diastereomers of oxaziridine (25) were separated by silica gel flash chromatography. The diastereoisomerism arises from the high inversion barrier of the oxaziridine nitrogen adjacent to the asymmetric center (Montanari, *et al.*, 1968, Biorgo and Boyd, 1973). A similar separation has been obtained for 2-(4',4'-diphenylheptan-5'-one-2'yl) oxaziridine (2)(Abbott, Slatter and Kang, 1986) and 2-[R-α-phenylethyl]-

3,3-dimethyloxaziridine (Belzecki and Mostowicz, 1975ab). The NMR spectrum of the oxaziridine was similar to that of 2-(4',4'-diphenylheptan-5'-one-2'yl) oxaziridine (2) and to 2-t-butyl oxaziridine (Crist, *et al.*, 1979). The upfield doublet (3.28 ppm and 3.46 ppm in the major and minor diastereomers of 25 respectively) is presumed to be the proton trans to the lone pair on nitrogen (Boyd, *et al.*, 1969, Jordan and Crist 1977).

The isomerization of oxaziridines to amides is well documented (Emmons, 1957, Lattes, *et al.*, 1982). Methylene oxaziridines afford formamides by thermal or Fe^{2+} mediated mechanisms. The formamide (12) in this study was obtained from the oxaziridine (25) on standing at room temperature or as a result of complete thermal degradation in the GC inlet. The identity of the product formamide derived from the oxaziridine by GC analysis was confirmed by comparison of mass spectra and NMR spectra of a peak collected off the GC column with authentic formamide synthesized by reaction of dinorrecipavrin (20) with ethyl formate (Slatter, 1983).

The synthetic method used here and the lability of the oxaziridine product indicates that a literature method for the synthesis of formamides from primary amines, formaldehyde and hydrogen peroxide (Seng and Ley, 1975) probably involves an oxaziridine intermediate which decomposes during distillation or sample workup.

G. Synthesis and Characterization of the isocyanide (65) and carbamates (66-69)

The isocyanide (65, $Ph_2CH-CH_2-CH(CH_3)-N\equiv C$) and carbamates (66-69) were synthesized as reference compounds for studies on possible formyl chloride or phosgene mediated artifacts that could arise from chloroform extraction of recipavrin metabolites.

$Ph_2CH-CH_2-CH(CH_3)-N(R_1)C(=O)OR_2$

66 $R_1=H$, $R_2=CH_3$

67 $R_1=H$, $R_2=CH_2CH_3$

68 $R_1=CH_3$, $R_2=CH_3$

69 $R_1=CH_3$, $R_2=CH_2CH_3$

The isocyanide had a strong C=N- stretch in the infrared at 2150 cm^{-1}. The NMR spectrum had resonances corresponding to the diphenylbutyl backbone. The isocyanide decomposed during GC analysis, however one component had the correct molecular weight (235 amu) with few other diagnostic ions.

The ethyl carbamate analogue of recipavrin (69) has been described previously as a test compound for derivatization of tertiary amines (Hartvig and Vessman, 1974). Spectral data was not supplied.

The mass spectra of ethyl and methyl carbamates of norrecipavrin and dinorrecipavrin had weak molecular ions and were dominated by the benzylic cleavage (m/z 167), and fragmentation of the C-C bond of the side chain to afford ions at m/z 130 and 116 for the norrecipavrin ethyl and methyl

carbamates (69,68) and m/z 116 and 102 for the dinorrecipavrin ethyl and methyl carbamates (67,66) respectively.

The NMR, mass and IR spectra were in accord with the carbamate structures and are included in the appendix.

H. Attempted synthesis and characterization of the formohydroxamic acid (61)

The hydroxamic acid (61, $Ph_2CH-CH_2-CH(CH_3)-N(OH)CHO$) was considered briefly as a precursor to the formamide on the basis that hydroxamic acid metabolites of aromatic amines are well known and the hydroxamic acid functional group is glucuronide conjugable. A weak point in the hypothesis was the mechanism whereby the hydroxamic acid could degrade to the formamide without a reduction step. The hydroxamic acid was also a possible precursor of an unidentified recipavrin metabolite that had a high mass ion at m/z 251 and base peak m/z 71 at relatively long retention time. Dehydration of the hydroxamic acid (M^+) in the source would be expected to result in these mass spectral characteristics. The Lossen rearrangement of hydroxamic acids to isocyanates is a similar transformation.

The synthesis was attempted by transformylation of the primary hydroxylamine (22) with ethyl formate. The results were not satisfactory. After derivatization with TMAH only one minor component in the mixture had a satisfactory mass spectrum for the methyl ether $(Ph_2CH-CH_2-CH(CH_3)-N(OCH_3)CHO$. A weak molecular ion at m/z 283 eliminates CH_3OH to give m/z 251. Presumably cleavage of the C-C bond adjacent to nitrogen in the 251 ion affords the base peak at m/z 72 (figure 33). In the infrared spectrum of the underivatized product there was a strong broad OH stretch between 3600 and 3000 cm^{-1}, and a carbonyl stretch at 1665 cm^{-1}. Both bands were diagnostic for the hydroxamic acid functional group (Socrates, 1980). Since no secondary formamide or m/z 251 compound was observed when the underivatized product was analyzed by GCMS, further purification and characterization of 61 was abandoned.

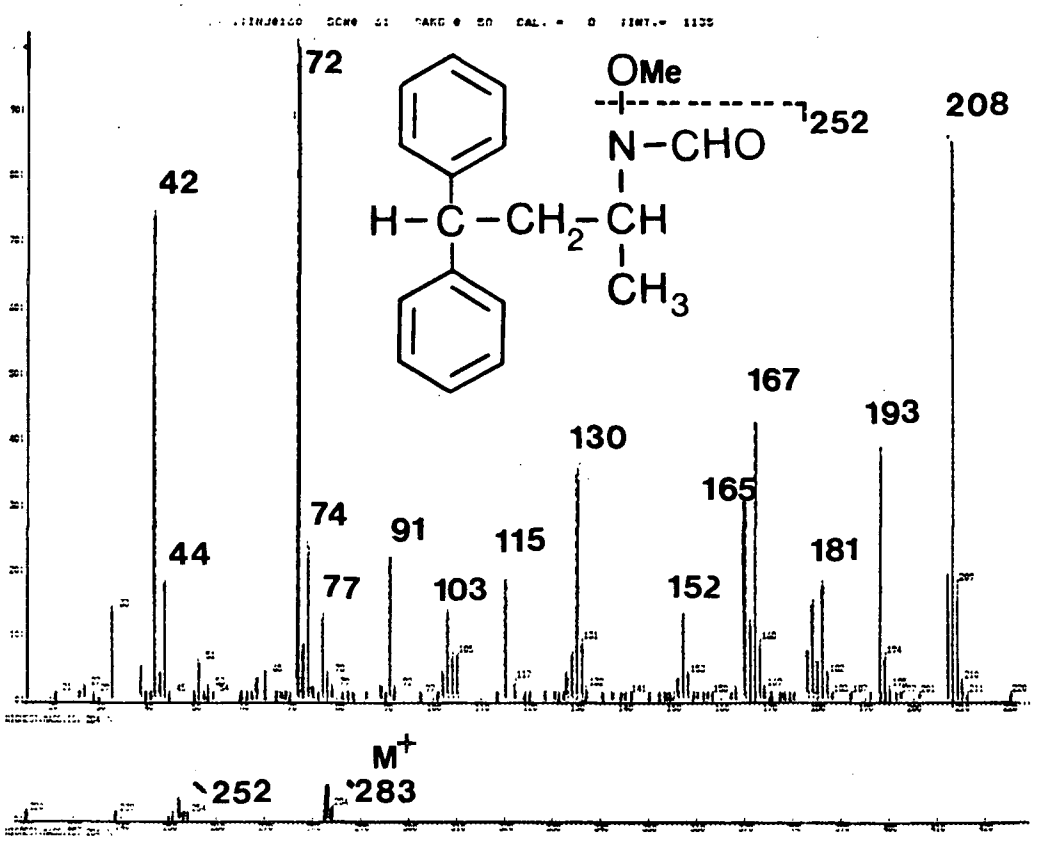
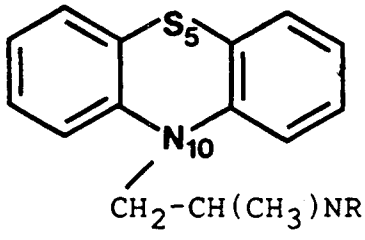


Figure 33. Mass spectrum of the suspected methyl ether of the formohydroxamic acid (61).

I. Synthesis and Characterization of the promethazine related formamides and their oxidation products.

The secondary formamide (72) was synthesized as a potential metabolite of the antihistamine promethazine (10)(see section 8 for mass spectral results of compounds in this section). The tertiary formamide (74) was synthesized for use in metabolism studies on arylaliphatic formamides.



10 R_1=CH_3, R_2=CH_3

72 R_1=H, R_2=CHO

74 R_1=CH_3, R_2=CHO

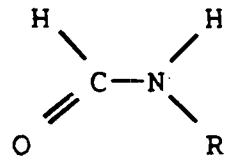
75 R_1=H, R_2=CHO, $^{+}N_{10}$-O^{-}

76 R_1=CH_3, R_2=CH_3, $^{+}N_{10}$-O^{-}

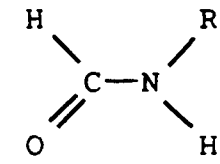
The secondary and tertiary formamide analogues of promethazine were synthesized by Leuckart reaction of 10-(2-propanone) phenothiazine with formamide and N-methyl formamide respectively.

In the secondary formamide (72) cis and trans rotamers were apparent in the proton and ^{13}C NMR spectra (figure 34 and 35). Cis and trans structures shown below were made according to LaPlanche and Rogers (1964). The formyl resonance appeared as a broadened singlet at 8.10 ppm for the trans rotamer and as a doublet at 7.88 (J=12 Hz) for the cis rotamer much like the secondary formamide analogues of recipavrin and methadone

(12 and 6 respectively). The cis isomer accounted for 20% of the total formamide. The literature value for the cis NH-CHO proton coupling constant of isopropylformamide in acid solution is 13.8 Hz. Trans NH-CHO couplings are approximately 2 Hz in the lower formamides. The larger NH-CHO proton coupling in the cis isomer reflects a trans relationship between the NH and CHO protons (Laplanche and Rogers, 1964). The partially overlapped NH resonances were found between 5.5 and 5.7 ppm and were broadened by the quadrupole relaxation of the ^{14}N nucleus. The relative amount of cis isomer in the secondary formamides increases with the bulk of the nitrogen substituent.



Trans (major)



Cis (minor)

The rotational isomerism arises from restricted rotation about the N-C=O bond (Laplanche and Rogers, 1964) which gives rise to hydrogen bonded cis dimers and trans polymers. As a result of hydrogen bonding, spectra are subject to concentration induced shifts. In the ^{13}C NMR spectrum trans and cis formyl resonances appeared at 161.04 and 163.81 ppm respectively. In the infrared spectrum (figure 36), the NH (3432 and 3400 cm^{-1}) and C=O (1692 and 1681 cm^{-1}) stretches were distinct for each rotamer in accord with literature IR data (Laplanche and Rogers, 1964, Hallam and Jones, 1970). A

pyrimidine (73) was identified by GCMS as a major byproduct of the reaction.

Peroxidation of the secondary formamide (72) with hydrogen peroxide to form the N_{10}-oxide (75) resulted in a compound with only one rotamer apparent by NMR (figure 37 and 38). The NH resonance was deshielded and resolved into a broad doublet at 6.61 ppm. The formyl proton resonance appeared as a doublet (J= 8.5 Hz) centered at 7.85 ppm suggesting a preference for the cis conformation. The large downfield shift of the CH_2 side chain resonance (one peak at 4.62 ppm from separate resonances at 3.8 and 3.95 ppm) and the N-O stretch at 1010 cm^{-1} in the infrared spectrum support the N_{10} oxide structure over that of the sulfoxide (figure 39).

The tertiary formamide (74) was also synthesized and characterized as a 3:1 mixture of cis and trans rotamers by NMR (figure 40). The IR (figure 41) and GCMS results are also shown (figure 42).

Promethazine N-oxide (76) has been described previously and characterized by mass spectrometry (Clement and Beckett, 1981b). This was a necessary reference compound for metabolism studies so it was synthesized and characterized by NMR, mass and IR spectrometry.

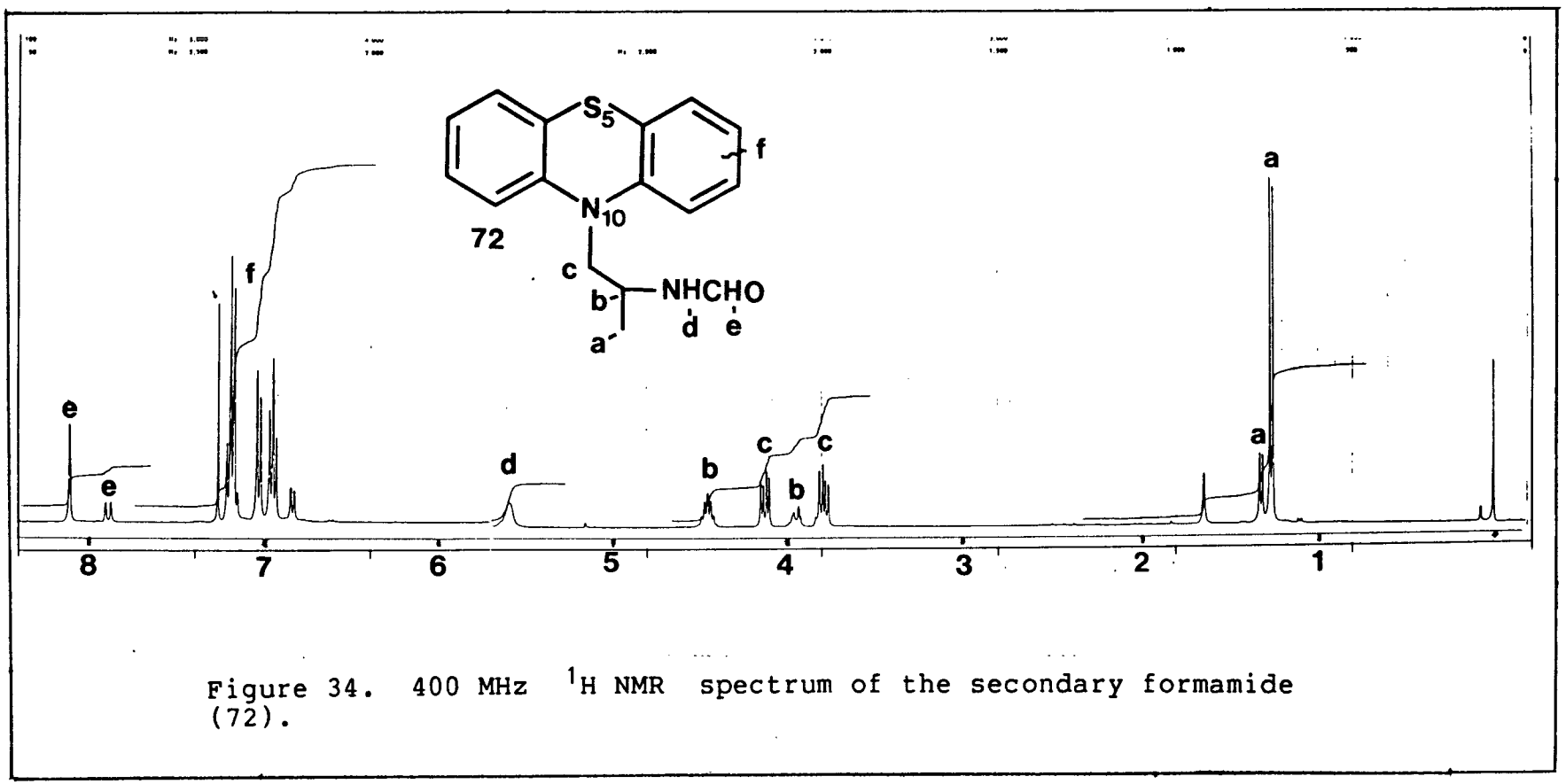


Figure 34. 400 MHz ^{1}H NMR spectrum of the secondary formamide (72).

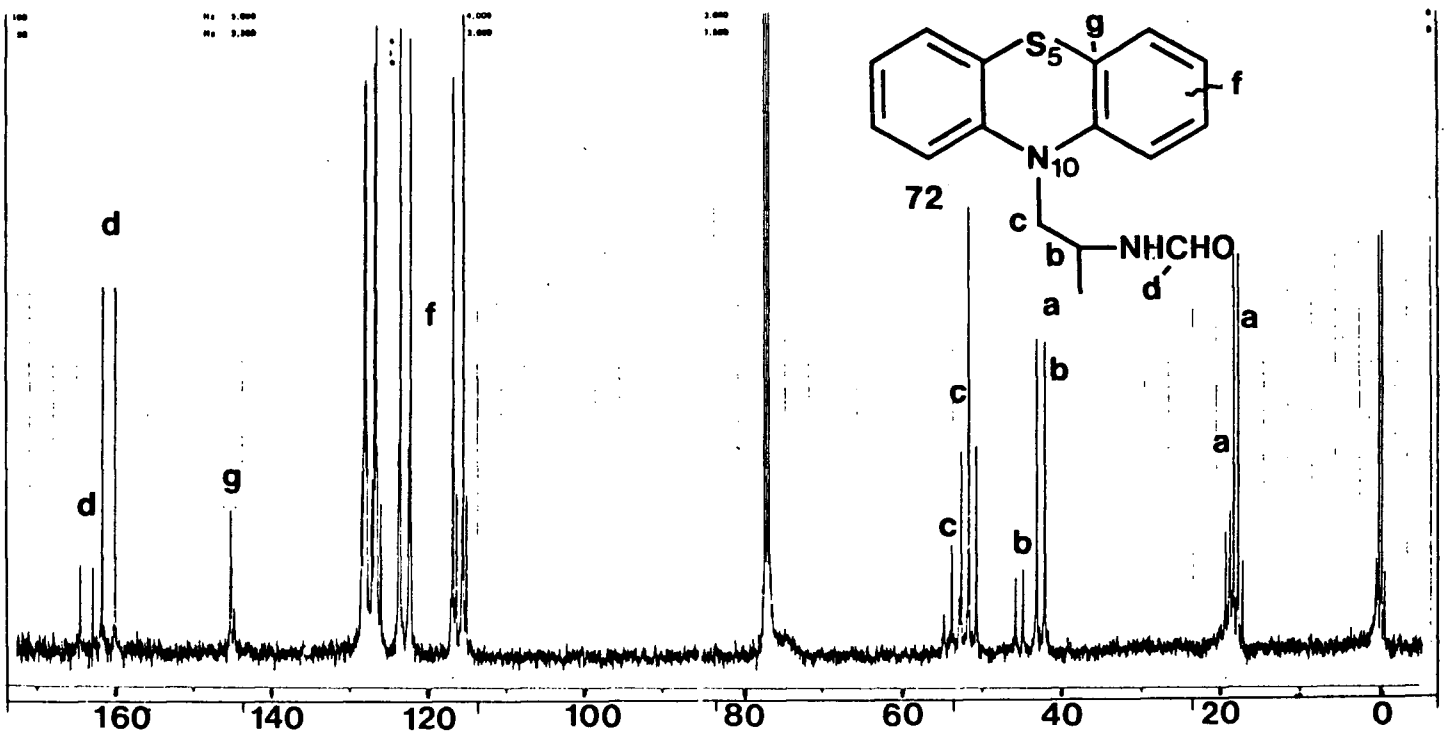


Figure 35. 100 MHz ^{13}C NMR SFORD spectrum of the secondary formamide (72).

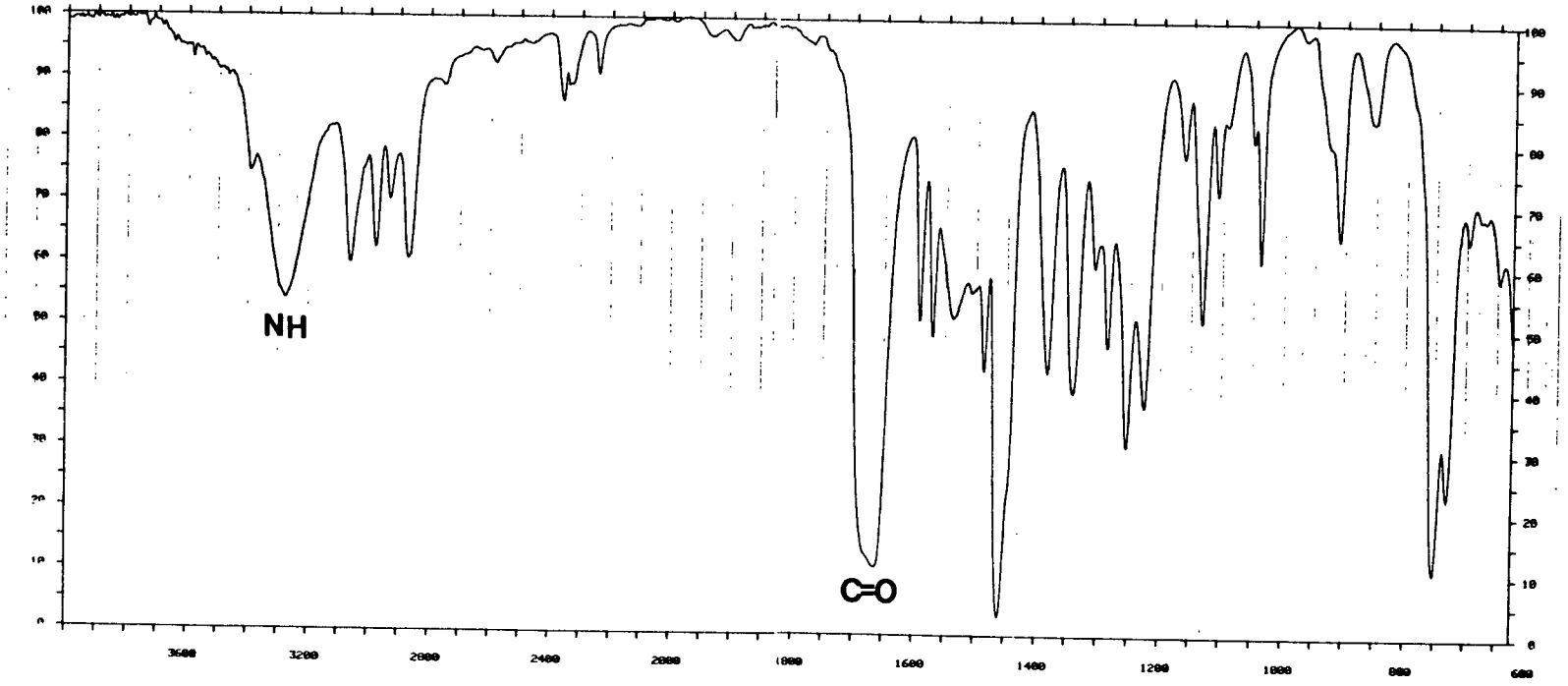


Figure 36. FTIR spectrum (film) of secondary formamide (72).

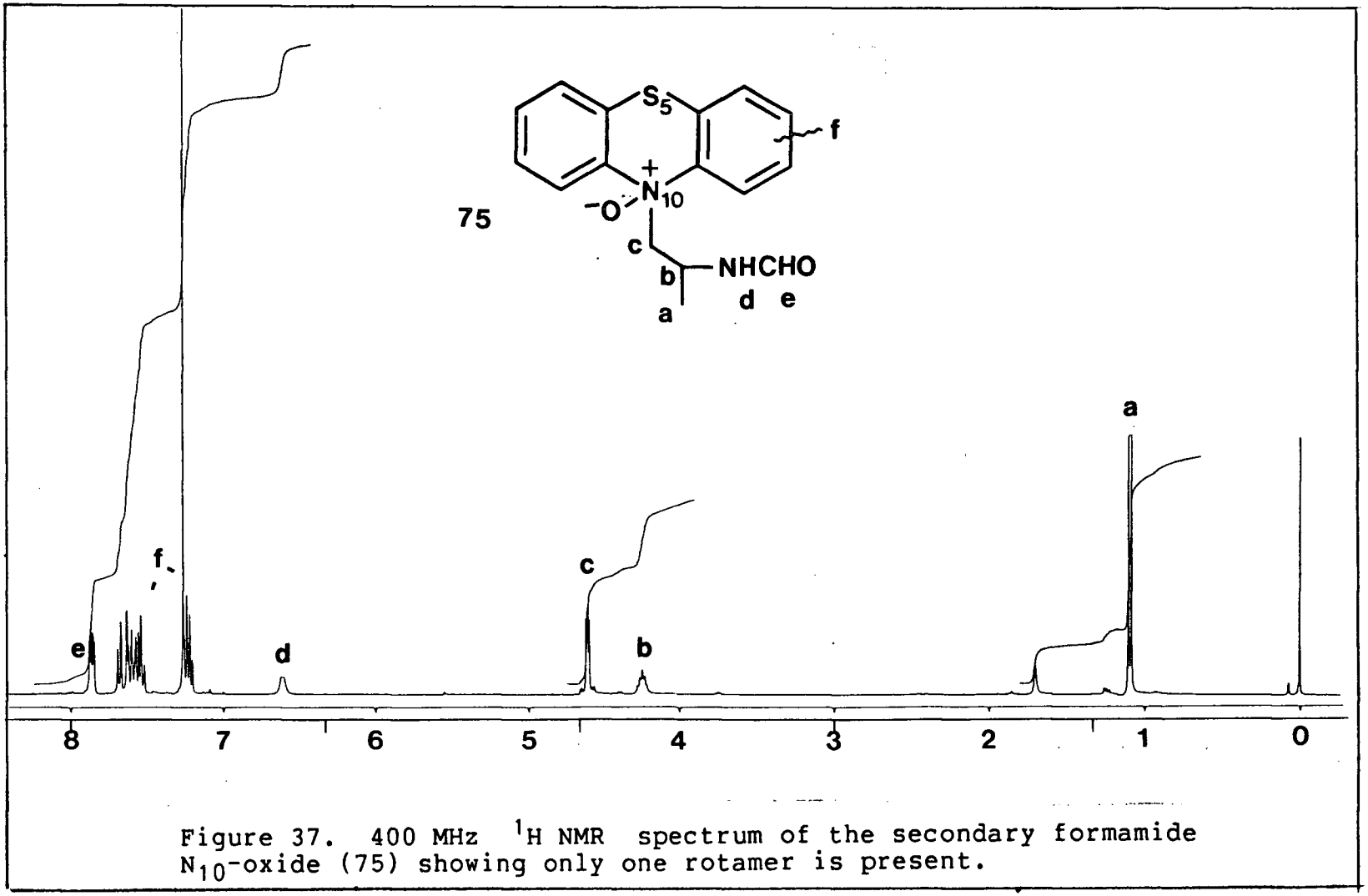
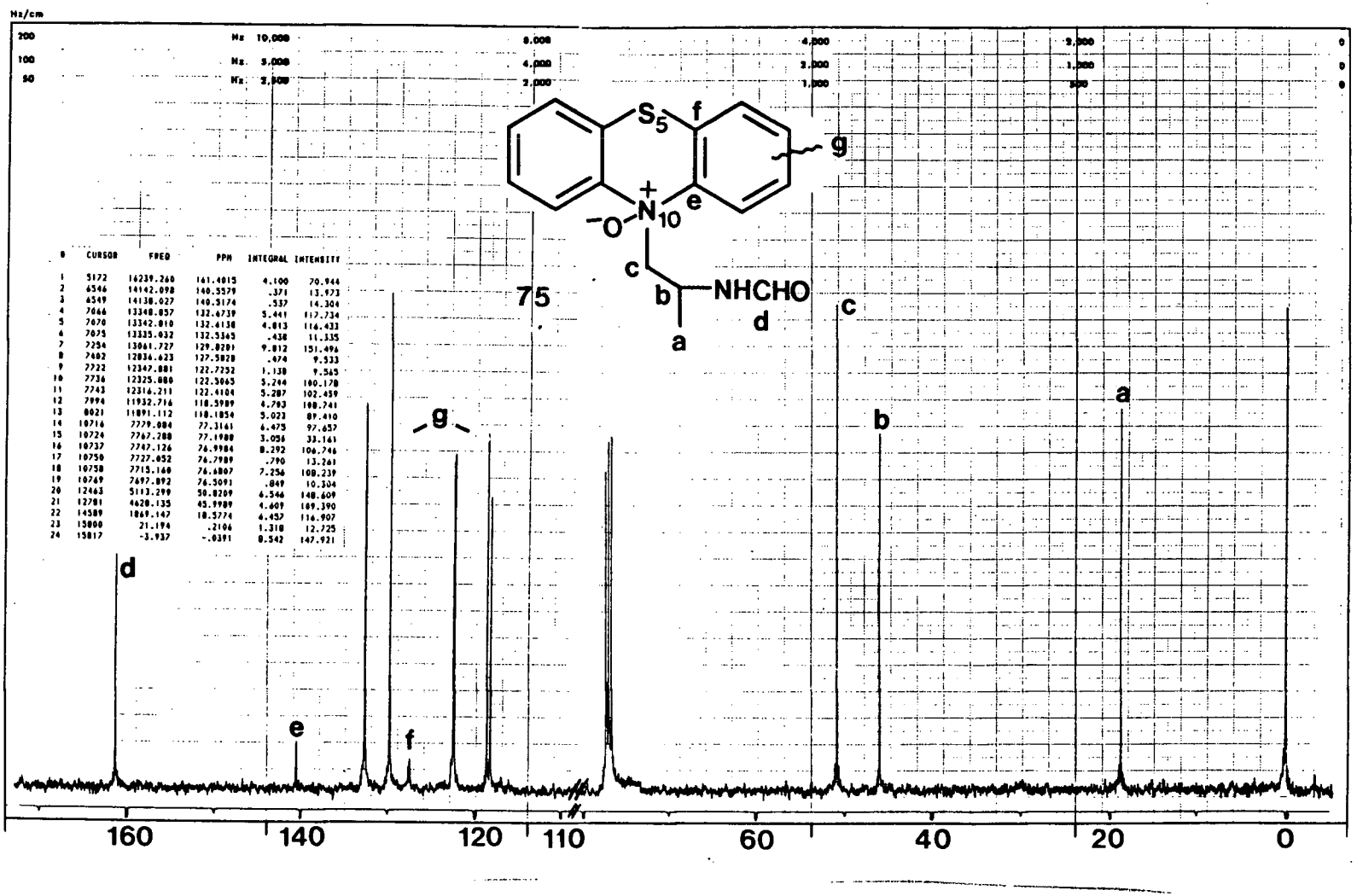


Figure 37. 400 MHz ^{1}H NMR spectrum of the secondary formamide N_{10}-oxide (75) showing only one rotamer is present.



#	CURSOR	FREQ	PPM	INTEGRAL	INTENSITY
1	5172	16239.260	161.4015	4.100	70.944
2	6546	14142.098	140.5579	.371	13.973
3	6549	14138.027	140.5174	.537	14.304
4	7046	13348.857	132.6739	5.441	117.734
5	7070	13342.810	132.4138	4.813	116.433
6	7075	13335.032	132.5365	.438	11.335
7	7294	13061.727	129.8291	9.812	151.496
8	7402	12834.623	127.5828	.474	9.533
9	7722	12347.881	122.7252	1.138	9.565
10	7736	12325.880	122.5065	5.244	100.178
11	7743	12314.211	122.4104	5.287	102.459
12	7994	11932.716	118.5989	4.793	108.741
13	8021	11891.112	118.1854	5.023	89.410
14	10716	7779.084	77.3161	4.475	97.657
15	10724	7767.288	77.1988	3.056	33.141
16	10737	7747.126	76.9984	8.292	106.746
17	10750	7727.052	76.7989	.790	13.261
18	10758	7715.160	76.6807	7.256	108.239
19	10769	7697.892	76.5091	.849	10.304
20	12463	5113.299	50.8209	4.546	148.609
21	12781	4628.135	45.9989	4.469	109.390
22	14589	1869.147	18.5774	4.457	116.907
23	15000	21.194	.2106	1.318	12.725
24	15017	-3.937	-.0391	8.542	147.921

Figure 38. 100 MHz broad band decoupled ^{13}C NMR spectrum of the secondary formamide N_{10}-oxide (75).

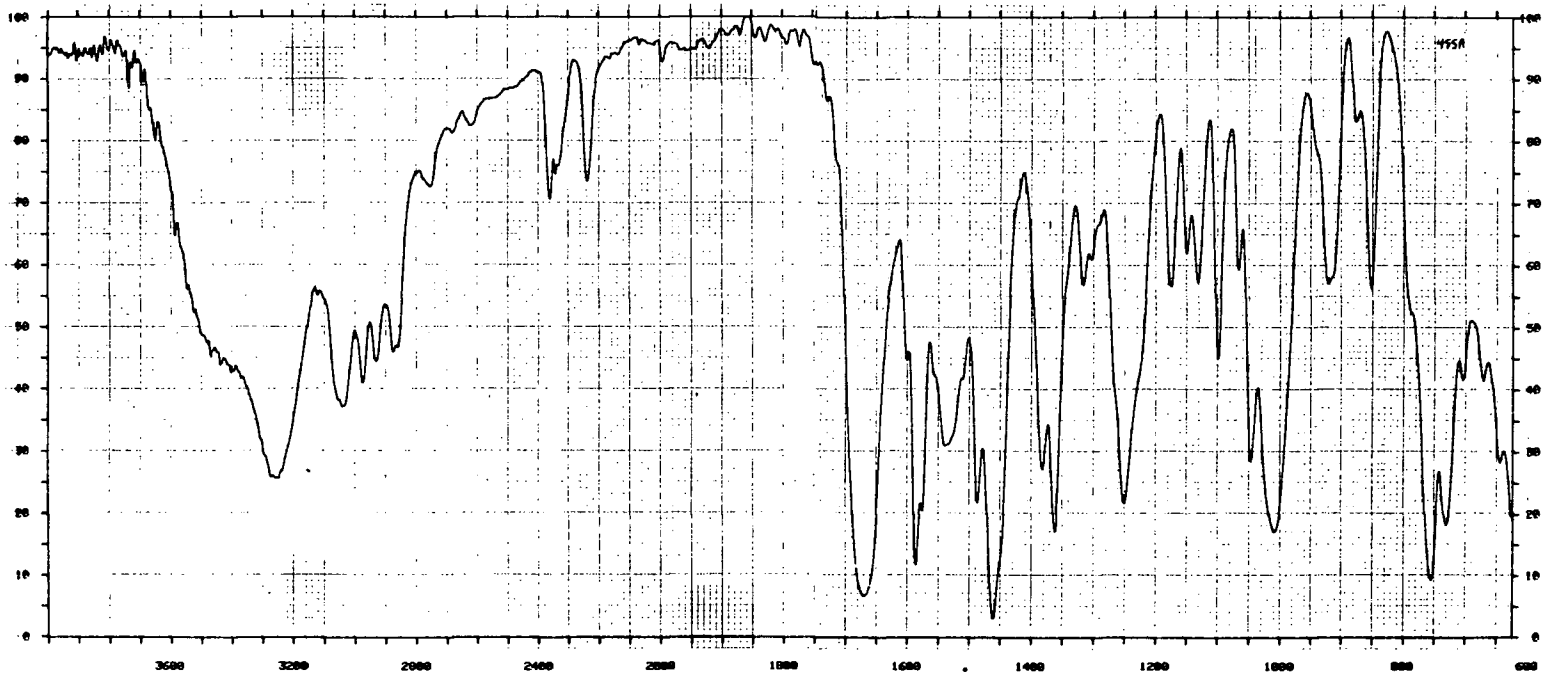


Figure 39. FTIR spectrum (film) of the secondary formamide N_{10}-oxide (75).

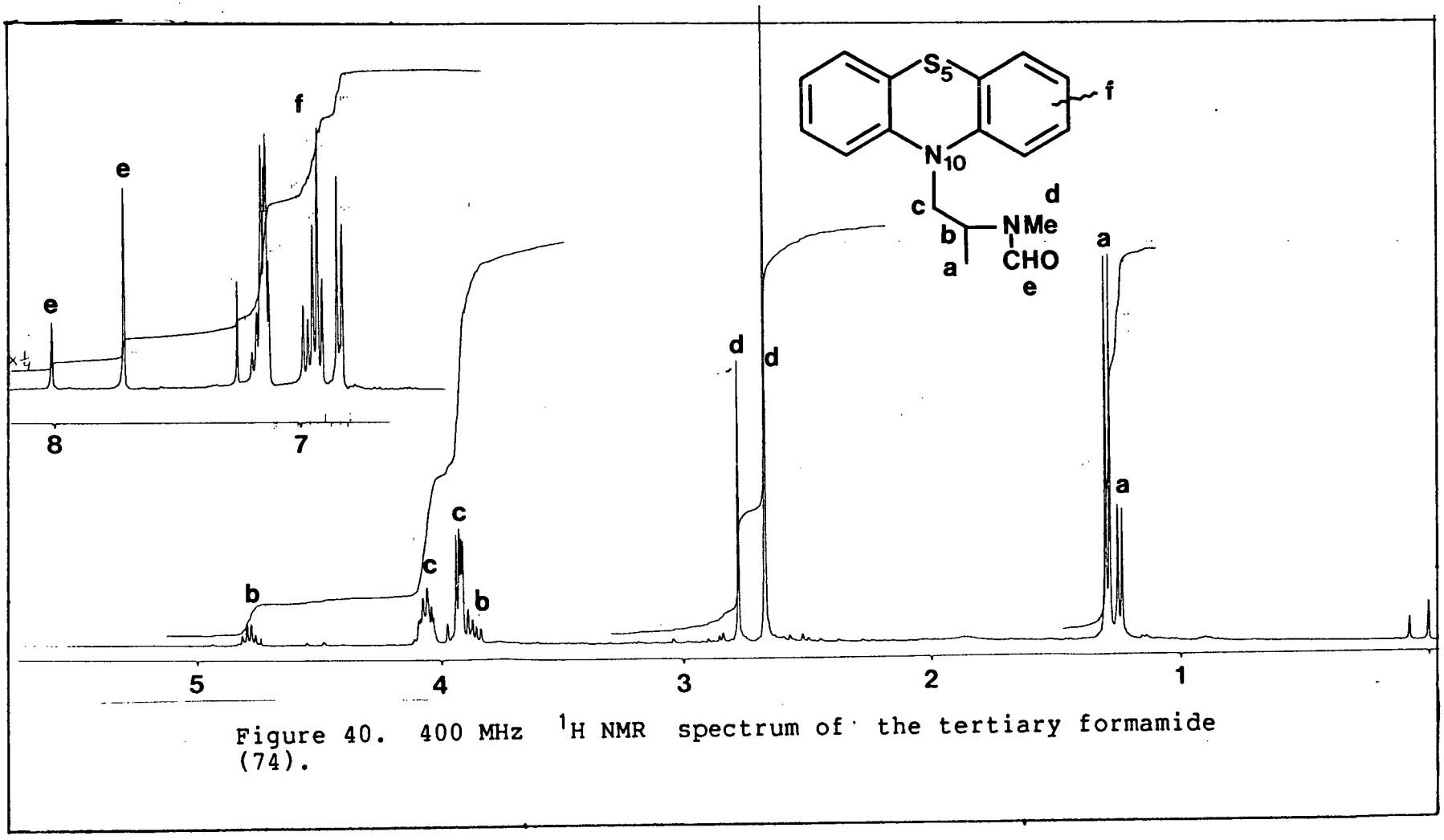


Figure 40. 400 MHz ^{1}H NMR spectrum of the tertiary formamide (74).

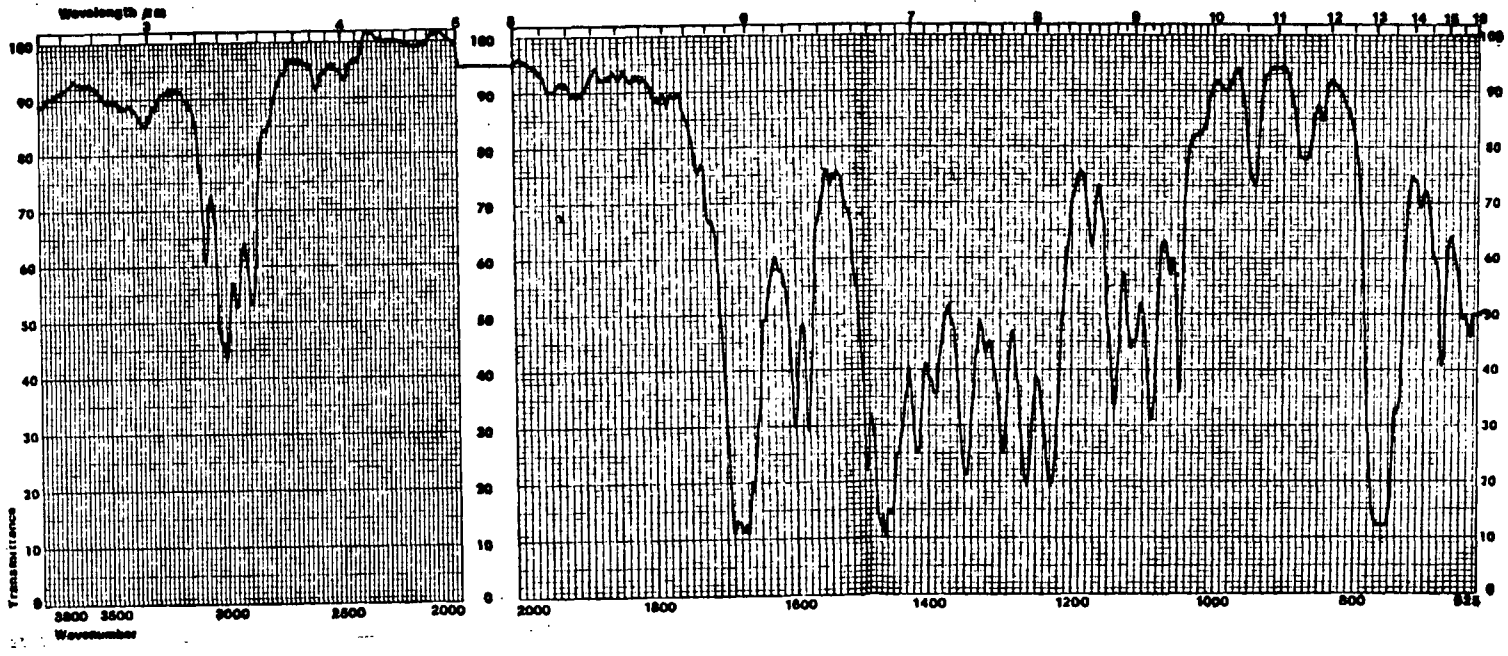


Figure 41. IR spectrum of a thin film of the tertiary formamide (74).

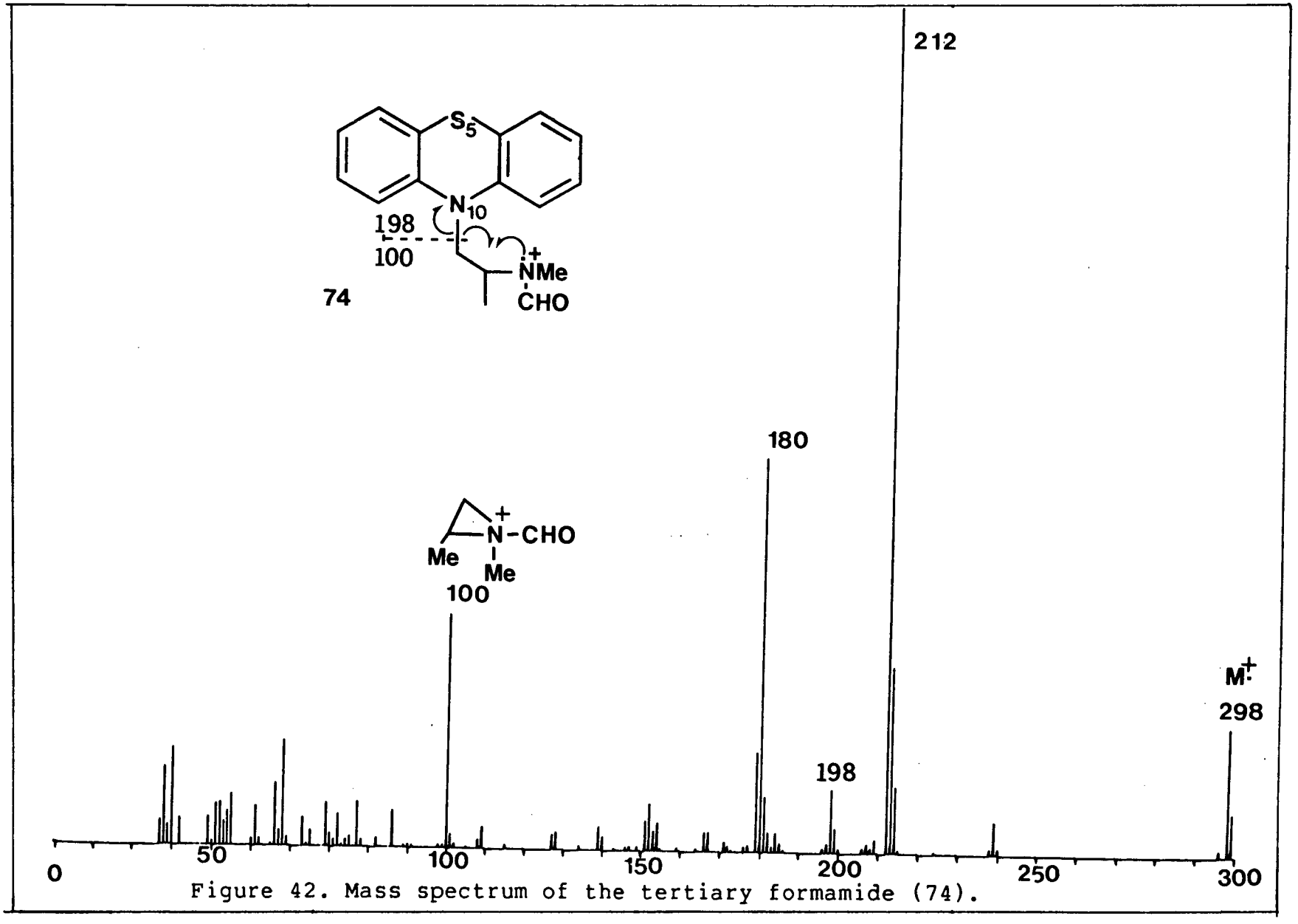


Figure 42. Mass spectrum of the tertiary formamide (74).

J. Synthesis and Characterization of the oxaziridine (82), methylene imine (78) and formamide (83) related to amphetamine

The methylene imine (78, $PhCH_2CH(CH_3)N=CH_2$) had similar spectral characteristics to the recipavrin imine (23, $Ph_2CHCH_2CH(CH_3)N=CH_2$). In the mass spectrum, the m/z 56 base peak for the monomer is one mass unit less than for the recipavrin imine. This implied that alpha cleavage was dominant. In the recipavrin imine, the availability of a gamma proton allowed a McLafferty rearrangement giving a m/z 57 base peak (Slatter, 1983). In the NMR spectrum (figure 43), the triazane (79), oxadiazane (80), and dioxazane (81) were distinct and present in a ratio of 3:2:5. Side chain resonances overlapped, with the exception of the CH_3 doublet of each component. Singlets for the ring protons were assigned based on ratios of integrated peaks. When the spectrum was rerun at 60°, the methyl and triazane ring resonances at 1.02 (d) and 3.75 (s) respectively disappeared and were replaced by singlets at 2.85 (methyl) and 2.77 (triazane ring) ppm. The other two components were unchanged by heating. Although temperature induced changes in the conformation of triazanes have been investigated (Bushweller *et al.* 1974), these results are not easily explained.

The amphetamine oxaziridine (82) was isolated in very low

yield from the peroxidation of the triazane (79). The product was characterized by NMR (figure 44). Two diastereomers were present in a ratio of 1.23:1. The oxaziridine ring resonances were present as pairs of doublets (J=10 Hz) centered at 3.65 and 4.00 ppm in the major diastereomer and 3.18 and 3.7 ppm in the minor diastereomer. The oxaziridine decomposed on standing and in the GC to the isomeric secondary formamide (83).

The secondary formamide (83, $PhCH_2CH(CH_3)NHCHO$) is well known as a Leuckart specific impurity of illegal amphetamine synthesis (Frank, 1983). The formamide was synthesized as a reference compound for comparison to the decomposition product of the isomeric oxaziridine (82). The mass spectrum of the formamide had a base peak at m/z 72 like the methadone secondary formamide but unlike the recipavrin secondary formamide which had a base peak m/z 73. This result supports the gamma proton transfer mechanism for fragmentation of the recipavrin secondary formamide (see section 3). The amphetamine and methadone formamides (83 and 6 respectively) lack the gamma proton and presumably fragment by cleavage of the alpha C-C bond.

The NMR spectrum of the formamide (83) showed cis and trans rotamers and was in accord with the results reported by Hess *et al.*, 1976 (figure 45).

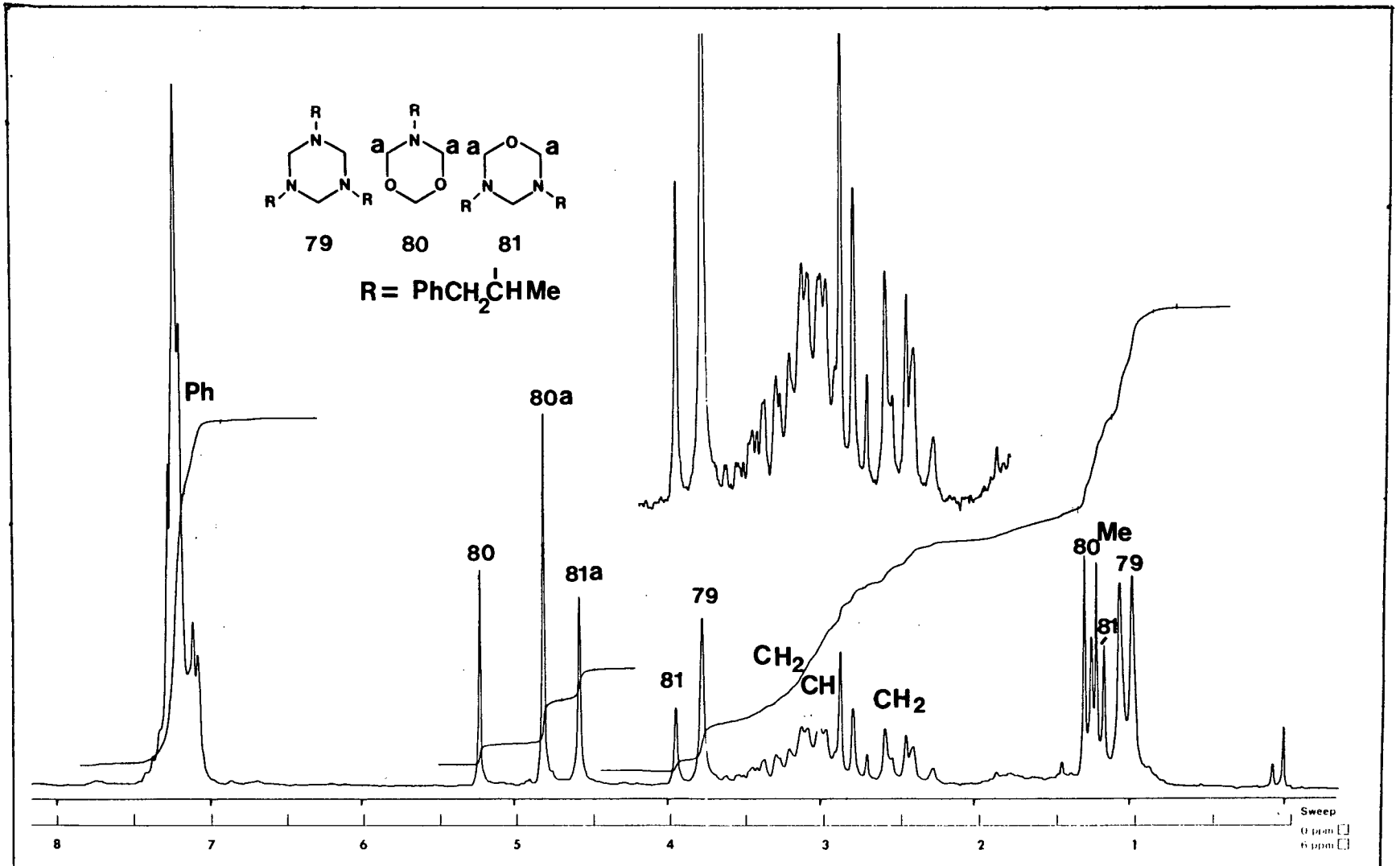


Figure 43. 80 MHz NMR spectrum of the amphetamine methylene imine derived polymers (79-81).

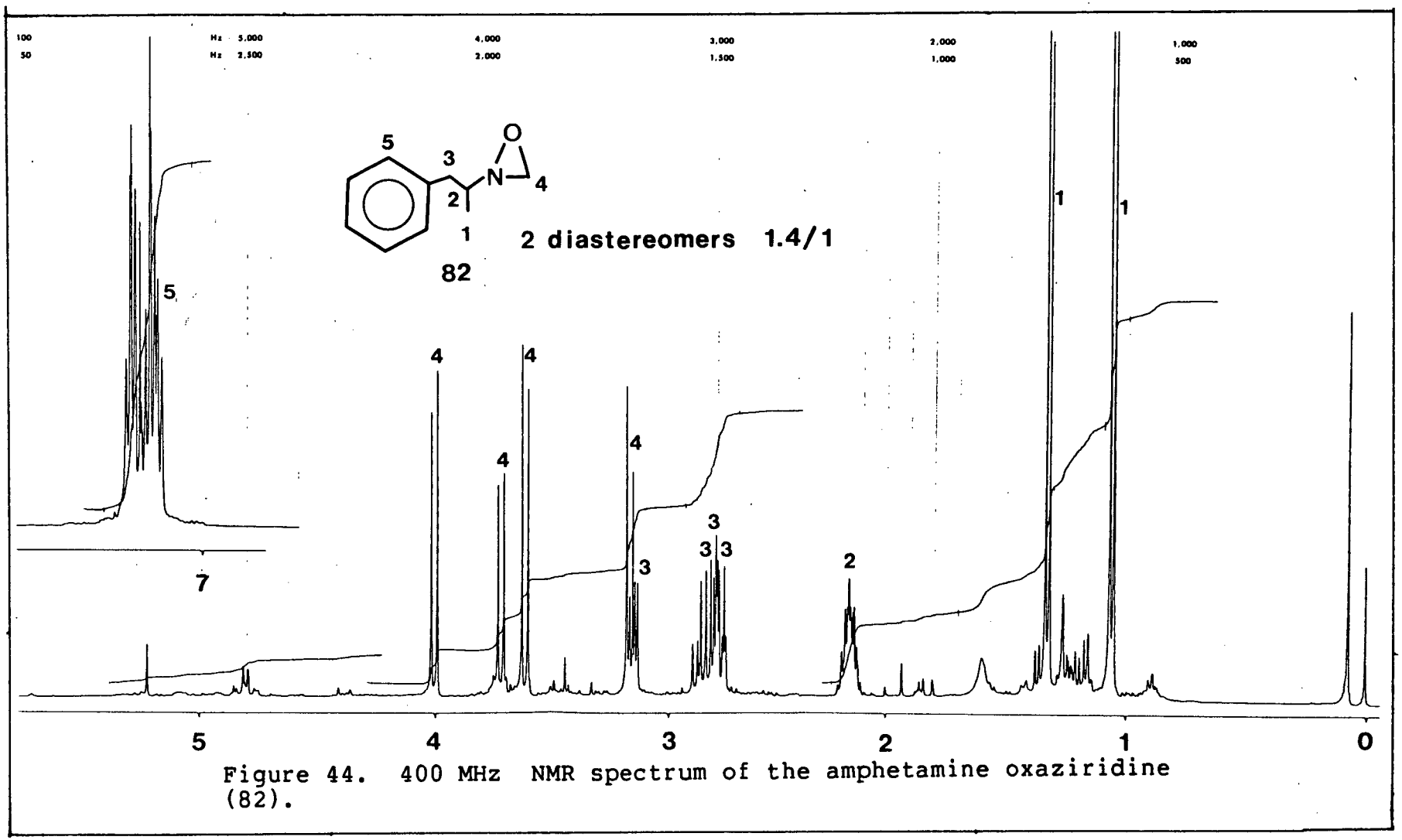


Figure 44. 400 MHz NMR spectrum of the amphetamine oxaziridine (82).

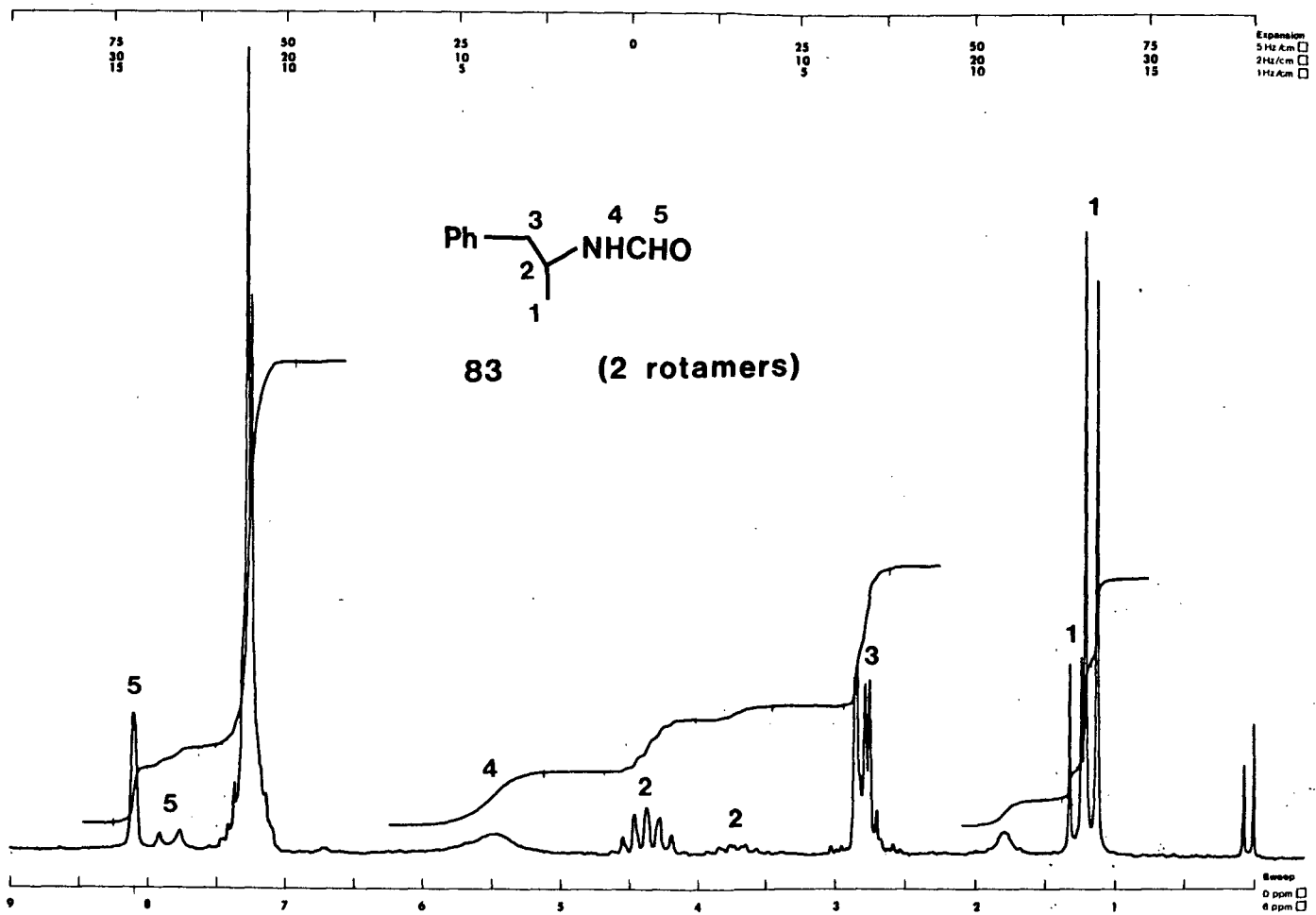


Figure 45. 80 MHz NMR spectrum of the amphetamine secondary formamide (83).

3. MASS SPECTROMETRY OF RECIPAVRIN RELATED COMPOUNDS

A.Fragmentation of the aliphatic side chain

The choice of a major fragmentation pathway in recipavrin (9) and other arylaliphatic compounds is determined by charge localization on either the phenyl rings or the heteroatom portion of the molecule. While pathways arising from both possible ionizations are always evident, factors such as electron density at nitrogen, or whether nitrogen or oxygen is singly or doubly bonded to the side chain, determine which of the two pathways predominate.

Generally a single bonded, basic nitrogen atom favors radical site initiation at N with alpha cleavage of the largest alkyl chain (Stevenson's Rule)(McLafferty, 1980). This cleavage generates base peak cations of the general formula A (figure 46, R_1,R_2=H or CH_3). Molecular ions and arylaliphatic fragments (m/z 208, 193, 179, 167, 165, 152, 130, 105, 91 etc.) are of low intensity as in norrecipavrin (figure 50 top).

When electron density decreases at the C-N single bond, as in the secondary formamide analogue of recipavrin,(R_1=H, R_2=CHO, Figure 50 bottom), a protonated alpha cleavage ion (m/z 73) is present as the base peak along with a less intense alpha cleavage ion. Rearrangement of the gamma hydrogen to the amide nitrogen with adjacent C-N cleavage and charge retention gives rise to the 1-methyl-2,2-diphenylcyclopropane cation

radical (m/z 208, figure 47) which can fragment to the diarylaliphatic series of ions (m/z 208, 193, 179, 167, 165, 152, 130, 105, 91,) that have been described in work on methadone (Kang, *et al.*,1979). Diarylaliphatic fragments in this series increase in intensity with decreasing mass to a maximum at the diphenylmethine cation (m/z 167).

In compounds containing nitrogen or oxygen doubly bonded to the diphenylbutane skeleton, i.e. diphenylbutanone (14, $Ph_2CH-CH_2-C(CH_3)=O$) and diphenylbutanone oxime (19, $Ph_2CH-CH_2-C(CH_3)=NOH$), the base peak arises by radical site initiation on the aromatic ring, with allylic (benzylic) cleavage to give a resonance stabilized diphenylmethine cation (m/z 167) (figure 48) (Noren *et al.*, 1985). Benzylic cleavage may compete with the McLafferty rearrangement shown in figure 49 especially in the phenyl ring oxidized diarylalkyl metabolites which form stable oxonium ions. Some alpha cleavage by radical site initiation at the unsaturated hetero atom occurs in diphenylbutanone and it's derivatives giving a m/z 43 peak. The oxime loses an OH radical to give M-17 ions (m/z 222).

Regardless of the intensity of fragments produced by cleavage alpha to the hetero atom, the diphenylmethyl cation (Ph_2CH^+) is always present and is the key ion in the detection of recipavrin metabolites with intact phenyl rings.

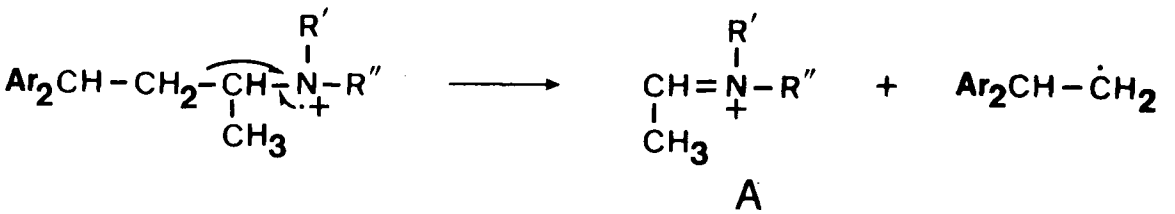


Figure 46. General mass spectral fragmentations of diarylbutanes: I. Radical site initiation at the saturated heteroatom with alpha cleavage. This gives rise to base peak in compounds with basic nitrogen atoms.

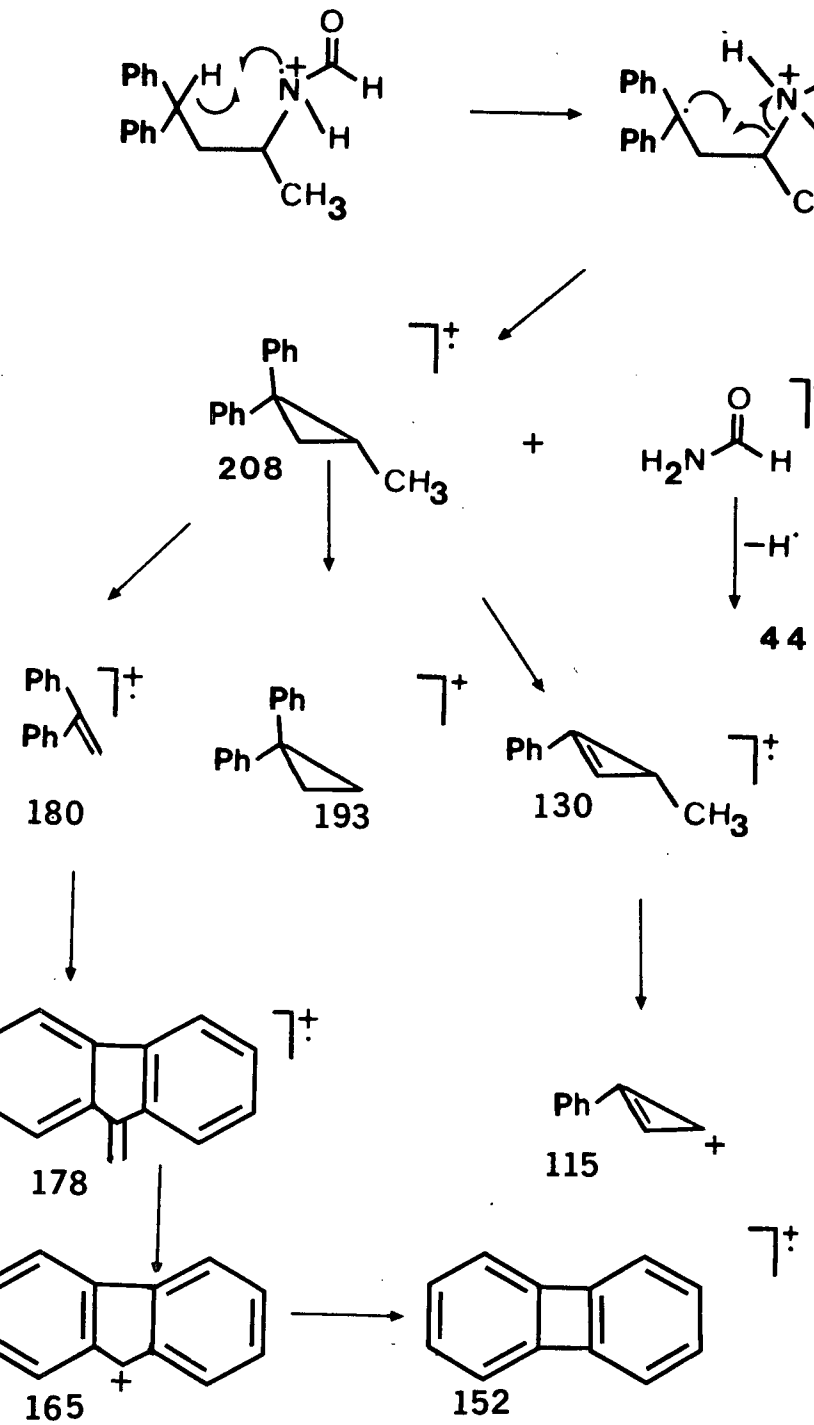


Figure 47. General mass spectral fragmentations of diarylbutanes: II. Rearrangement of the gamma hydrogen to the saturated hetero atom with adjacent cleavage and charge retention. This gives rise to m/z 208 cascade common to all phenyl ring intact metabolites.

a.

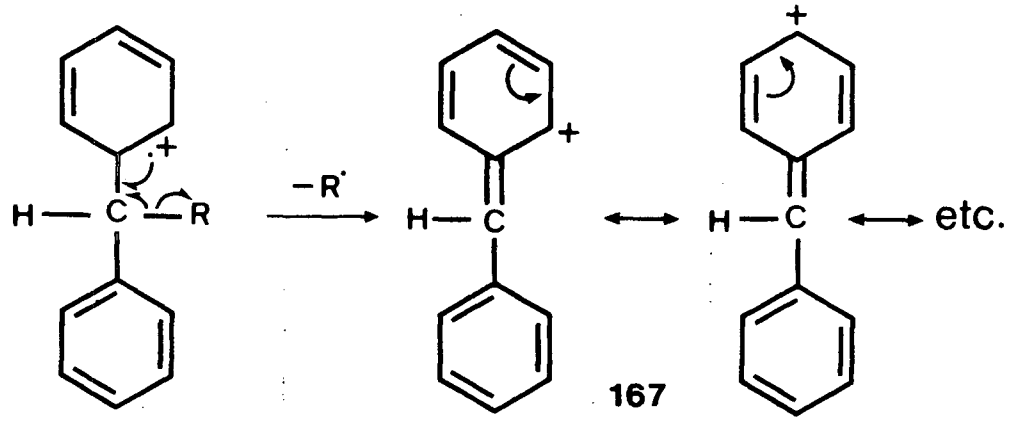


Figure 48. General mass spectral fragmentations of diarylbutanes: III. Radical site initiation on the aromatic ring with allylic (benzylic) cleavage. This gives rise to base peak diagnostic ions in the ketones and oximes.

b.

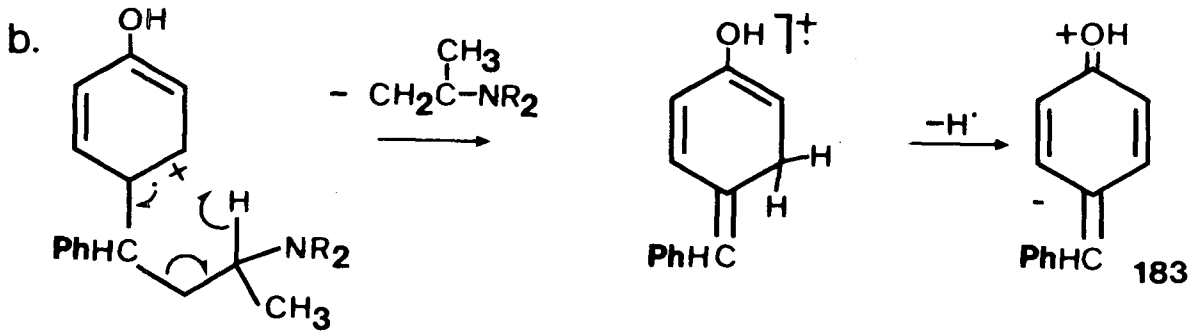


Figure 49. General mass spectral fragmentations of diarylbutanes: IV. Rearrangement of gamma hydrogen with benzylic cleavage and loss of a hydrogen radical to afford diagnostic oxonium ions in phenyl ring oxidized metabolites and their derivatives.

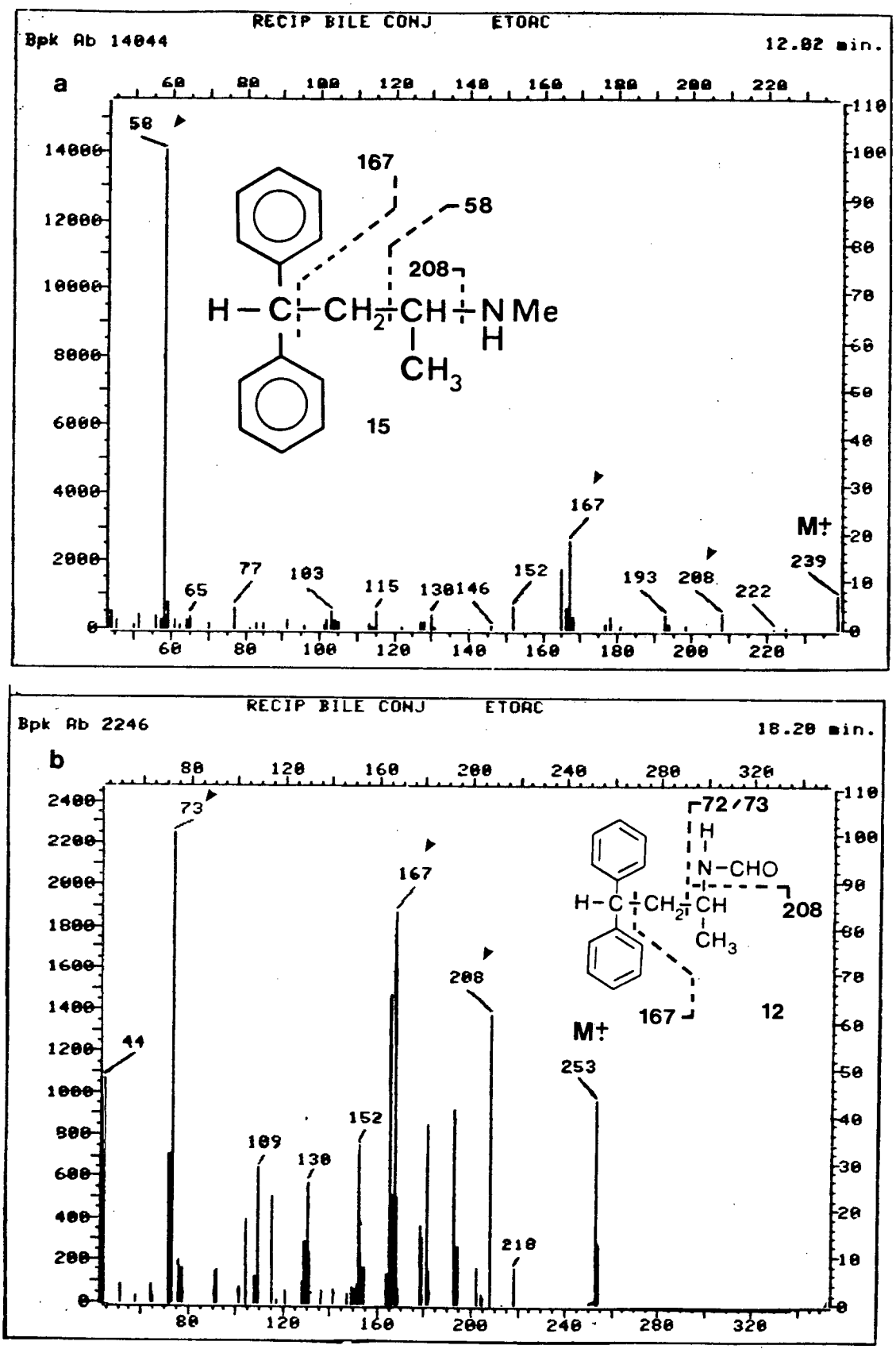


Figure 50. Mass spectra of recipavrin metabolites illustrating the three major fragmentation schemes in figure 46,47,48. A. Norrecipavrin (15). B. Secondary formamide (12). A proton transfer is involved in the formation of m/z 208 (figure 47).

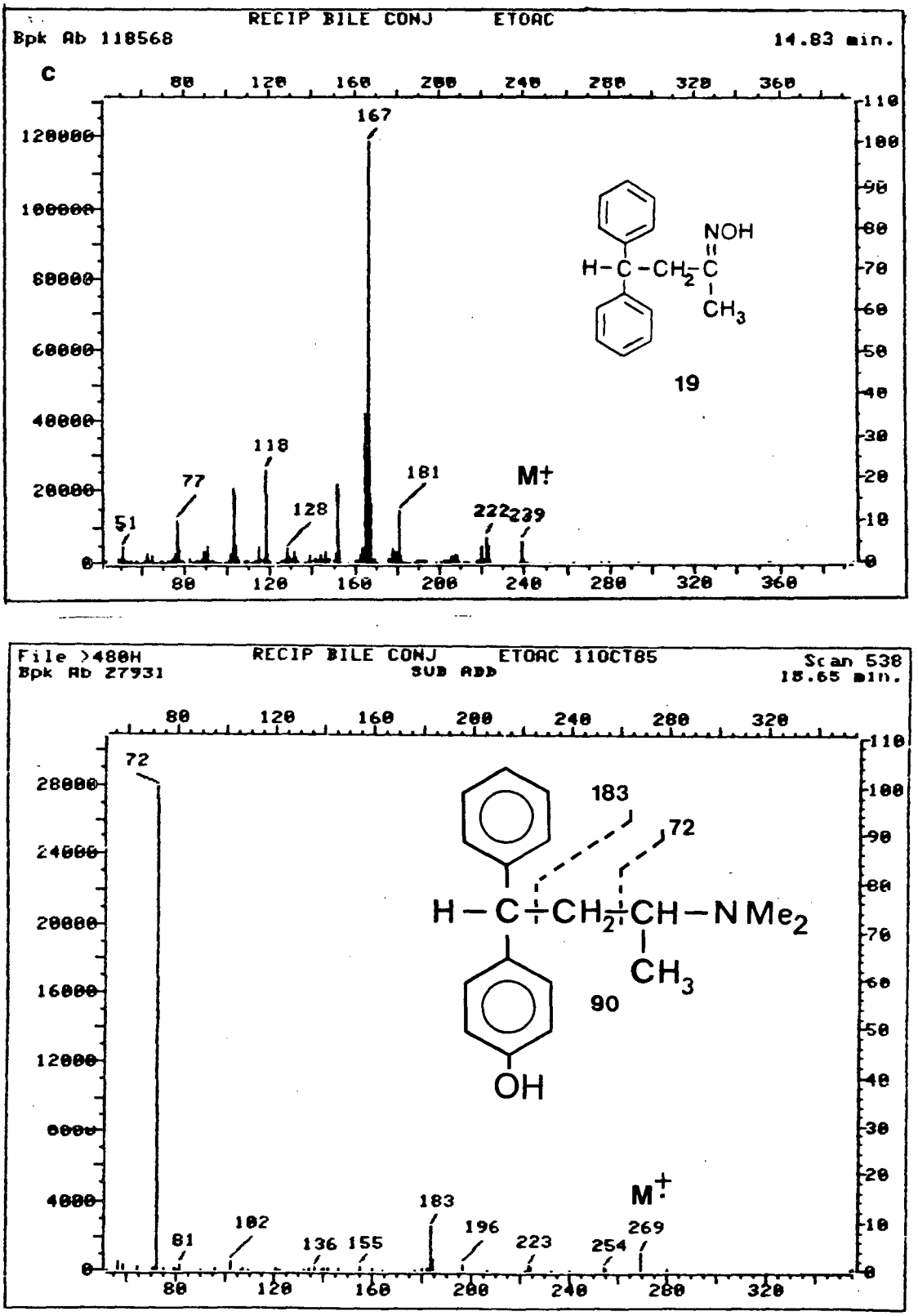


Figure 50. Mass spectra of recipavrin metabolites illustrating the three major fragmentation schemes in figures 46,47,48. C. anti-diphenylbutanone oxime (19). D. Recipavrin phenol (90).

B. Fragmentation of the substituted diphenylbutane moiety

Phenyl ring metabolic oxidation results in shifts in the masses of some of the arylaliphatic fragments (Benthe and Thieme,1983). Phenolic and O-methylcatechol metabolites are easily detected in underivatized, TMS and permethylated forms by calculating the appropriate mass increments induced by oxidation and derivatization of the diarylmethine cation (table 4). The analogous peaks for the 1-methyl-2,2-diphenylcyclopropanyl cation radical (m/z 208), the 2,2-diphenylcyclopropanyl cation (m/z 193) and a diphenylethyl cation (m/z 181) are also present in low relative abundance. The other ions in the diarylbutyl cascade are generally absent, having been replaced by minor aryl ring substituent directed fragmentations. Sensitivity is greatest when the charge is localized in the diphenyl region as in compounds fragmenting according to figure 48. Nonetheless all recipavrin related compounds described here can be easily located by monitoring the substituted diarylmethyl cation.

In phenyl ring oxidized metabolites and their derivatives, the fluorenyl cation (m/z 165) and the diphenylethyl cation (m/z 181) are usually present as minor fragments. The permethylated catechols lose a methyl radical to afford a diagnostic ion at m/z 212.

Phenyl Ring Substitution	Mass Increment	Major Diagnostic Ion (MDI)	Major diarylalkyl cation radical	MDI + CH_2	MDI-R_2R_3	Other diagnostic ions
R_2=H,R_3=H	0	167	208	181	-	208 cascade
R_2=H,R_3-OH	16	183	224	197	165(-H_2O)	155(-C≡$\overset{+}{O}$)
R_2=H,R_3-OTMS	88	255	296	269	165(-TMSO)	-
R_2=H,R_3=OCH_3	30	197	238	211	165(-CH_2O)	-
R_2=OCH_3,R_3=OH	96	213	254	223	181(-CH_3OH)	165
R_2=OCH_3,R_3=OTMS	118	285	326	299	255(-CH_2O)	165,181,223
R_2=OCH_3,R_3=OCH_3	60	227	268	241	196(-CH_3O)	165,212

$Ph(R_2R_3Ar)C^+H$ | $Ph(R_2R_3Ar)CHCH{=}CHCH_3$]‡ | $Ph(R_2R_3Ar)CHC^{\ddagger}H_2$

Table 4. Diagnostic masses of the diarylmethyl cation for phenyl ring oxidized metabolites and their derivatives.

4. METABOLISM OF RECIPAVRIN

This section details the structure elucidation by GCMS of the biliary metabolites of recipavrin (9) in male Wistar rats. Complete characterization of recipavrin metabolites by GCMS was required to show that the secondary formamide (12) was present in the bile extract and to characterize any potential metabolic or chemical precursors of the formamide.

The systematic and trivial names, substituents and formulas of compounds of general structure A and B (figure 51), are summarized in Tables 5 and 6.

GCMS data for all metabolites, derivatives and reference compounds are tabulated as follows: Table 7; Metabolites with intact phenyl rings and their derivatives. Table 8; Phenolic metabolites and their derivatives. Table 9; O-methylcatechol metabolites and their derivatives. Mass spectra of all metabolites, derivatives and reference compounds are included in the appendix.

Total ion current and selected ion chromatograms used to locate recipavrin metabolites and their TMS derivatives are shown in figures 52-55.

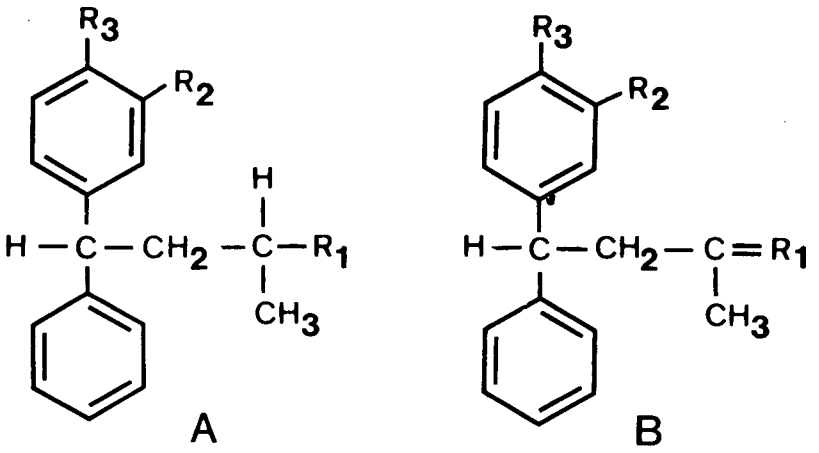


Figure 51. General structures for metabolites: A. Single bonded R_1 substituent. B. Double bonded R_1 substituent.

Table 5. Names structure and formulae for recipavrin, recipavrin metabolites and synthetic reference compounds. Legend: A. Observed in both conjugated and nonconjugated extracts. B. Also a terodiline metabolite (Noren *et al.* 1985a,b). C. Possibly associated with ethyl acetate extraction. D. Synthetic reference compound. E. TMAH derivative, also arises in bile from prolonged storage. F. Decomposes in GC inlet.

Compound Number	Systematic Name	Trivial Name	R_1	R_2	R_3	Empirical Formula
9	(±) N,N, α-trimethyl-γ-phenyl-benzenepropanamine	Recipavrin[A]	$-N(CH_3)_2$	H	H	$C_{18}H_{23}N$
15	(±) N, α-dimethyl-γ-phenyl-benzenepropanamine	Norrecipavrin[A]	$-NHCH_3$	H	H	$C_{17}H_{21}N$
14	1,1-diphenyl-3-butanone	Diphenylbutanone[AB] (DPB)	$=O$	H	H	$C_{16}H_{16}O$
19	1,1-diphenyl-3-butanone oxime	DPB oxime[A]	$=NOH$ (cis and trans)	H	H	$C_{16}H_{17}NO$
12	(±)N-formyl-α-methyl-γ-phenyl benzenepropanamine	2^O Formamide	$-NHCHO$	H	H	$C_{17}H_{19}NO$
86	(±) Unidentified	Unidentified		H	H	$C_{18}H_{19}N$
63	(±)N-acetyl-α-methyl-γ-phenyl-benzenepropanamine	Acetamide[C]	$-NHC(=O)CH_3$	H	H	$C_{18}H_{21}NO$
20	(±)α-methyl-γ-phenyl benzenepropanamine	Dinorrecipavrin	$-NH_2$	H	H	$C_{16}H_{19}N$
26	(±)N-formyl-N,α-dimethyl-γ-phenylbenzenepropanamine	3^O Formamide[E]	$-N(CH_3)CHO$	H	H	$C_{18}H_{21}NO$
64	(±)N-ethylidene-α-methyl-phenylbenzenepropanamine	Ethanimine[D]	$-N=CHCH_3$	H	H	$C_{18}H_{21}N$
53	(±)N,N,α-trimethyl-γ-phenyl benzenepropanamine-N-oxide	N-oxide[F]	$-\overset{O^-}{\overset{\vert}{\overset{+}{N}}}(CH_3)_2$	H	H	$C_{18}H_{23}NO$

Table 6. Names, structures and formulae for phenol and O-methyl catechol metabolites of recipavrin. Legend: A. Observed in both conjugated and nonconjugated extracts. B. Also a terodiline metabolite (Noren *et al.* 1985a,b). C. Possibly associated with ethyl acetate extraction. D. Synthetic reference compound. E. TMAH derivative, also arises in bile from prolonged storage.

Compound Number	Systematic Name	Trivial Name	R_1	R_2	R_3	Empirical Formula
87	(±)1-(4-hydroxyphenyl)-1-phenyl-3-butanone	DPB-phenol[AB]	=O	H	OH	$C_{16}H_{16}O_2$
88	(±)N,α-dimethyl-γ-(4-hydroxyphenyl)-benzenepropanamine	Norrecipavrin phenol	$-NHCH_3$	H	OH	$C_{17}H_{21}NO$
89	(±)1-(4-hydroxyphenyl)-1-phenyl-3-butanone oxime	DPB-oxime phenol[A]	=NOH	H	OH	$C_{16}H_{17}NO_2$
90	(±)N,N-α-trimethyl-γ-(4-hydroxyphenyl)-benzenepropanamine	Recipavrin phenol	$-N(CH_3)_2$	H	OH	$C_{18}H_{23}NO$
91	(±)1-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-butanone	DPB O-methyl catechol[A] (DPB-OMC)	=O	OCH_3	OH	$C_{17}H_{18}O_3$
92	(±)1-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-butanol	diphenybutanol-OMC	-OH	OCH_3	OH	$C_{17}H_{20}O_3$
93	(±)1-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-butanone oxime	DPB oxime-OMC[A]	=NOH	OCH_3	OH	$C_{17}H_{19}NO_3$
94	(±)N,N,α-trimethyl-γ-(4-hydroxy-3-methoxyphenyl)-benzenepropanamine	Recipavrin-OMC	$-N(CH_3)_2$	OCH_3	OH	$C_{19}H_{25}NO_2$

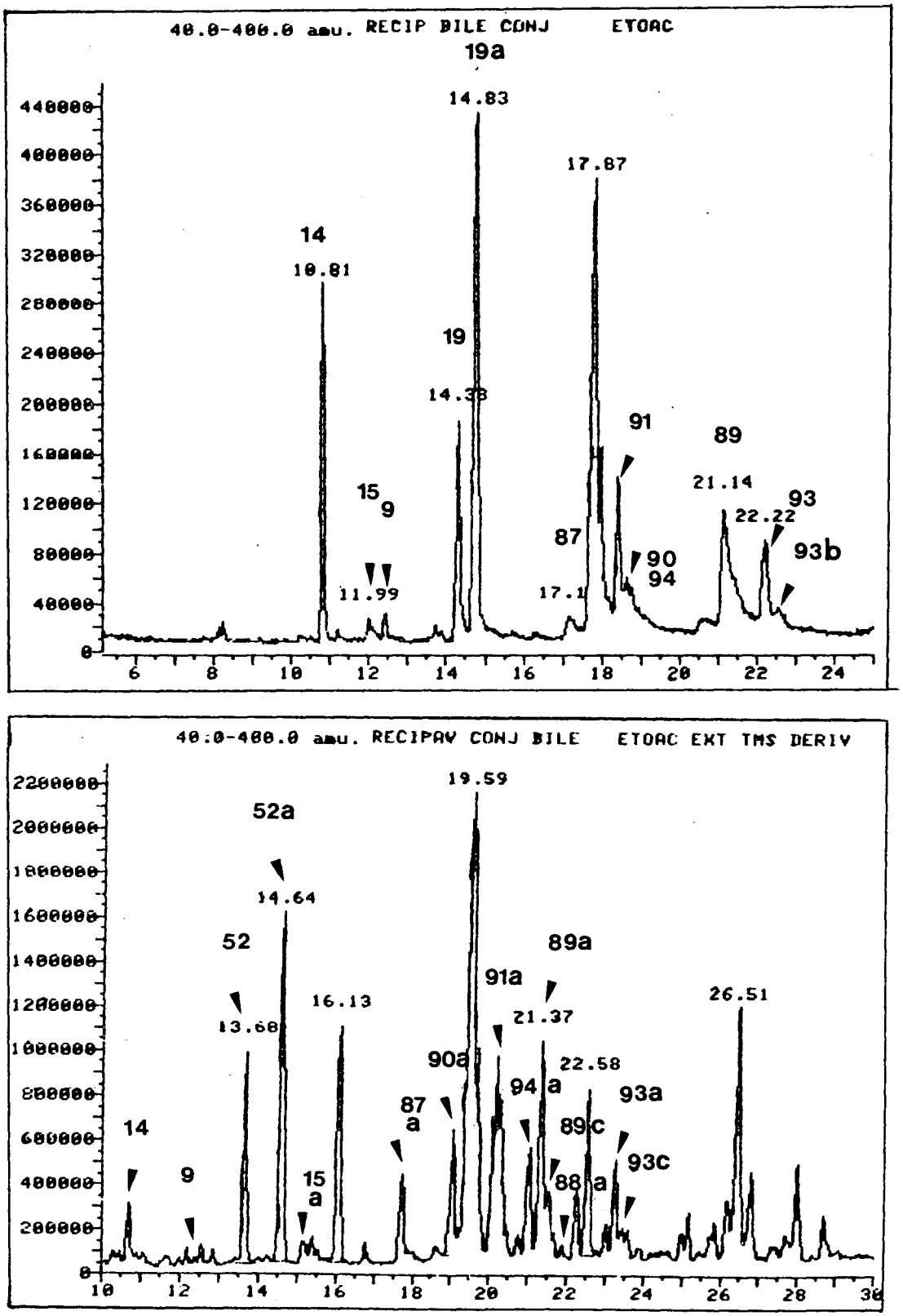


Figure 52. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.I. Total ion current for all metabolites (above) and their TMS derivatives (below).

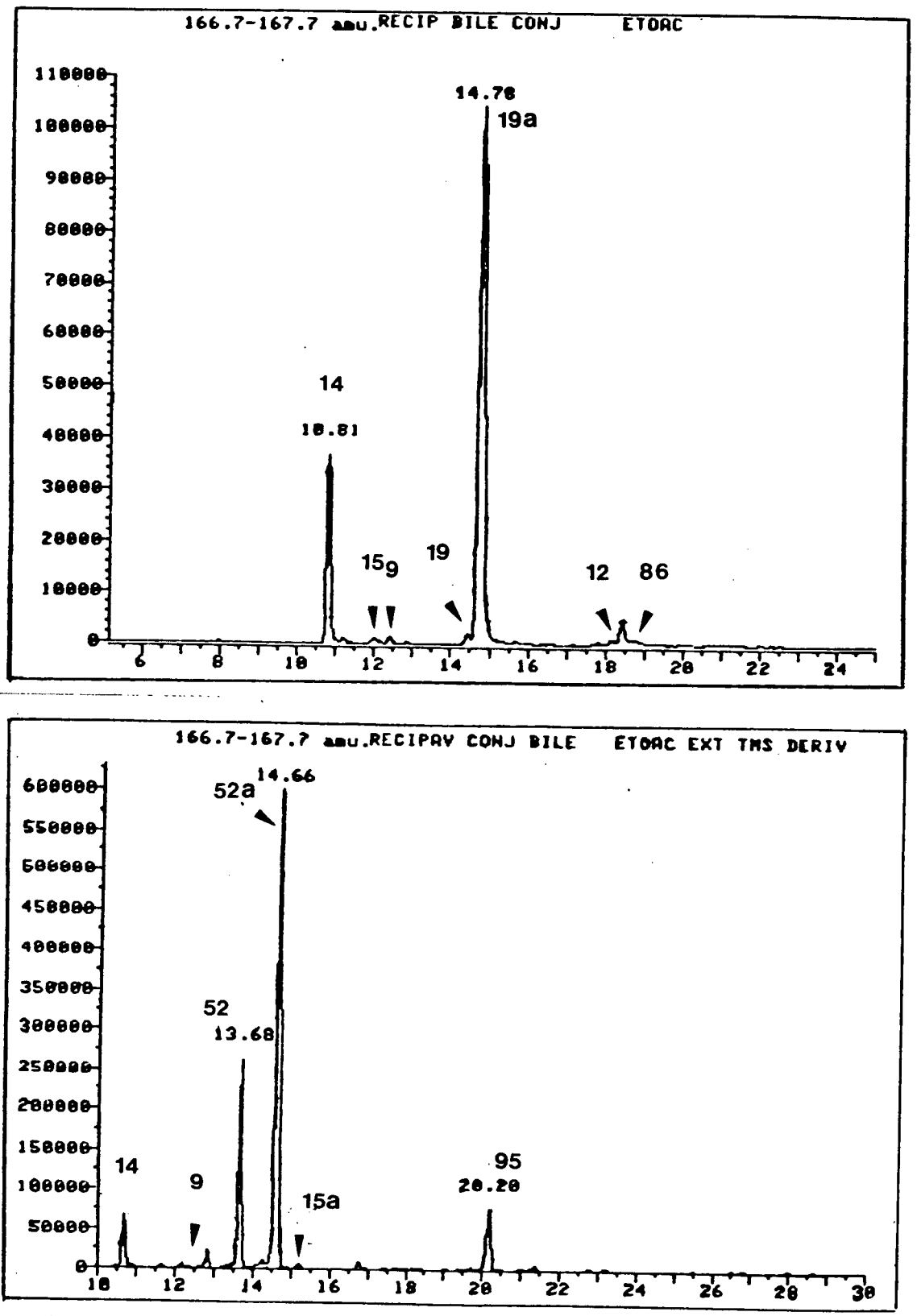


Figure 53. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.II. Ion chromatogram m/z 167 showing phenyl ring intact metabolites (above) and their TMS derivatives (below).

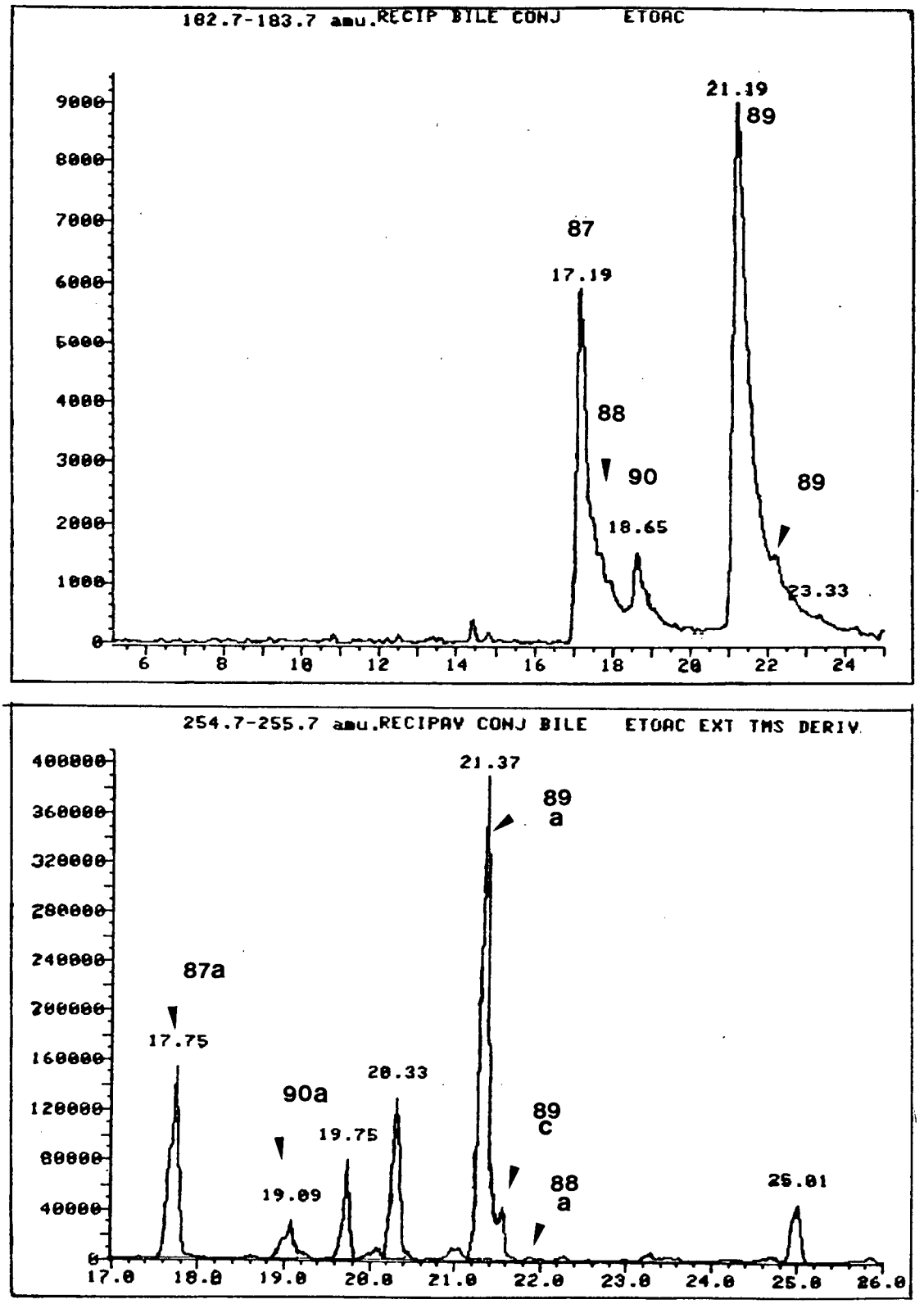


Figure 54. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.III. Ion chromatogram m/z 183 showing intact phenol metabolites (above) and m/z 255 showing TMS derivatized phenols (below).

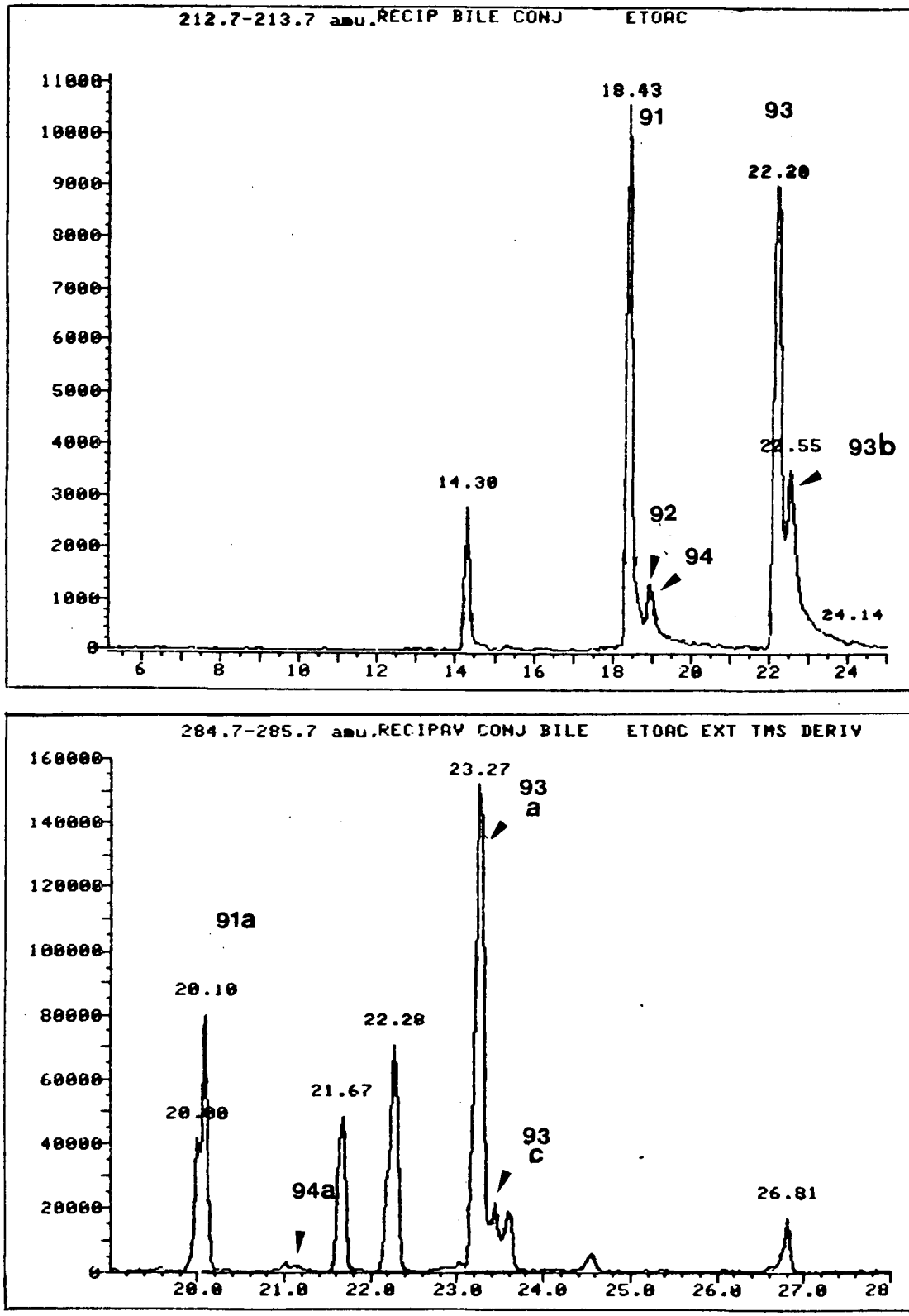


Figure 55. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary recipavrin metabolites.IV. Ion chromatogram m/z 213 showing intact O-methylcatechol metabolites (above) and m/z 285 showing TMS derivatized O-methylcatechols (below).

Table 7. GCMS data for phenyl ring intact recipavrin metabolites and their derivatives. Legend as in table 5. G. TMAH derivative.

Compound Number	Trivial Name	Retention Time (Minutes)	$M^{\dagger}$ (% Intensity)	Base Peak 100%	Other Diagnostic Ions ((m/z)(% of base peak))					
					M_1	M_2	M_3	M_4	M_5	M_6
9	Recipavrin[A]	12.42	253 (3)	72	167 (12)	165 (6)	152 (3)	115 (3)	91 (3)	73 (3)
15	Norrecipavrin[A]	12.02	239 (7)	58	167 (18)	165 (12)	152 (5)	208 (4)	103 (4)	193 (4)
15a	Norrecipavrin N-TMS	15.17	311 (5)	130	167 (19)	193 (31)	73 (25)	105 (20)	270 (13)	165 (7)
14	Diphenylbutanone (DPB)[AB]	10.79	224 (35)	167	43 (10)	103 (32)	165 (32)	181 (28)	152 (18)	77 (14)
19	DPB-oxime (minor[A])	14.43	239 (0)	167	165 (57)	105 (32)	180 (24)	209 (20)	91 (20)	222 (10)
52	DPB oxime (minor)(O-TMS)	13.68	311 (1)	167	165 (36)	75 (11)	103 (10)	220 (6)	296 (2)	152 (2)
19a	DPB oxime[A] (major)	14.83	239 (8)	167	165 (25)	152 (15)	103 (18)	42 (12)	118 (11)	181 (11)
52a	DPB-oxime (major-O-TMS)	14.64	311 (8)	167	166 (60)	165 (20)	118 (20)	222 (8)	220 (8)	221 (8)
16	DPB-O-methyloxime[G] (major)	12.03	253 (8)	167	165 (32)	152 (18)	118 (16)	103 (12)	181 (8)	77 (5)
16a	DPB-O-methyloxime (minor)	11.69	253 (0)	167	165 (34)	180 (28)	152 (18)	168 (12)	115 (6)	77 (6)
12	2^{o} Formamide	18.20	253 (44)	73	167 (84)	165 (66)	208 (60)	44 (50)	193 (42)	181 (38)
86	Unidentified	18.78	251 (0)	70	167 (32)	71 (66)	165 (50)	91 (36)	208 (32)	130 (22)
95	Unidentified -TMS	20.18	323 (5)	144	167 (84)	165 (50)	193 (38)	251 (36)	118 (32)	91 (28)
63	Acetamide[C]	19.16	267 (12)	87	167 (14)	44 (61)	72 (16)	167 (14)	86 (13)	208 (10)
20	Dinorrecipavrin	10.78	225 (2)	44	208 (12)	165 (5)	58 (5)	115 (4)	193 (3)	222 (2)
104	Dinorrecipavrin-N-TMS	13.51	297 (3)	116	73 (22)	167 (16)	118 (12)	102 (8)	154 (8)	152 (6)
26	3^{o} Formamide[E]	19.41	267 (10)	87	86 (70)	58 (28)	72 (25)	167 (22)	165 (20)	208 (18)
64	Ethanimine[D]	11.41	251 (8)	71	165 (46)	236 (43)	167 (30)	70 (26)	152 (24)	105 (23)
53	Recipavrin N-oxide[F] (as cis (minor) and trans (major) diphenyl-but-2-enes)	7.92	208 (6)	167	165 (26)	152 (18)	115 (10)	208 (6)	193 (5)	128 (4)
		8.26	208 (68)	115	193 (58)	178 (42)	91 (38)	178 (38)	130 (38)	165 (36)

Table 8. GCMS data for phenolic metabolites of recipavrin and their derivatives. Legend as in table 5. G. TMAH derivative. H. Diazomethane derivative. I. Also a norrecipavrin phenol derivative.

Compound Number	Trivial Name	Retention Time (Minutes)	M^+ (% Intensity)	Base Peak 100%	Other Diagnostic Ions ((m/z)(% of base peak))					
					M_1	M_2	M_3	M_4	M_5	M_6
87	Diphenylbutanone phenol[B]	17.19	240 (18)	183	43 (12)	185 (8)	103 (6)	119 (5)	153 (5)	225 (2)
87a	DPB phenol TMS ether	17.75	312 (13)	255	257 (22)	73 (14)	165 (8)	179 (4)	103 (4)	43 (4)
87b	Diphenylbutanone [G,H], O-methyl phenol	15.94	254 (16)	197	198 (16)	165 (16)	152 (10)	182 (6)	103 (4)	115 (3)
88	Norrecipavrin phenol	17.71	255 (5)	58	183 (7)	224 (6)	59 (5)	165 (2)	115 (2)	152 (2)
88a	Norrecipavrin phenol (N-TMS, O-TMS)	21.87	399 (5)	130	255 (22)	73 (38)	131 (15)	75 (10)	296 (8)	58 (4)
88b	Norrecipavrin O-methyl phenol[H]	17.08	269 (10)	197	58 (28)	238 (24)	153 (20)	165 (14)	207 (10)	222 (8)
89	DPB-oxime phenol (major)	21.19	255 (2)	183	165 (26)	196 (16)	184 (12)	199 (10)	152 (10)	121 (10)
89a	DPB-oxime phenol-di-TMS ether (major)	21.37	399 (2)	255	73 (20)	259 (18)	269 (10)	103 (4)	165 (4)	206 (4)
89b	DPB-oxime phenol (minor)	22.20	255 (0)	183	Shoulder, poor mass spectrum obtained					
89c	DPB-oxime phenol-di-TMS ether (minor)	21.54	399 (14)	255	73 (56)	268 (44)	165 (24)	295 (21)	257 (20)	193 (16)
89d	Diphenylbutanone-O-methyloxime-O-methyl phenol (major and minor)[G]	17.4 17.24	283 (0)		Good mass spectral data not obtained due to overlapping peaks					
90	Recipavrin phenol		269 (5)	72	183 (12)	196 (2)	102 (2)	254 (1)	155 (1)	136 (1)
90a	Recipavrin phenol TMS ether	19.09	341 (6)	72	255 (12)	73 (14)	165 (6)	296 (6)	115 (2)	179 (2)
90b	Recipavrin[G,I] phenol O-methyl ether	17.57	283 (10)	72	197 (22)	283 (10)	153 (10)	238 (8)	165 (8)	207 (6)

Table 9. GCMS data for O-methyl catechol metabolites of recipavrin and their derivatives. Legend as in table 5. G. TMAH derivative. H. Diazomethane derivative. I. Also a norrecipavrin phenol derivative.

Compound Number	Trivial Name	Retention Time (Minutes)	$M^{\dagger}$ (% Intensity)	Base Peak 100%	Other Diagnostic Ions ((m/z)(% of base peak))					
					M_1	M_2	M_3	M_4	M_5	M_6
91	DPB-OMC[B]	18.43	270 (26)	213	43 (38)	103 (20)	152 (18)	153 (16)	181 (12)	72 (10)
91a	DPB-OMC TMS ether	20.10	342 (23)	285	286 (24)	144 (18)	73 (14)	167 (10)	255 (9)	103 (4)
91b	DPB-dimethyl catechol[G,H]	18.85	284 (32)	227	228 (18)	165 (12)	196 (11)	103 (10)	181 (8)	73 (6)
92	Diphenylbutanol-OMC	18.95	272 (26)	213	44 (72)	182 (15)	151 (8)	-	-	-
93	DPB-oxime-OMC (major)	22.20	285 (0)	213	225 (50)	152 (30)	151 (24)	105 (16)	91 (14)	239 (6)
93a	DPB-oxime-OMC (major) di-TMS ether	23.27	429 (0)	285	286 (22)	73 (16)	103 (6)	181 (5)	255 (5)	325 (4)
93b	DPB-oxime-OMC (minor)	22.55	285 (3)	213	153 (18)	214 (12)	183 (10)	91 (9)	108 (8)	268 (6)
93c	DPB-oxime-OMC (minor) di-TMS ether	23.44	429 (0)	285	73 (52)	286 (20)	373 (16)	295 (15)	223 (14)	120 (14)
93d	Diphenylbutanone-O-methyloxime-dimethyl catechol (minor)[G]	19.74	313 (6)	227	229 (14)	240 (14)	196 (8)	139 (7)	165 (6)	181 (5)
93e	Diphenylbutanone-O-methyloxime-dimethyl catechol (major)[G]	20.05	313 (6)	227	228 (14)	196 (10)	165 (10)	72 (9)	241 (6)	181 (6)
93f	DPB-oxime dimethyl catechol[I]	18.89	299 (10)	227	228 (14)	196 (8)	165 (8)	241 (8)	282 (6)	152 (4)
94	Recipavrin OMC	18.96	299 (10)	72	213 (9)	152 (4)	58 (3)	254 (3)	115 (3)	75 (3)
94a	Recipavrin OMC TMS ether	21.16	371 (14)	72	good mass spectrum not obtained					
94b	Recipavrin dimethyl catechol[H,I]	19.54	313 (8)	72	227 (20)	237 (8)	165 (8)	91 (6)	115 (6)	152 (4)

A. Biliary metabolites of recipavrin

Based on the metabolites observed by GCMS, recipavrin follows four general metabolic routes. The pathways of oxidative deamination, N-dealkylation, N-oxidation and phenyl ring oxidation are common to structurally related compounds in the amphetamine series (Caldwell, 1976, Coutts and Beckett, 1977 (reviews), Beckett and Al Sarraj, 1972), promethazine (10)(Clement and Beckett, 1981), terodiline (Noren, *et al.*, 1985a,b) and related compounds. One metabolite, the secondary formamide (12) could not be assigned to any one pathway. Experiments on the origin of the formamide are discussed in section 7.

i. GCMS observation of the intact formamide

The secondary formamide (12) was observed by GCMS as a minor metabolite in the conjugated fraction of bile. The mass spectrum (figure 56) and retention time were similar to the synthetic product. The chemical ionization mass spectrum shows the M^+ +1 ion at m/z 254 and associated alkane adducts $M^+ + C_2H_5$ and $M^+ + C_3H_7$ from the methane reagent gas (Figure 57). The secondary formamide metabolite was methylated with TMAH to give the tertiary formamide (figure 58 (top)). Obtaining a TMS derivative of the secondary formamide was not as straightforward. Firstly the derivatization of the synthetic standard was slow and afforded an apparent mono derivative (60, $Ph_2CHCH_2CH(CH_3)N(TMS)CHO$) with a retention time of 15.36

minutes (see figure in section 1). In the TMS derivatized bile extract, the underivatized formamide was not seen.

A new peak (95) appeared at 20.2 minutes had a base peak at m/z 144 suggesting silylation at nitrogen and an apparent molecular ion at m/z 341 (figure 58 (bottom)) which corresponds to an underivatized molecular weight of 269. The loss of $TMSOH^+$ from m/z 341 could account for the m/z 251 peak. The mass spectrum of 95 is in accord with derivatization of the unidentified compound (86), since loss of TMSOH+ or H_2O in 95 and 86 respectively, affords m/z 251 from molecular ions presumed to be at m/z 341 (derivatized) and m/z 269 (underivatized). The m/z 251 ion could correspond to an isocyanate structure since alpha cleavage affords a m/z 70/71 ion doublet in the underivatized compound.

The mass spectrum of unidentified compound 95 (figure 58 bottom) has a base peak m/z 144 like the amphetamine secondary formamide TMS derivative (84, figure 59). The reason the amphetamine formamide TMS derivative lacks the m/z 145 ion present in the mass spectrum of the synthetic recipavrin formamide TMS derivative (60, figure 23)) is the absence of a gamma proton.

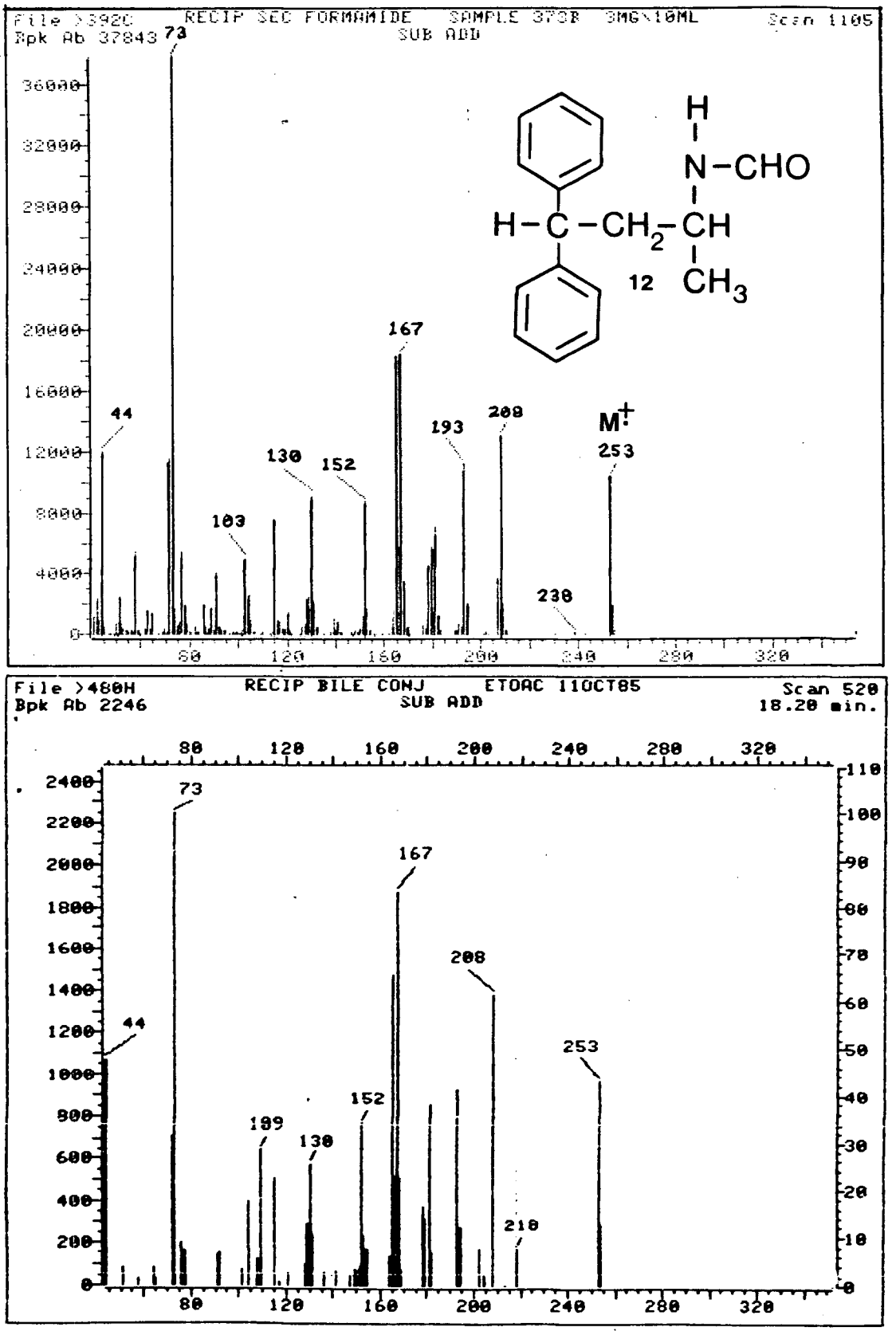


Figure 56. (top) Mass spectrum of the synthetic secondary formamide (12). (bottom) Mass spectrum of the underivatized secondary formamide metabolite.

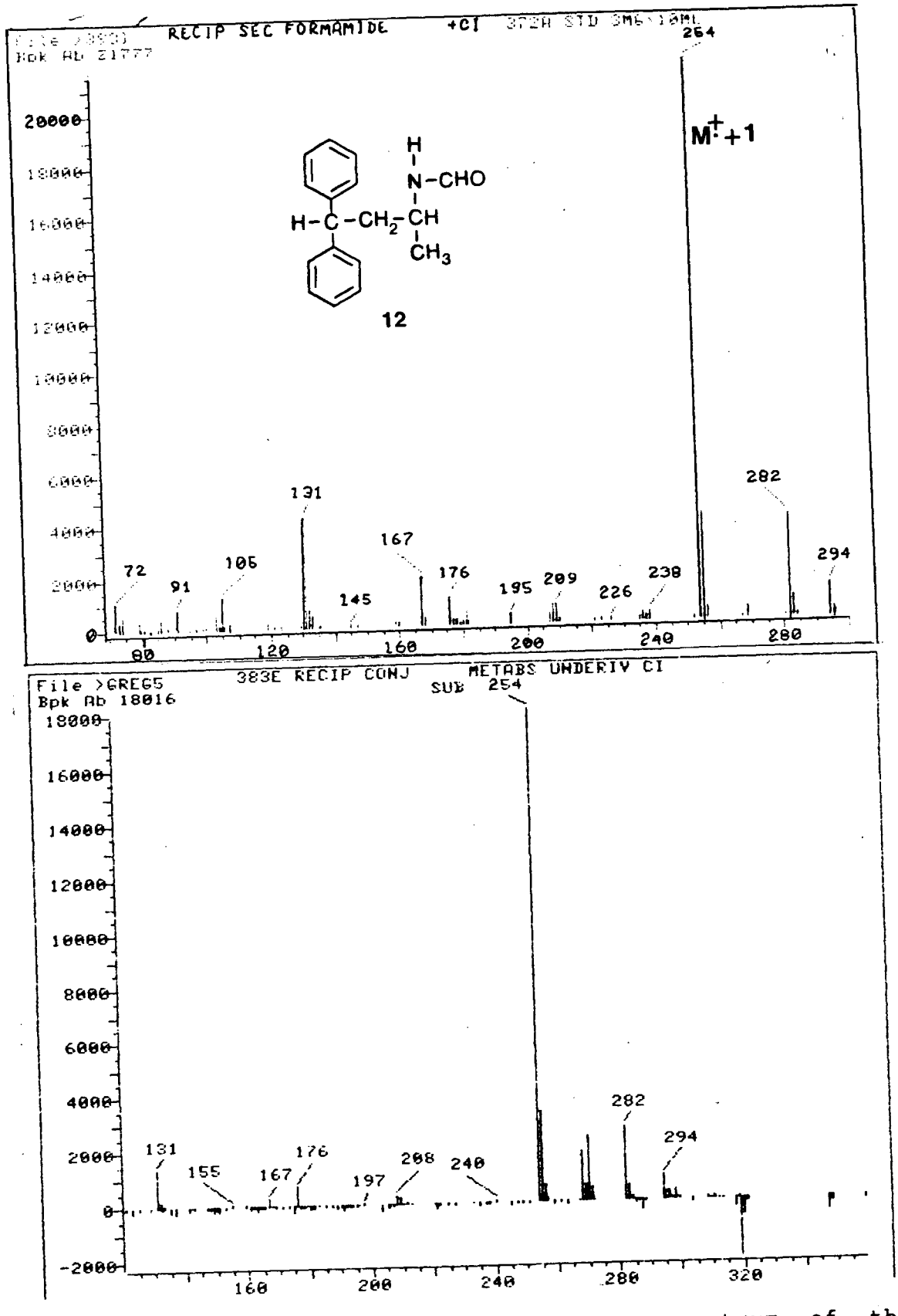
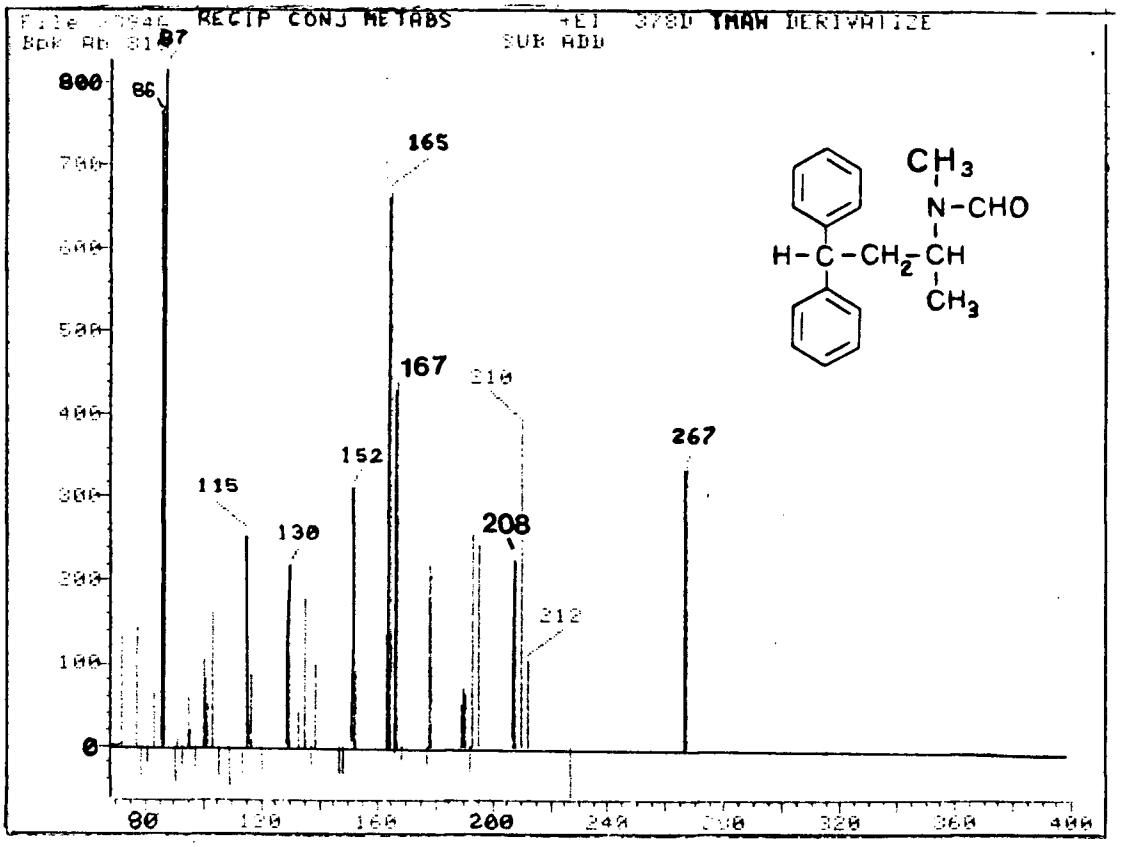


Figure 57. (top) Chemical ionization mass spectrum of the synthetic secondary formamide (12). (bottom) Chemical ionization mass spectrum of the secondary formamide metabolite.



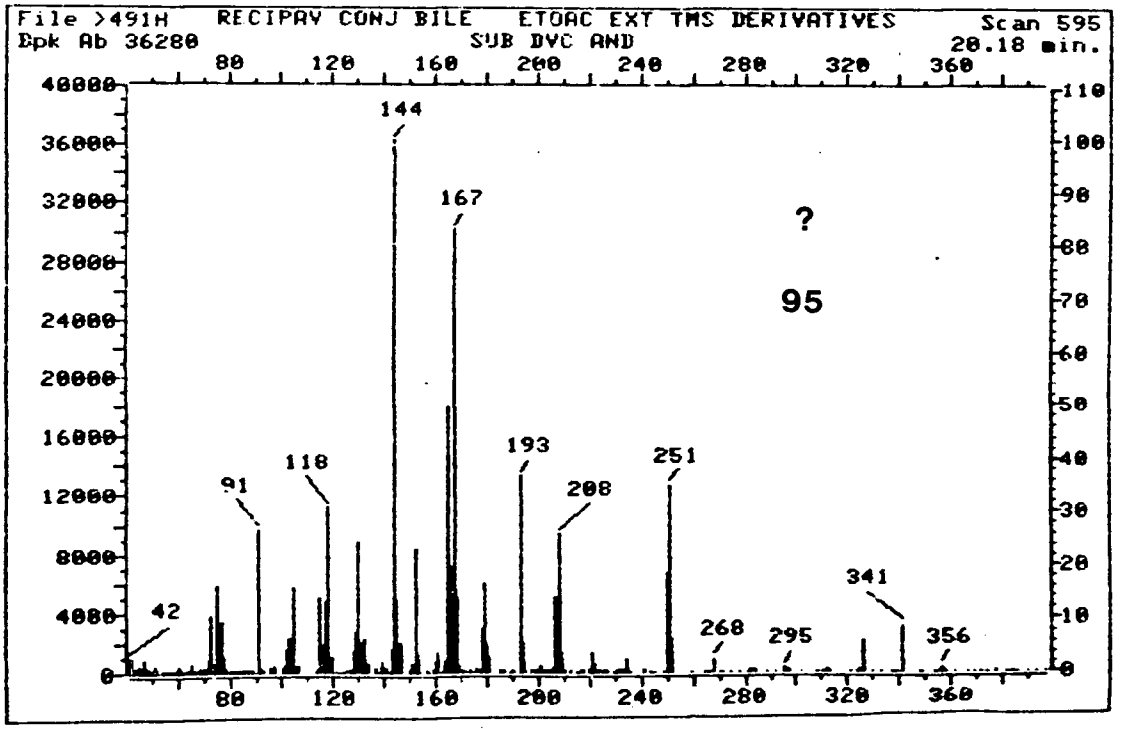


Figure 58. (top) Mass spectrum of the TMAH derivatized secondary formamide. (bottom) Mass spectrum of the unidentified TMS derivative (95).

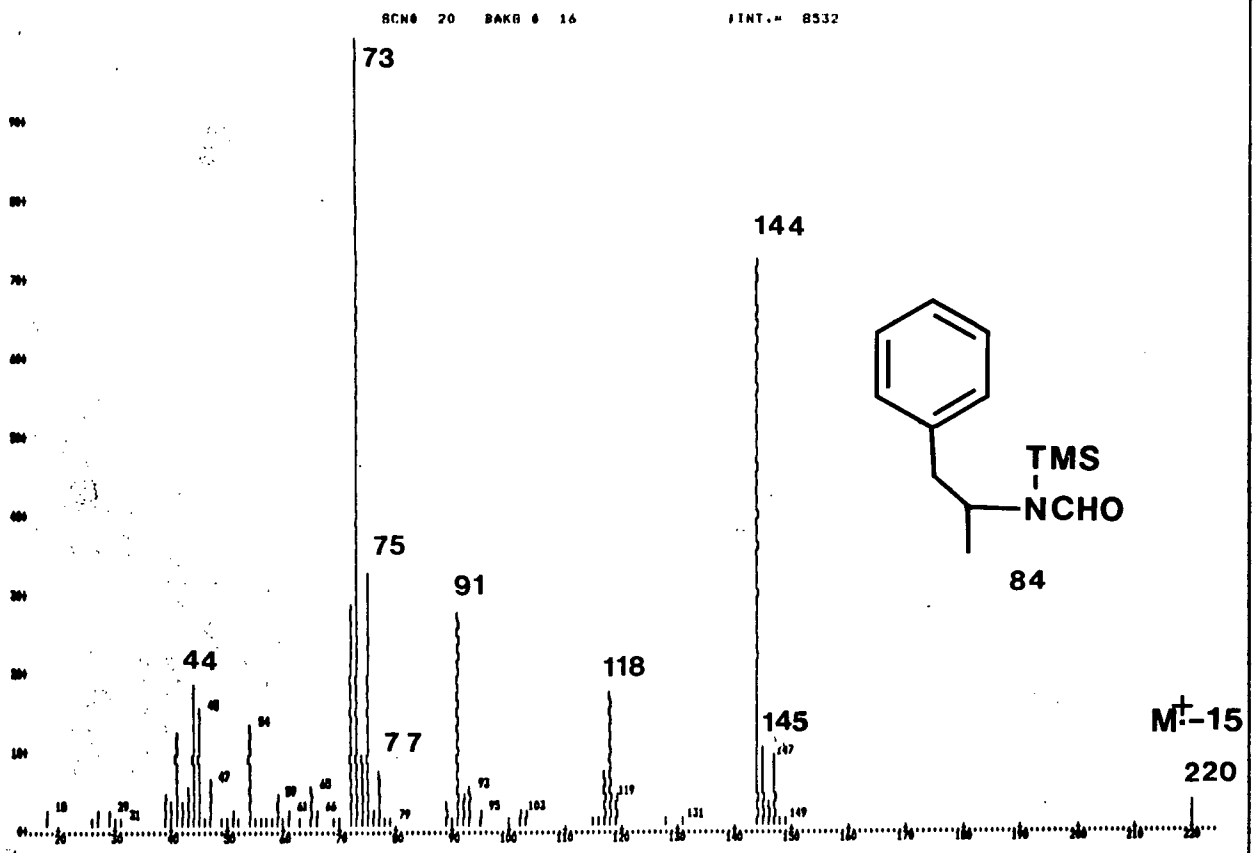


Figure 59. Mass spectrum of the TMS derivative of the amphetamine secondary formamide (84).

ii. LCMS Demonstration of the secondary formamide metabolite

Liquid chromatography-mass spectrometry was used to determine whether the secondary formamide (12) or the thermally unstable isomeric methylene nitrone (24) was responsible for the formamide observed by GCMS of β-glucuronidase-hydrolyzed bile from recipavrin dosed rats. Using the direct liquid introduction (DLI) interface for the HP-5987 mass spectrometer, synthetic samples of the nitrone and formamide were chromatographed on a HP 1090 LC equipped with a semi micro RPC_{18} HPLC column. Retention times and mass spectra of the standards were recorded for 200 ng (100 ng/ul) in scan mode (figure 60). The instrument was then put in selected ion monitoring mode for the ions m/z 254 (M+1), 238 (M+1-16) and 295 (M+1+CH_3CN). The loss of oxygen from the molecular ion (m/z 238) is common in the mass spectra of nitrones (Coutts *et al.*, 1978). The standards were rerun at one tenth concentration (20 ng). A blank injection showed no carryover from the standard injection. The bile sample was then run under identical conditions. The m/z 254 ion chromatogram in figure 61 shows that the secondary formamide (12) is present in the bile extract. Monitoring the base peak m/z 238 failed to reveal any methylene nitrone. The m/z 238 peaks at shorter retention times were not identified.

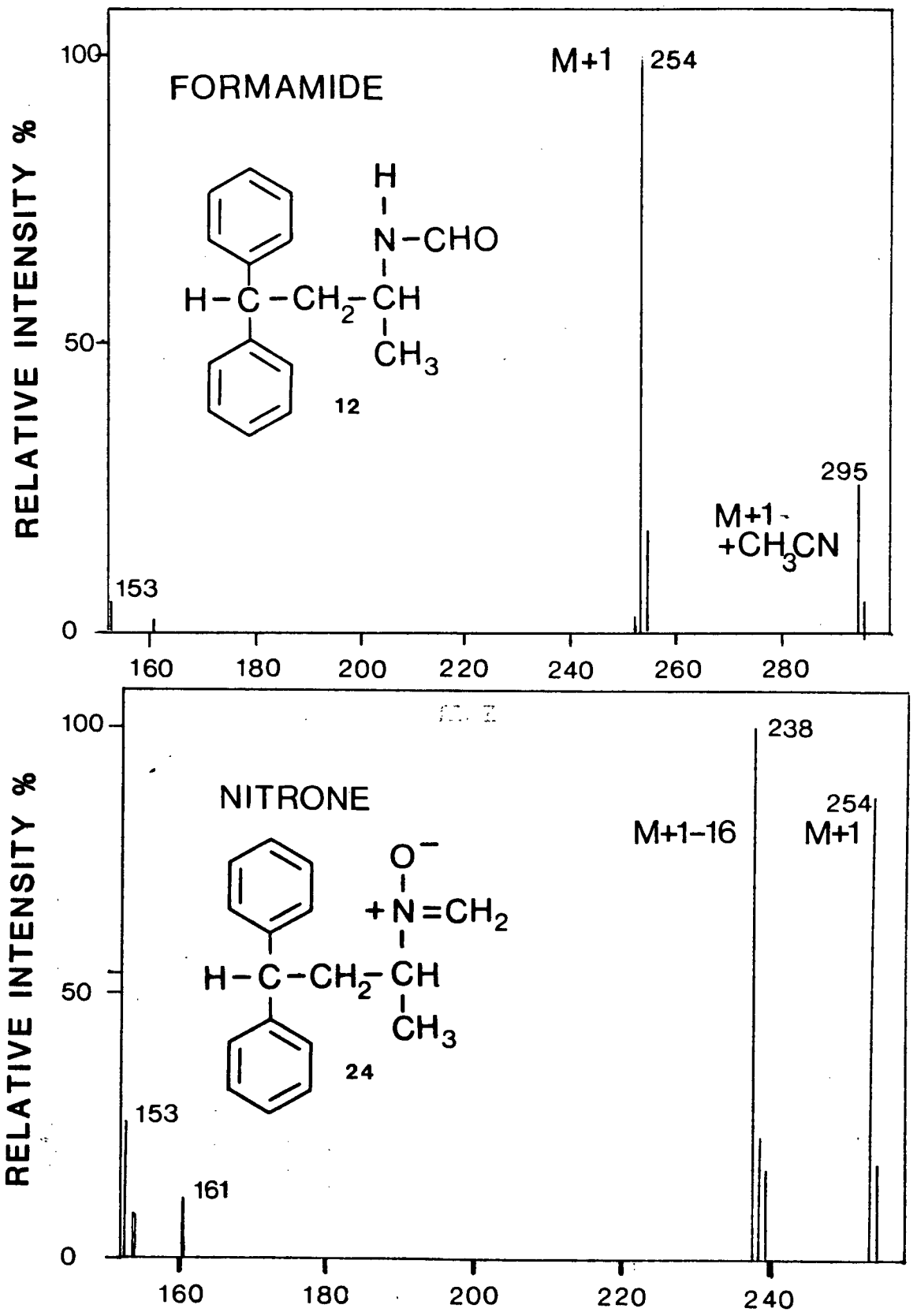


Figure 60. LCMS mass spectrum of the secondary formamide (12)(top) and the methylene nitrone (24)(bottom) in the acetonitrile/ water solvent system.

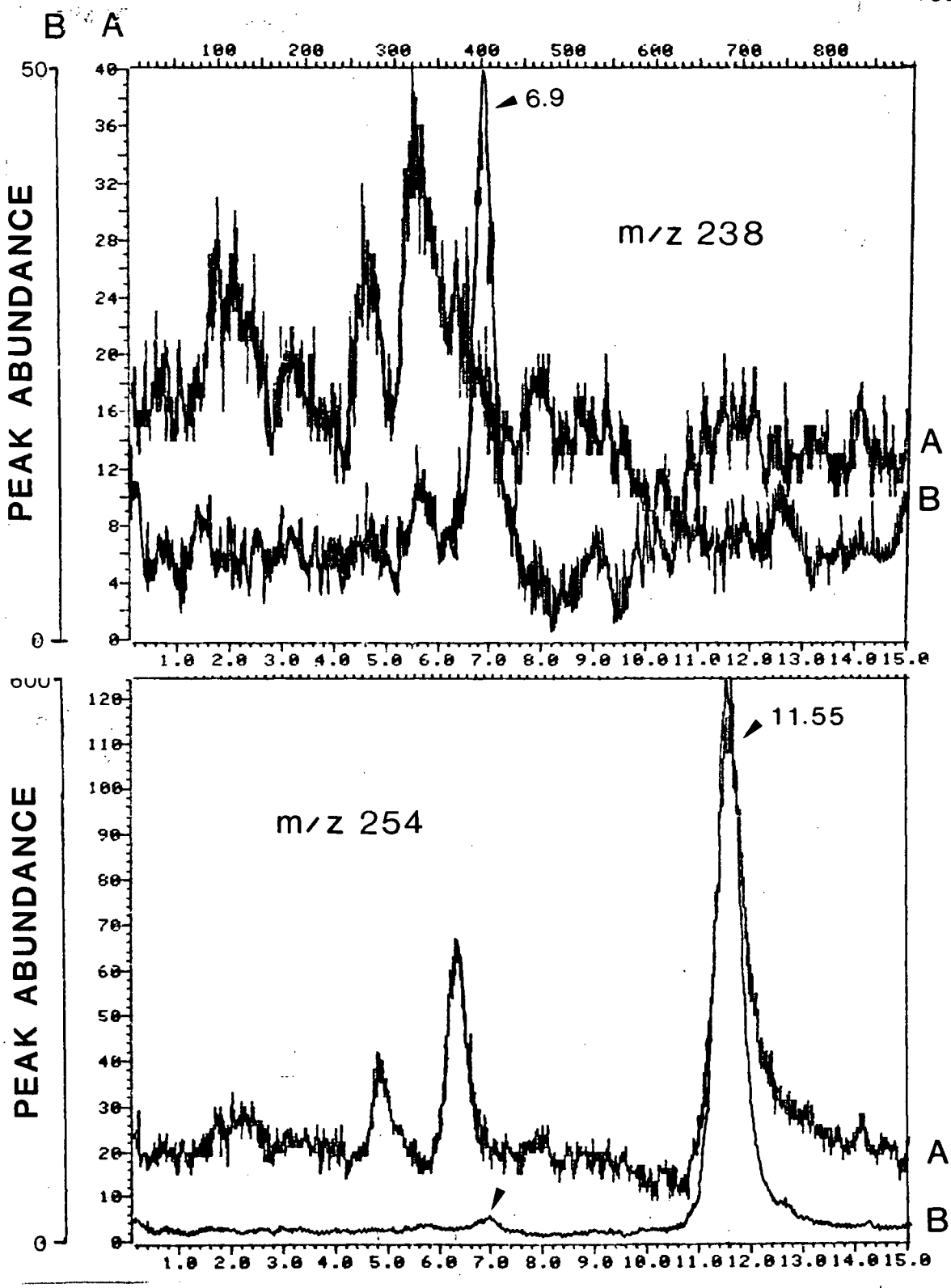


Figure 61. Superimposed LCMS selected ion monitoring results for A. A β-glucuronidase-hydrolyzed bile extract from a recipavrin dosed rat. B. A mixture of the synthetic methylene nitrone (24) and secondary formamide (12)(10 ng each) standards. Bottom frame shows the M^++1 m/z 254 of both components and the top frame shows the M^++1-16 m/z 238 diagnostic ion of the nitrone. Only the formamide is present in the bile extract.

iii. Oxidative deamination

The mixed function oxidase responsible for the oxidative deamination of amphetamines was first described by Axelrod (1955). The formation of diphenylbutanone (14, $Ph_2CHCH_2C(CH_3)=O$) from recipavrin (9, $Ph_2CHCH_2CH(CH_3)N(CH_3)_2$) is in direct analogy to the production of phenylacetone from dimethylamphetamine (Beckett and Al Sarraj, 1972). O-methylcatechol and phenolic derivatives of diphenylbutanone have been described as metabolites of terodiline (Noren, *et al.*, 1985a,b). Surprisingly, 10-(2-propanone) phenothiazine (71) has not been observed among the *in vitro* metabolites of promethazine (10)(Clement and Beckett, 1981) and the diketone (3) has not been observed as a metabolite of methadone (8)(Abbott, *et al.*, 1985). This may involve instability of a key intermediate in the case of methadone, or preferential oxidation of the ring heteroatoms in the case of promethazine.

Diphenylbutanone (14) is present in both conjugated and nonconjugated extracts and is the second most abundant metabolite. The mechanism of oxidative deamination is a matter of some controversy. It is likely that diphenylbutanone arises from several possible mechanisms, which may include spontaneous hydrolysis of the alpha methine carbinolamine ($Ph_2CHCH_2COH(CH_3)N(CH_3)_2$), hydrolysis of an imino cation $Ph_2CHCH_2C(CH_3)=N^+(CH_3)_2$) arising by dehydration of the carbinolamine or dehydrogenation of the amine or by hydrolysis of the oxime (19, $Ph_2CHCH_2C(CH_3)=NOH$). Experimental evidence

supports a combination of two or more of the above precursors (Caldwell, 1976 (review)). The occurrence of diphenylbutanone in the conjugated fraction can be attributed to either the post-enzymatic acid hydrolysis of an oxime precursor during workup or by the existence of an enol sulfate or glucuronide of diphenylbutanone. The enol sulfate of phenylacetone has been characterized by NMR spectroscopy of amphetamine metabolic extracts from rabbits (Dring, et. al., 1970). The glucurase^{R} used in this study was not a sulfatase free preparation. The metabolic reduction of diphenylbutanone (14) to diphenylbutanol was shown only by the presence of the O-methylcatechol of diphenylbutanol (92). No phenolic diphenylbutanol, a metabolite of terodiline (Noren,et. al., 1985a,b) or intact diphenylbutanol was detected.

No benzylic carbinols were observed in this study, although the benzylic oxidation of terodiline (Noren,1985a,b) and related compounds has been observed. Benzophenone was observed and may be a metabolic or chemical oxidation product of a diphenylcarbinol metabolite.

iv. N-dealkylation

Recipavrin (9, $Ph_2CHCH_2CH(CH_3)N(CH_3)_2$) is dealkylated to norrecipavrin (15, $Ph_2CHCH_2CH(CH_3)NHCH_3$) *in vivo*. Small amounts of norrecipavrin were observed in unhydrolyzed and hydrolyzed bile and urine extracts by GCMS. Norrecipavrin is a more important nonconjugated component of the urine where it was observed along with the major components, recipavrin and

recipavrin phenol. The lack of a plausible glucuronide precursor to norrecipavrin indicates that it could be carried over from incomplete extraction of the nonconjugated fraction, or arise from a chemolabile precursor such as a secondary hydroxylamine which could form a glucuronide conjugate.

Trace quantities of underivatized dinorrecipavrin (20, $Ph_2CHCH_2CH(CH_3)NH_2$) were detected in bile and urine. Detection was difficult because of the low intensity of the diphenylmethyl cation and the poor diagnostic value of the m/z 44 base peak. Dinorrecipavrin was more easily located as its TMS derivative which has a m/z 116 base peak. The low levels of dinorrecipavrin are in accord with the general decrease in reactivity towards N-dealkylation that occurs in secondary amines relative to tertiary amines. This may be further complicated by the stereoselectivity that is a feature of secondary, but not tertiary amine dealkylation (Henderson, *et al.*, 1974).

The observation of dinorrecipavrin in the conjugated fraction could also result from carryover or chemical breakdown of a N-oxidized precursor. Dinorrecipavrin is an assumed intermediate in the occasional observation of the secondary acetamide (63, $Ph_2CHCH_2CH(CH_3)NHC(=O)CH_3$). The acetamide is an acceptable metabolic N-acetyl conjugate of a primary arylaliphatic amine in the rat (Dring, et. al., 1970). The N-acetyl compound was only seen at trace levels in ethyl acetate extracted samples.

The ethanimine (64, $Ph_2CHCH_2CH(CH_3)N{=}CHCH_3$), a synthetic condensation product of acetaldehyde and dinorrecipavrin (20) was not observed in the bile extracts. The unidentified compound (86) has a similar mass spectrum to the ethanimine but was eluted at much longer retention time.

The metabolic dealkylation of amines is generally thought to proceed via an unstable carbinolamine intermediate, which in the case of basic amines, spontaneously dissociates to the desalkyl compound and an aldehyde. Exceptions to this spontaneous hydrolysis occur in the pyrrolidine series where the alpha carbinolamine intermediate is further oxidized to a lactam (Rose and Castagnoli, 1983 (review)) or when the nitrogen atom is non basic. Although an initial four electron metabolic oxidation of a basic tertiary amine to a tertiary N-methyl formamide is unlikely, the possibility of a glucuronide conjugated carbinolamide precursor of the secondary formamide was ruled out by studying the metabolism of the tertiary formamide (see later section).

The desalkyl metabolite, norrecipavrin was also oxidized to a phenol (88). N-oxidation of norrecipavrin to a secondary hydroxylamine (17, $Ph_2CHCH_2CH(CH_3)N(OH)CH_3$) or nitrones 24 or 44 was not observed by GCMS, probably due to the sample workup conditions. The potential secondary hydroxylamine and nitrone involvement in formamide production will be discussed in a later section.

v. Phenyl ring oxidation

Phenols and O-methylcatechols of intact recipavrin (90 and 94) and certain compounds (87,88,89,91,92,93) in all three metabolic pathways were observed in bile by GCMS. Only norrecipavrin phenol (88) and recipavrin O-methylcatechol (94) were observed exclusively in the conjugated fraction. The other phenols were also evident as minor components of the nonconjugated fraction.

In the amphetamine series, biliary excretion of phenyl ring oxidized metabolites is a relatively major metabolic pathway in the rat (Caldwell, *et al.*, 1972a), but of less importance in most other species (Caldwell, *et al.*, 1972b). Phenol formation is thought to occur exclusively in the 4' position with subsequent 3' oxidation to the catechol. Attempts were made to detect catechol or dihydrodiol metabolites of recipavrin by monitoring for the phenylmethine-3',4'-catechol or phenylmethine-3',4'-benzenedihydrodiol moieties in the underivatized (m/z 199 and 201 respectively) and TMS derivatized (m/z 343 and 345) bile extracts. No metabolites with these ions were detected. The mass spectrum of an underivatized catechol metabolite of terodiline has been described (Noren,*et al.*, 1985a,b). Therefore instability can be ruled out as an explanation for the absence of catechol metabolites. The catechol metabolites were undoubtedly intermediates in the formation of the four 3'-O-methylcatechol metabolites (91-94) by catechol-O-methyltransferase.

vi. N-oxidation

Only two N-oxidized metabolites of recipavrin, the oxime (19) and N-oxide (53), were observed in the bile extract. The N-oxide decomposed during GCMS analysis to the Cope rearrangement products, cis and trans 1,1-diphenylbut-2-ene (54 and 55).

The most abundant metabolite was the oxime (19), which was observed in conjugated and nonconjugated extracts in both bile and urine. The oxime was further transformed to phenolic and O-methylcatechol metabolites (89 and 93). Syn (Z) and anti (E) geometric isomers of the oxime were observed in the extracts. The major component occurred at long retention time and corresponds to the anti (E) isomer. The minor isomer only afforded a detectable molecular ion as the TMS derivative. The predominance of the anti isomer in metabolic extracts may be due to metabolic or chemical factors. Beckett (1971) has indicated that the syn isomer of phenylacetone oxime is more susceptible to acid hydrolysis than the anti isomer.

In this study, bile was exposed to pH 5 for the duration of the glucuronidase incubation. This condition could contribute to the selective hydrolysis of syn diphenylbutanone oxime to diphenylbutanone. Alternatively, metabolic oxime formation may be stereoselective, as it is in the metabolism of acetophenone imine (Gorrod and Christou, 1986). The order of elution of major and minor isomers is reversed in the phenyl ring oxidized oximes and their TMS derivatives. The

mass spectrum of the underivatized syn oxime isomer is very similar to the spectrum of the unidentified metabolite XIII of terodiline (Noren, *et al.*,1985b).

Oxime metabolites of alpha methyl tertiary amines are well known. Dimethylamphetamine affords phenylacetone oxime in both free and conjugated forms *in vivo* (Beckett and Al Sarraj, 1972). It is difficult to determine the precursor to the oxime (19) *in vivo* since it is also a major metabolite of norrecipavrin (15) and dinorrecipavrin (20)(see next section). It is possible that at least some of the oxime can be accounted for by oxidation of the primary or secondary hydroxylamines (22 and 17) or nitrones (24 and 44)(Clement and Beckett, 1981, Coutts and Beckett, 1977 (review), Lindeke, 1982 (review)). The oxime of phenylacetone glucuronide (synthesized with immobilized glucuronyl transferase) has been characterized in its intact form by mass spectrometry (Fenselau and Yellet, 1986). Since oximes of this type are good substrates for glucuronyltransferase, it is likely that the bulk of the oxime metabolite of recipavrin arises from an oxime glucuronide rather than by post hydrolysis oxidation of a primary hydroxylamine glucuronide.

Secondary arylaliphatic hydroxylamines (RN(OH)R') are *in vitro* metabolites of other basic tertiary (Clement and Beckett, 1981, Beckett, *et al.*, 1983) and secondary amines (Coutts and Beckett, 1977). Demonstrating the existence of hydroxylamines *in vivo* is not always possible (Beckett, *et*

al., 1983). The reason for the difficulties in characterizing *in vivo* hydroxylamines was presumed to be the ease with which the hydroxylamines undergo oxidation, especially under the aerobic and mildly alkaline conditions commonly employed to extract other more basic metabolites (Beckett, *et al.*, 1977) and due to the facile condensation of hydroxylamine metabolites with any available aldehyde to afford nitrones (Beckett, *et al.*, 1979). The hydroxylamines (17 and 22) were derivatized with BSTFA or TMAH for GCMS analysis using methods described by Beckett and Achari (1977).

No hydroxylamines were detected in the hydrolyzed bile extracts. The sample workup conditions employed here are likely to have oxidised any glucuronidase liberated primary hydroxylamine (22) to the oxime (19), or allowed a condensation of the hydroxylamine with formaldehyde present in the extract to afford a methylene nitrone (24). A glucuronidase liberated secondary hydroxylamine (17, $Ph_2CHCH_2CH(CH_3)N(OH)CH_3$) would be oxidized during workup to afford two possible nitrones, 24 ($Ph_2CHCH_2CH(CH_3)N^+(O^-)=CH_2$) and 44 ($Ph_2CHCH_2C(CH3)=N^+(O^-)CH_3$) both of which are isomeric with the secondary formamide (12, $Ph_2CHCH_2CH(CH_3)N(H)CHO$). A N-methylidene nitrone has been documented as a tertiary arylaliphatic amine metabolite in the in vitro metabolism of promethazine (Clement and Beckett, 1981b), and as a metabolite of many secondary arylaliphatic amines (Coutts and Beckett, 1977, (review)). The potential of the nitrones as intermediates in the observation of the formamide will be discussed in the nitrone decomposition section.

B. Nonconjugated biliary metabolites of recipavrin

GCMS results for the underivatized nonconjugated fraction of bile from recipavrin dosed rats are presented in figure 62. Nonconjugated metabolites include recipavrin (9); norrecipavrin (15); diphenylbutanone (14); diphenylbutanone oxime (19); and detectable amounts of phenolic diphenylbutanone (87); diphenylbutanone oxime (89), and recipavrin (90); and O-methylcatechol derivatives of diphenylbutanone (91) and diphenylbutanone oxime (93). The formamide metabolite was not detected in the nonconjugated fraction.

C. Recipavrin metabolism conclusions

In conclusion recipavrin is extensively metabolized in the rat via four major pathways, deamination, N-dealkylation, N-oxidation and phenyl ring oxidation. These pathways (summarized in figure 63) are common to most tertiary and secondary arylaliphatic amines. Mass spectral evidence for the proposed metabolites and their derivatives has been presented along with synthetic data for derivatives of the phenyl ring intact metabolites. One novel compound observed as a minor component of the conjugated biliary extract has been identified by GCMS as the secondary formamide (12). Possible origins of formamide (12) will be discussed in section 7.

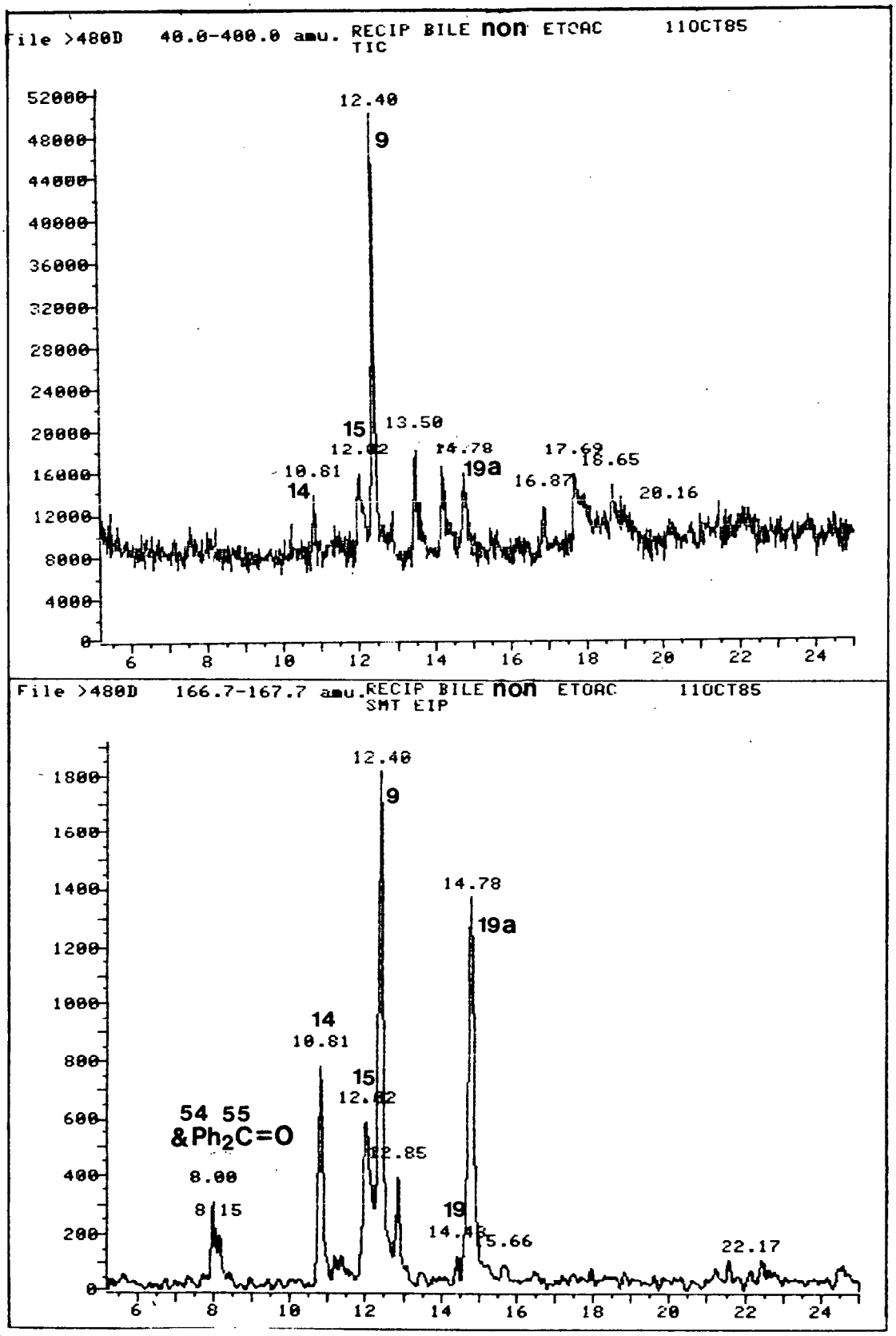


Figure 62. Total ion current and selected ion chromatograms for a nonconjugated extract of biliary recipavrin metabolites. (top) Total ion current for all metabolites. (bottom) Ion chromatogram m/z 167 showing phenyl ring intact metabolites.

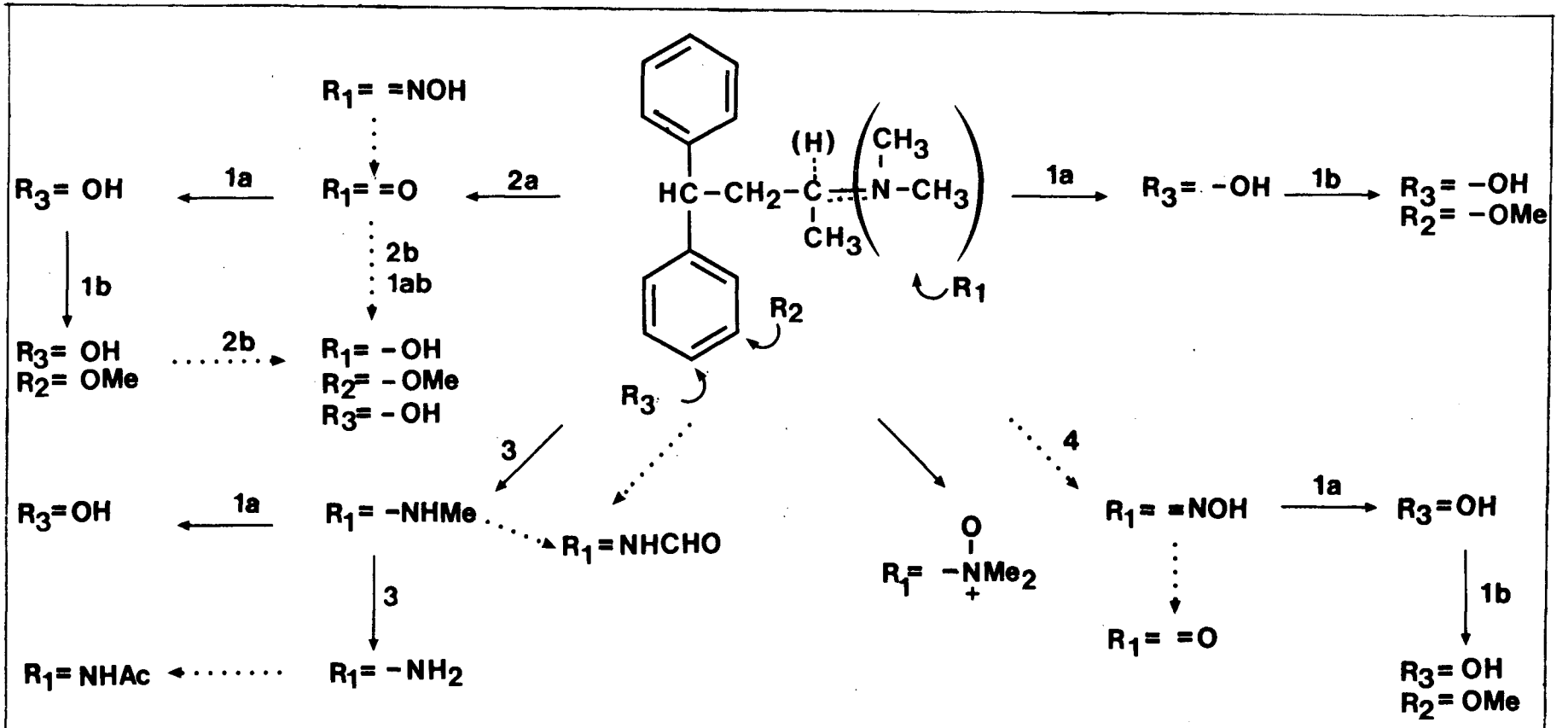


Figure 63. Metabolic pathways for recipavrin based on the metabolites observed by GCMS. 1a. Phenyl ring oxidation. 1b. Oxidation to the catechol and 3'-O-methylation. 2a. Oxidative deamination. 2b. Ketone reduction. 3. N-dealkylation. 4. N-oxidation. Dotted arrows = uncertain origin.

5. Metabolism of Norrecipavrin

The metabolism of norrecipavrin (15) was investigated to determine whether 15 was an intermediate in the conversion of recipavrin (9) to the secondary formamide (12). This was an experiment that was not possible with methadone (8) because of the instability of N-desalkyl methadone, a compound assumed to spontaneously cyclize to the pyrrolidine EDDP (1). Based on the similarity to N-methylamphetamine, norrecipavrin is an excellent candidate for N-oxidation to hydroxylamine and nitrone metabolites, and metabolism was expected to follow the pathways of N-oxidation, N-dealkylation, phenyl ring oxidation and oxidative deamination.

A. Conjugated metabolites

The resemblance of the total ion current (figure 64) for the conjugated fraction of norrecipavrin metabolites to that of recipavrin is evidence for desalkylation of recipavrin as the major metabolic pathway.

Ion monitoring for the unsubstituted (m/z 167, figure 64) and substituted (m/z 183, m/z 213, figure 65) ions of the phenyl ring portion resulted in the observation of the metabolites summarized in table 10.

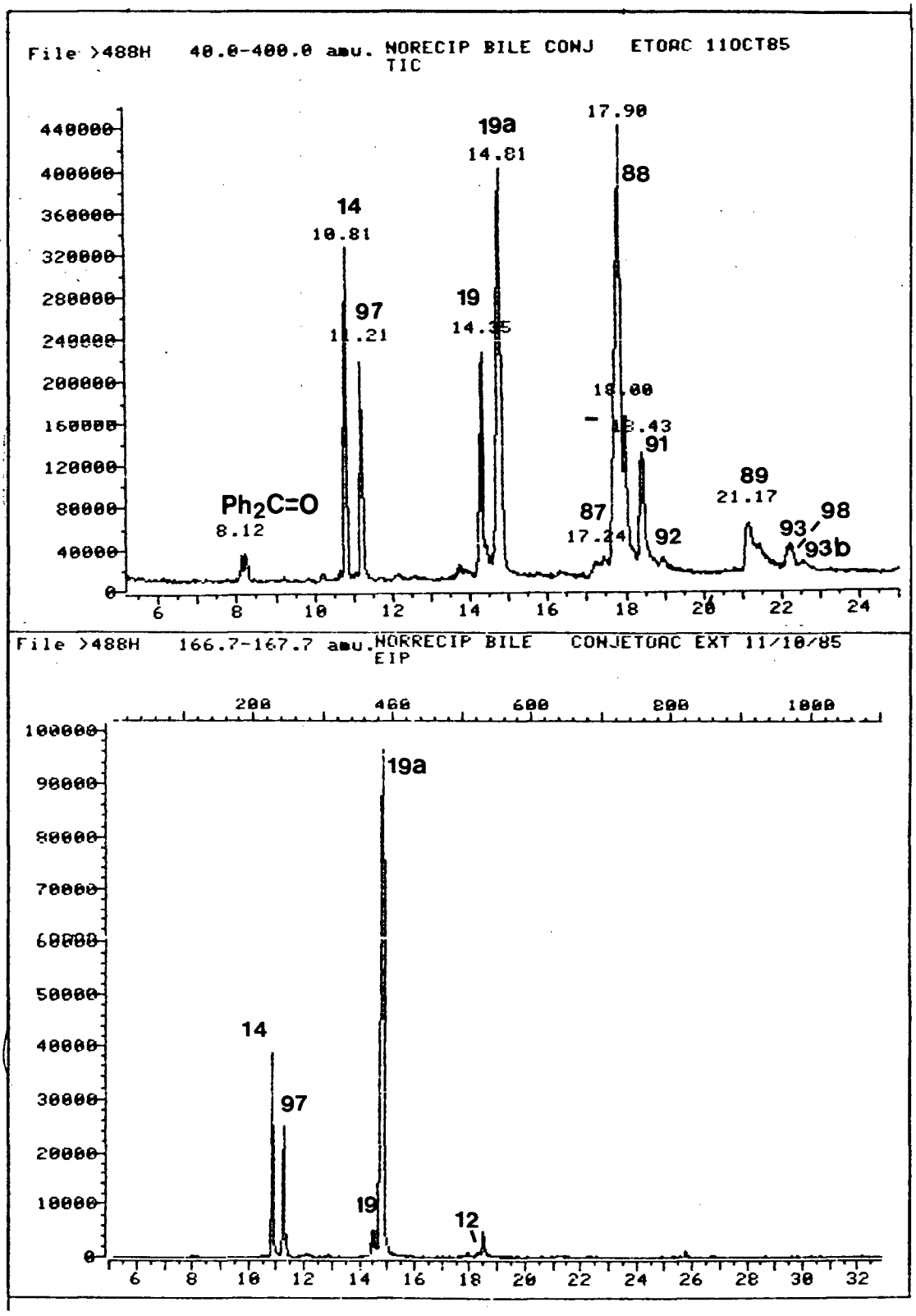


Figure 64. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary norrecipavrin metabolites. I. Total ion current for all conjugated norrecipavrin metabolites (above) Ion chromatogram m/z 167 showing phenyl ring intact metabolites (below).

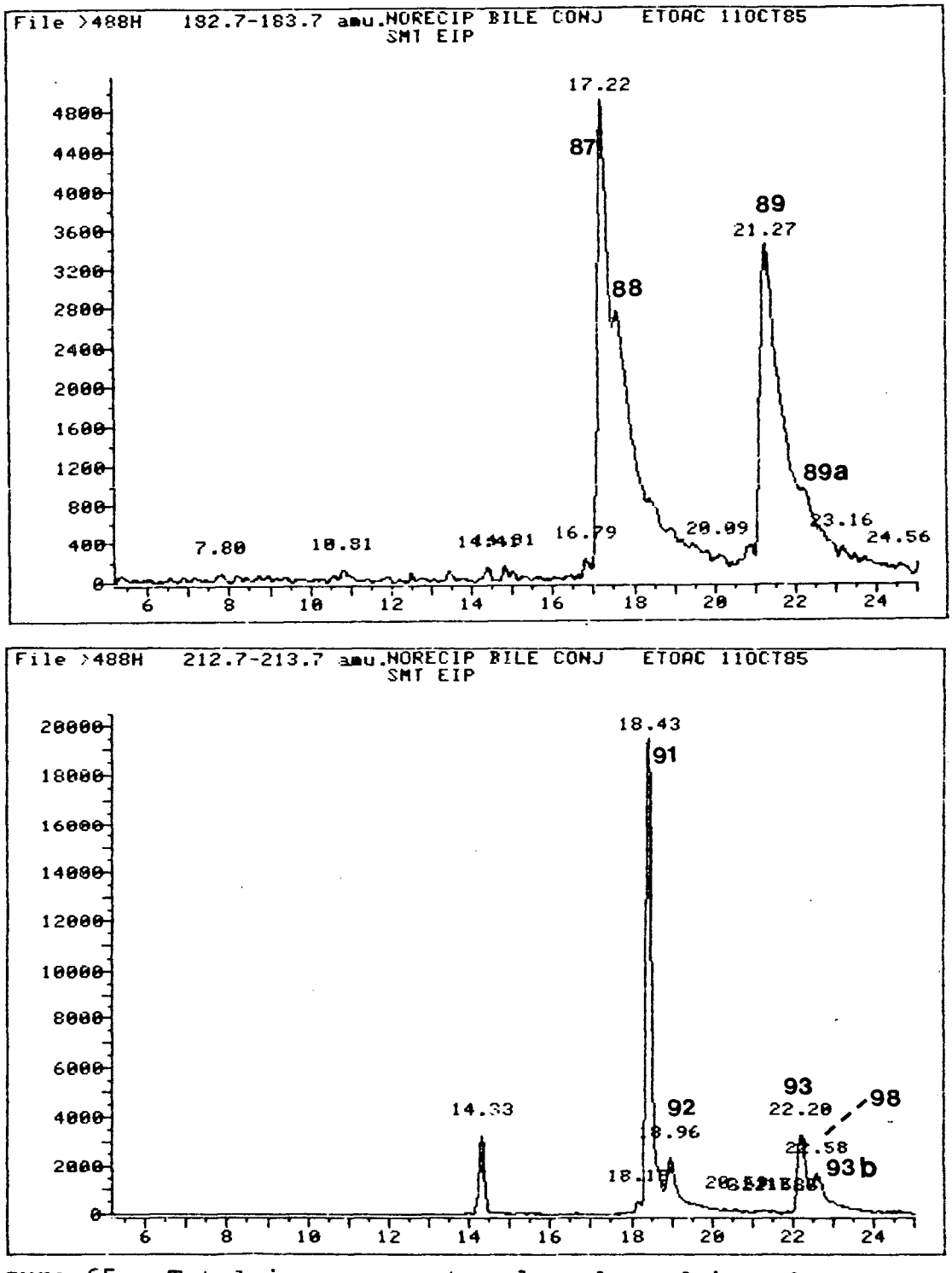


Figure 65. Total ion current and selected ion chromatograms for β-glucuronidase-hydrolyzed extracts of biliary norrecipavrin metabolites. II. Ion chromatogram m/z 183 showing conjugated norrecipavrin phenol metabolites (above). Ion chromatogram m/z 213 showing O-methylcatechol metabolites (below).

Table 10. Retention times and diagnostic ion abundances for the conjugated norrecipavrin metabolites.

Phenyl ring Substitution	Retention Time	Metabolite	Peak Height (diag. ion)
Intact			
14	10.81	Diphenylbutanone	40000
97	11.21	Diphenylbutanol	25000
19	14.53	Cis oxime	4000
19a	14.81	Trans oxime	96000
12	18.20	Sec. Formamide	<1000
Phenol			
87	17.22	Diphenylbutanone	4800
88	17.80	Norrecipavrin	2500
89	21.27	Oxime	3200
O-methylcatechol			
91	18.43	Diphenylbutanone	19000
92	18.96	Diphenylbutanol	2000
93	22.20	Trans Oxime[1]	3000
98	22.28	Nitro Compound	1000
93b	22.58	Cis Oxime	2000

[1] The predominant oxime isomer has been assigned the trans configuration. The reason for the reversal in the order of elution is not clear.

In the conjugated fraction of bile, the secondary formamide (12), while not readily apparent by ion monitoring, was detected and gave a satisfactory mass spectrum. (figure 66 top). Phenyl ring intact diphenylbutanol (97) had not been detected among the recipavrin metabolites. It was identified by the mass spectrum (figure 66 bottom) wherein it was dehydrated to diphenylbutene (55) on electron impact (M^+=226). The diphenylbutene m/z 208 ion follows the arylaliphatic cascade. The metabolic reduction of phenyl ketones is well documented (Smith *et al*. 1954)

The oxime O-methylcatechol (93) was apparently further oxidized to a nitro compound (98) which was identified on the basis of a molecular ion at m/z 301 (figure 67 (top)). Nitro compounds are metabolites of arylaliphatic oximes (Matsumoto and Cho, 1982)

In urine, the phenyl ring intact conjugated metabolites included diphenylbutanone (14), norrecipavrin (15)(11.9 min.) and diphenylbutanone oxime (19) but at much lower concentrations than bile (figure 68). Only traces of diphenylbutanone phenol (87) and diphenylbutanone O-methylcatechol (91) were detected in the conjugated urine fraction. Diphenylbutanol (97) and the formamide (12) were not detected in the urine. The presence of norrecipavrin (15) in the conjugated fraction was unusual (it was also present in recipavrin conjugated fractions). A possible explanation is that it arises from thermal decomposition of the secondary

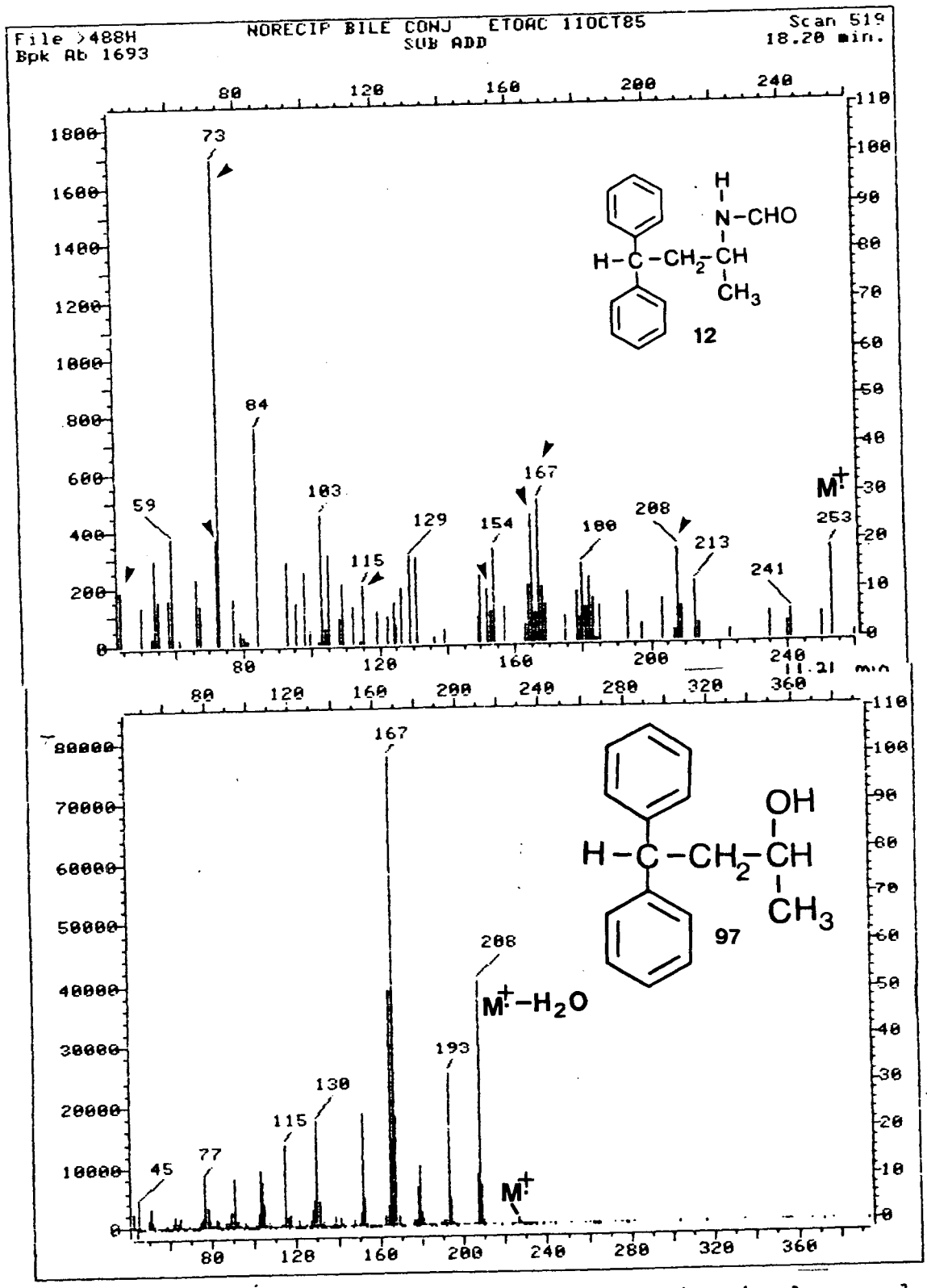


Figure 66. (top) Mass spectrum of the underivatized secondary formamide metabolite (12) of norrecipavrin (15). The intensity of the m/z 73 peak is enhanced by an overlapping endogenous compound with major peaks at m/z 73 and 129 (bottom) Mass spectrum of the norrecipavrin metabolite 1,1-diphenyl-3-butanol (97).

hydroxylamine aglycone in underivatized samples. This hypothesis was not born out by BSTFA derivatization of the recipavrin conjugated fractions (Slatter, 1983).

Decomposition of synthetic secondary hydroxylamine (17) in the GC inlet mainly produced norrecipavrin. The more acidic urine (pH 5) may favor the survival of the hydroxylamine conjugate (42), whereas in bile where the conjugate is exposed to slightly alkaline conditions (pH 8.2) capable of oxidizing the aglycone or the conjugate to a methylene nitrone (24). The nitrone (24) is a possible source of the formamide (12) (see later section). Thus the presence of norrecipavrin in the conjugated fraction may be an indirect indication of the N-oxidation pathway to methylene nitrone (24).

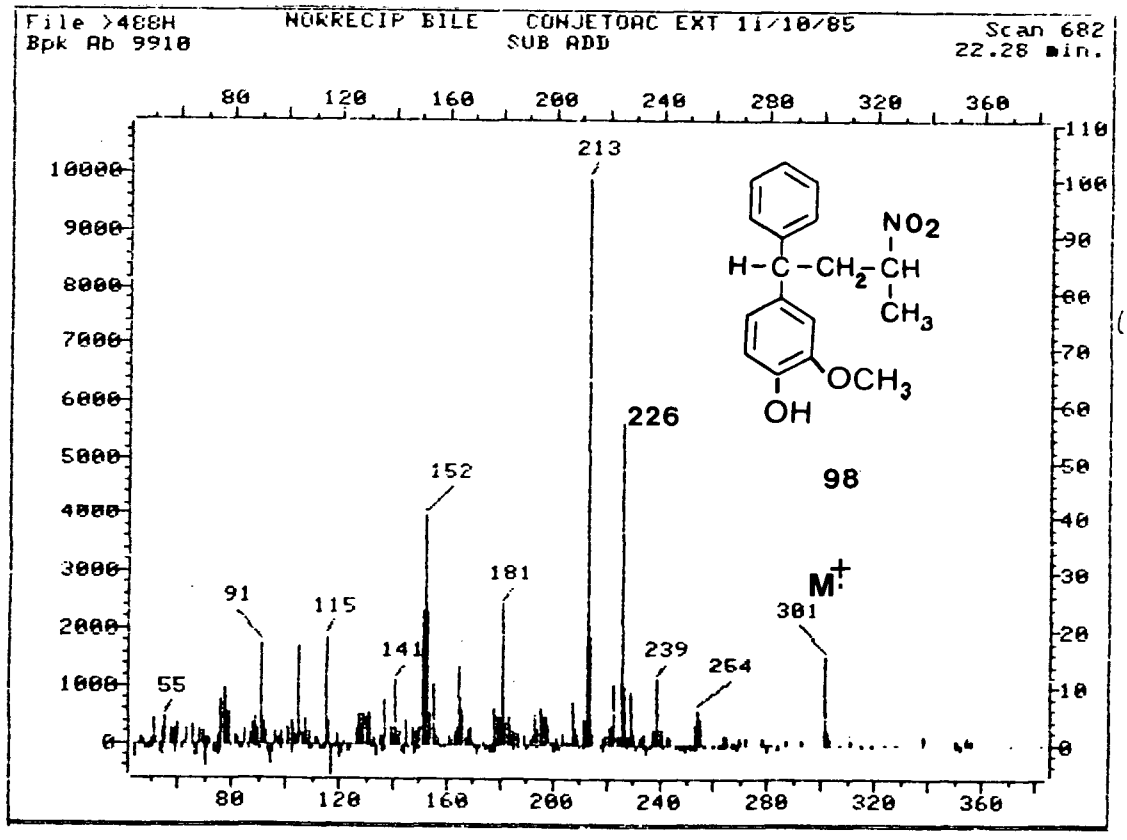


Figure 67. Mass spectrum of the apparent nitro compound O-methylcatechol metabolite (98) of norrecipavrin.

B. Nonconjugated metabolites

The major nonconjugated biliary metabolite was the oxime, with small amounts of diphenylbutanone (14), diphenylbutanol (97), diphenylbutanone phenol (87) and diphenylbutanone O-methylcatechol (91) (figure 69 (top)). In the urine extracts, norrecipavrin (15) was the major component along with small amounts of diphenylbutanone (14), diphenylbutanone phenol (87) and diphenylbutanone O-methylcatechol (91) (Figure 69 (bottom)).

C. Conclusions regarding norrecipavrin metabolism

The pathways of oxidative deamination (diphenylbutanone), N-oxidation (oxime), and phenyl ring oxidation (phenols and O-methylcatechols) are common to all arylaliphatic amines (Caldwell, 1976, Coutts and Beckett, 1977). Dealkylation of norrecipavrin was not observed however, facile oxidation of dinorrecipavrin (20) to the oxime (19) or hydroxylamine (22) may account for the absence of dinorrecipavrin.

The trace appearance of the secondary formamide (12) in conjugated extracts of norrecipavrin suggests that dealkylation of recipavrin is the first step in the genesis of the formamide metabolite. The formamide was still a minor component of the conjugated bile fraction only.

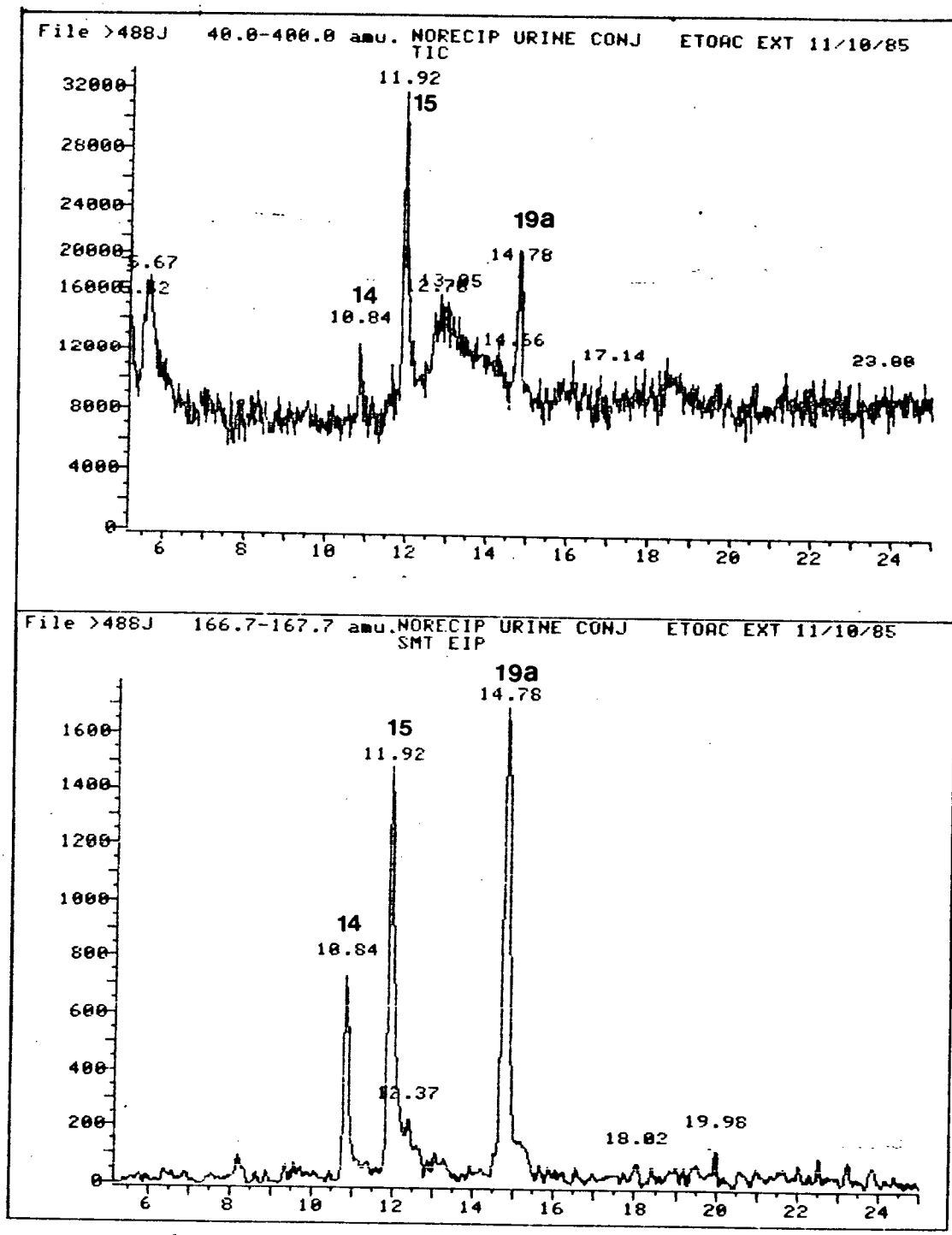


Figure 68. (top) Total ion current for the extract of β-glucuronidase-hydrolyzed fraction of urine from norrecipavrin dosed rats. (bottom) Mass chromatogram m/z 167 for the same sample. Standard GC conditions.

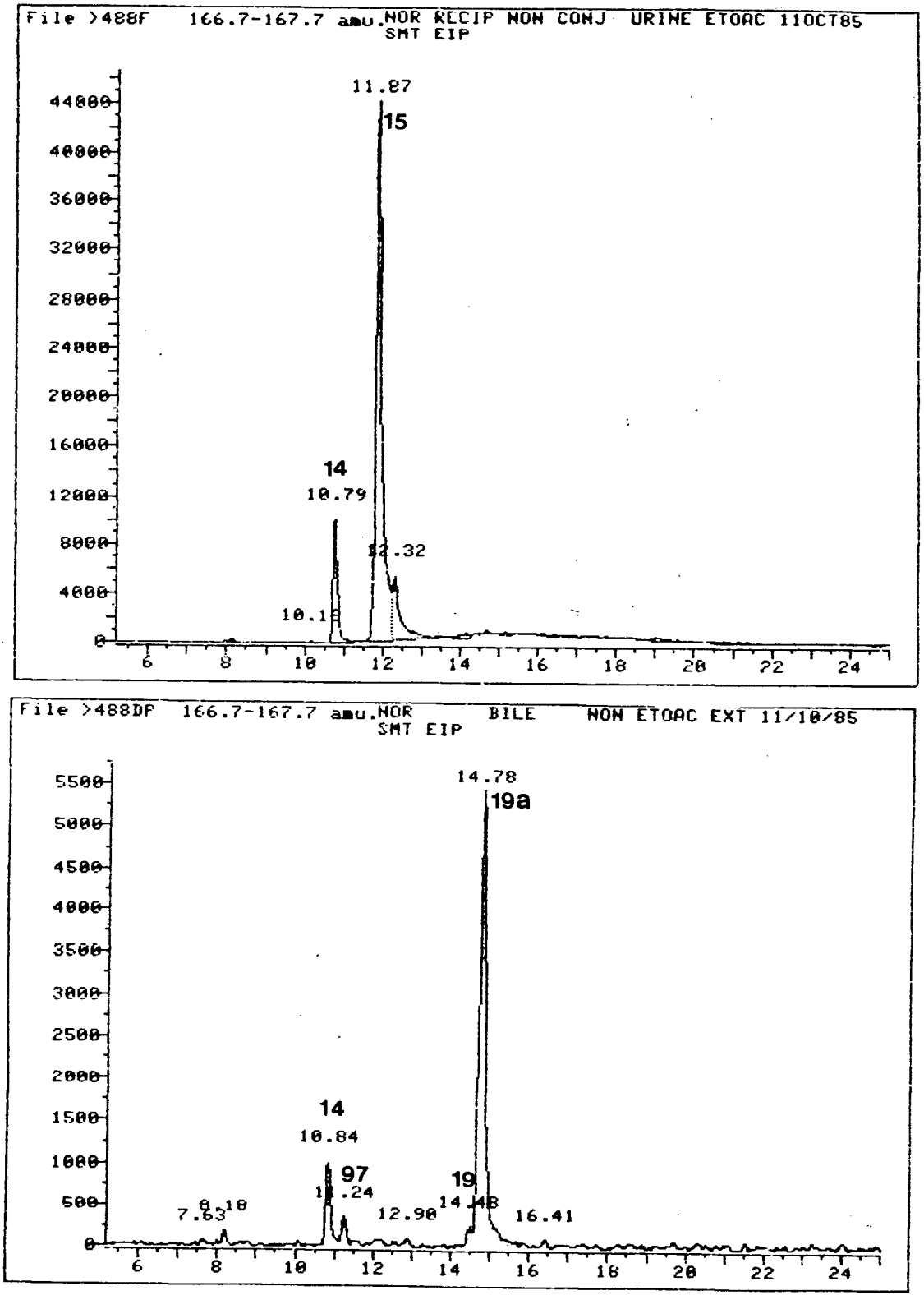


Figure 69. (top) Mass chromatogram m/z 167 for the extract of nonconjugated metabolites of urine from norrecipavrin dosed rats. (bottom) Mass chromatogram m/z 167 for the extract of nonconjugated metabolites of bile.

6. Metabolism of dinorrecipavrin

Dinorrecipavrin (20) is a trace metabolite of recipavrin. The metabolism of dinorrecipavrin was investigated to determine whether a secondary formamide metabolite (12) was among the conjugated metabolites. If this was the case it could be considered an intermediate in the genesis of the recipavrin formamide metabolite.

A. Metabolites of dinorrecipavrin

The total ion current and mass chromatograms 167, 183 and 213 for the β-glucuronidase-hydrolyzed fraction of bile from a dinorrecipavrin dosed rat are shown in figure 70 and 71. The metabolites were identified as summarized in table 11.

Table 11. Retention times and diagnostic ion abundances for the conjugated dinorrecipavrin metabolites.

Phenyl ring Substitution	Retention Time	Metabolite	Peak Height (diag. ion)
Intact			
14	13.56	Diphenylbutanone	14000
97	14.16	Diphenylbutanol	2500
19	18.17	Oxime	20000
Phenol			
87	19.96	Diphenylbutanone	320
89	24.67	Oxime	1800
O-Methylcatechol			
91	21.88	Diphenylbutanone	800
92	22.45	Diphenylbutanol	600
98	25.67	Nitro Compound	1000
93	29.12	Oxime	1800

No formamide (12) was detected in any dinorrecipavrin extract. In the nonconjugated bile fraction, only diphenylbutanone (14) and diphenylbutanone oxime (19) were detected.

TMS derivatization did not reveal any primary hydroxylamine (22). The parent amine dinorrecipavrin was only detected in the nonconjugated urine fraction. Diphenylbutanol (97) formed a TMS derivative (tr=15.42 min.). The mass spectrum had m/z 73/75 ion pair and retention time change, otherwise the mass spectrum was similar to underivatized diphenylbutanol due to the loss of $TMSOH^+$ from the molecular ion to give an intense m/z 208 cascade.

Three possibile sources of a formamide artifact were also investigated. These were based on known synthetic conversions of a primary amine or hydroxylamine to a secondary formamide or nitrone. Although dinorrecipavrin is unlikely to exist as a glucuronide conjugate, the formylation of dinorrecipavrin during sample preparation was attempted by adding excess formic acid to the bile sample prior to extraction. Secondly, the condensation of dinorrecipavrin with formaldehyde, followed by peroxidation to the thermolabile oxaziridine would give rise to the formamide. The last possible reaction involves a hydroxylamine aglycone condensation with

formaldehyde to give rise to the methylene nitrone. The nitrone has been shown to give rise to the formamide under certain GC conditions and in mild alkali (discussed later). These last two possibilities were tested by adding formaldehyde or the antioxidant BHT to the bile sample prior to workup.

Treatment of the β-glucuronidase-hydrolyzed bile extracts with BHT, formic acid or formaldehyde did not significantly alter the metabolite profile. Formate and formaldehyde did not give rise to the secondary formamide. (mass chromatograms in appendix).

On the basis of the metabolism of dinorrecipavrin and its treatment with two sources of formaldehyde, dinorrecipavrin and its metabolites are not precursors in the generation of a secondary formamide metabolite of recipavrin or norrecipavrin.

The pathways of deamination and N-oxidation supported by the array of dinorrecipavrin metabolites are those expected for the homologue amphetamine. The relative amounts of conjugated metabolites and biliary metabolites are probably greater in dinorrecipavrin because, unlike amphetamine it is above the minimum molecular weight (200-250 amu) for biliary excretion in the rat.

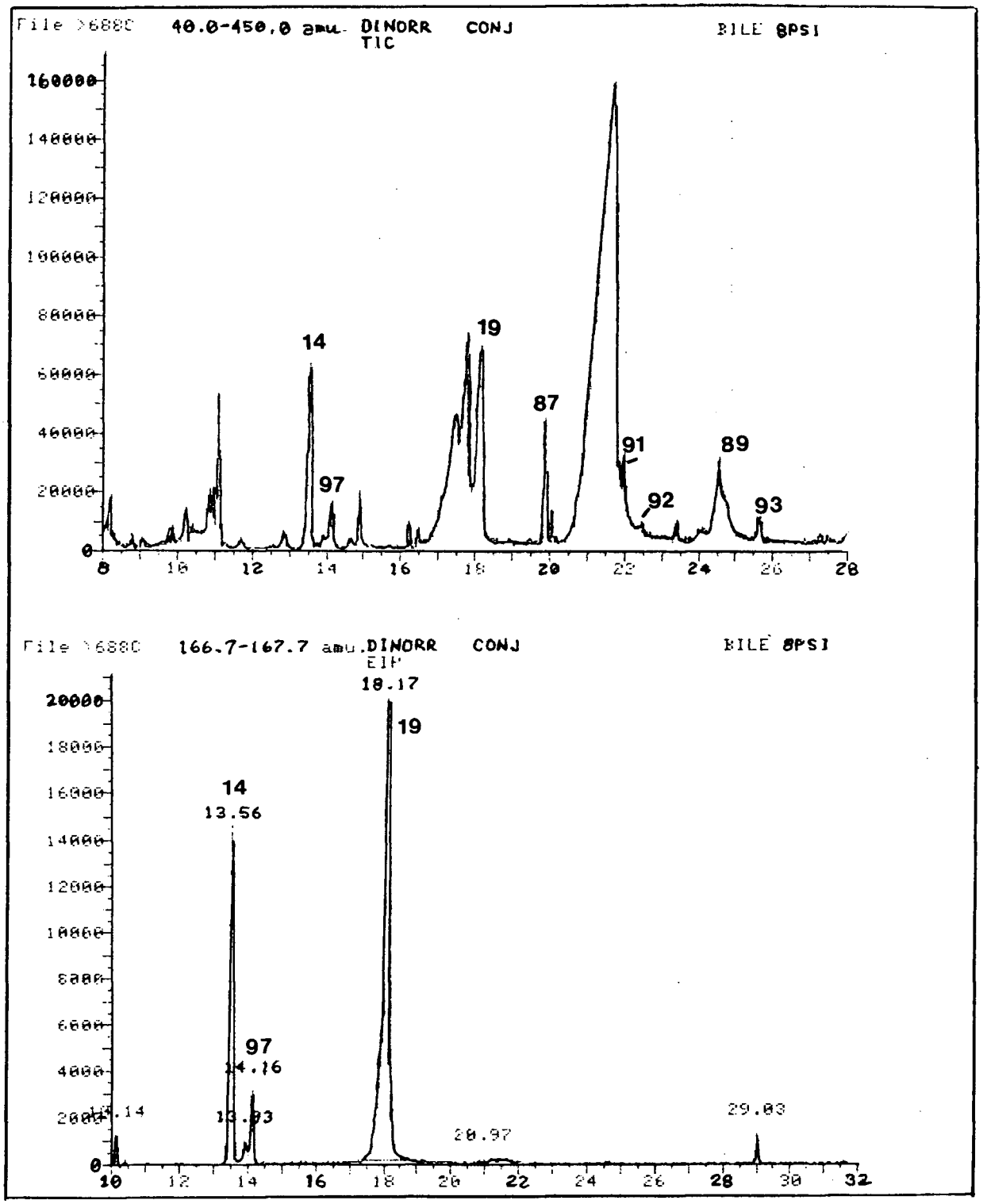


Figure 70. Total ion current for the extract of β-glucuronidase-hydrolyzed fraction of bile from dinorrecipavrin dosed rats. (bottom) Mass chromatogram m/z 167 for the same sample. GC condition B.

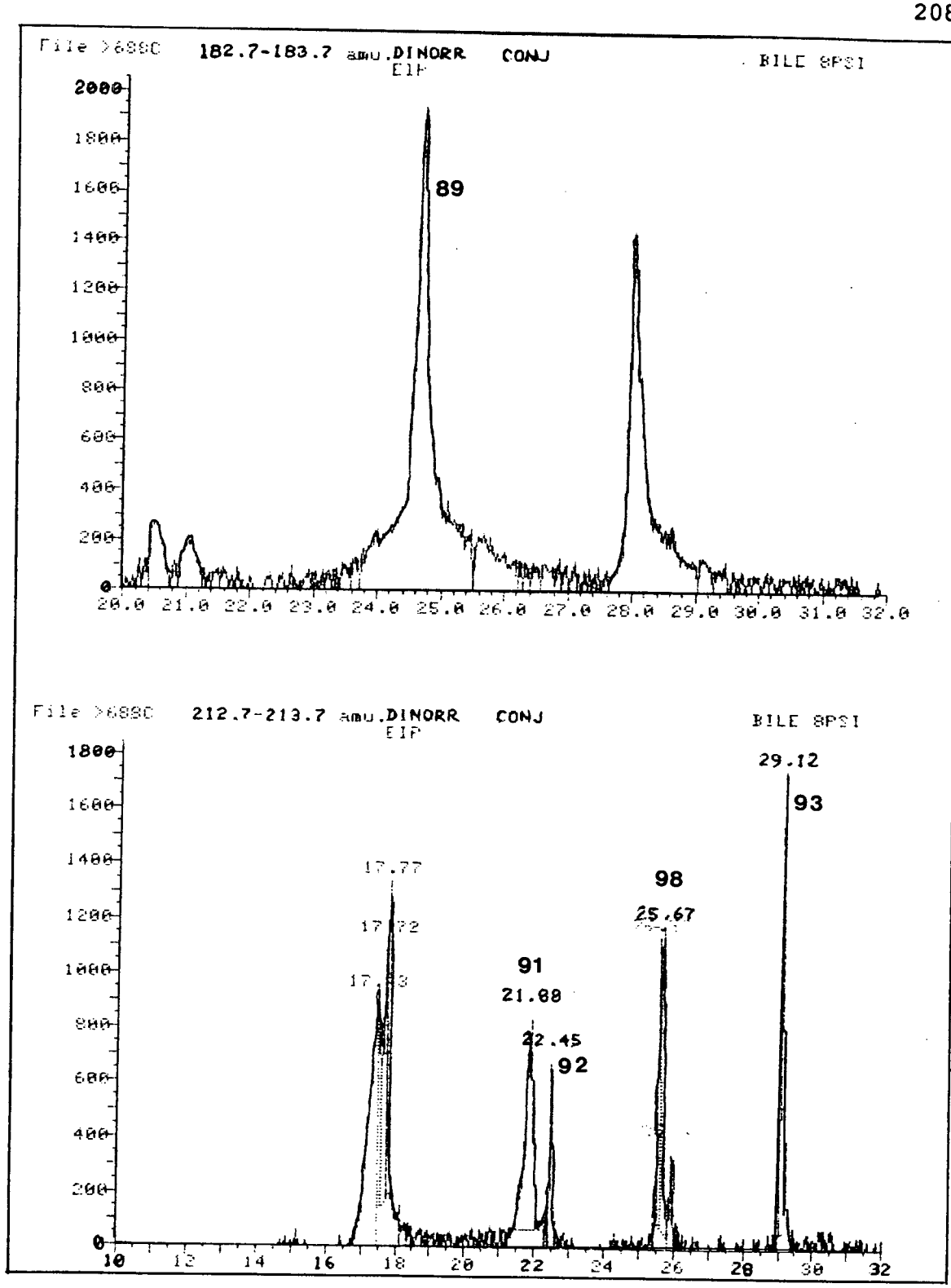


Figure 71. Mass chromatogram m/z 183 (top) and 213 (bottom) for the extract of β-glucuronidase-hydrolyzed fraction of bile from dinorrecipavrin dosed rats. GC condition B.

7. EXPERIMENTS TO DETERMINE THE SOURCE OF THE FORMAMIDE METABOLITE

A. Dilution of bile extracts with the synthetic secondary formamide (12)

Early experiments demonstrating the secondary formamide in the conjugated fraction of bile from recipavrin dosed rats were often confusing due to peak overlap with a major aliphatic bile component, and with recipavrin phenol (90) which has an interfering m/z 72 base peak. This was a problem when steeper gradient temperature programs were employed since peak overlap resulted in poor quality mass spectra even with tedious manual background subtractions. The retention time was also a problem since a small (5-7 second) difference was observed between the synthetic formamide and the bile extract peak.

The spiking experiment compared the intensities of two diagnostic ions of the formamide metabolite (m/z 253, and 167) with the corresponding ions of the overlapping metabolite, recipavrin phenol (m/z 269, and 183), in the presence of increasing amounts of added synthetic secondary formamide. The peak shape and retention time of the formamide were compared from sample to sample to demonstrate co-elution of the metabolic and standard formamide, and the effect on retention time and peak shape induced by co-elution with endogenous bile components. Table 12 shows the effects of exogenous formamide on peak height ratios, peak shape and retention time.

Table 12. The effects of added synthetic secondary formamide standard on peak height ratios, peak shape and retention time of the formamide metabolite present in a β-glucuronidase-hydrolyzed bile extract

Sample number	ng std[1] / ul bile.	tr (m/z 253)[2] rel. to std.	ratio m/z[3] 253/269	ratio m/z[4] 167/183	peak[5] shape
1	0/1	+0.16	0.88	1.08	sh,as
2	30/0	same	-	-	sh,as
3	15/0.5	+0.17	2.15	2.7	ra,as
4	27.3/0.09	+0.02	6.17	7.5	ra,as

1. Synthetic formamide (ng) on column / uL bile on column.
2. Retention time in minutes relative to the synthetic secondary formamide (tr=15.68 min.)
3. Ratio of peak heights of molecular ions of the secondary formamide and recipavrin phenol (m/z 253, m/z 269 respectively).
4. Ratio of peak heights of diarylmethyl cations of the secondary formamide and recipavrin phenol (m/z 167, m/z 183 respectively).
5. Peak shape: sh= sharp, ra= ramped, as= asymmetrical.

The presence of added secondary formamide (12) did not give rise to a second peak in the spiked samples. The presence of bile components resulted in a shift to slightly longer retention times, an effect that was abolished with the lower concentration of bile extract (sample 4, figure 72). The formamide peak was sharp in the undiluted bile and acquired a ramped appearance as the concentration of formamide relative to bile was increased. The ratio of ions 253 and 167 increased relative to the corresponding ions of the other conjugated metabolite recipavrin phenol. This evidence supports co-elution of the metabolite and the synthetic standard. Retention time differences are apparently associated with the co-elution of bile components.

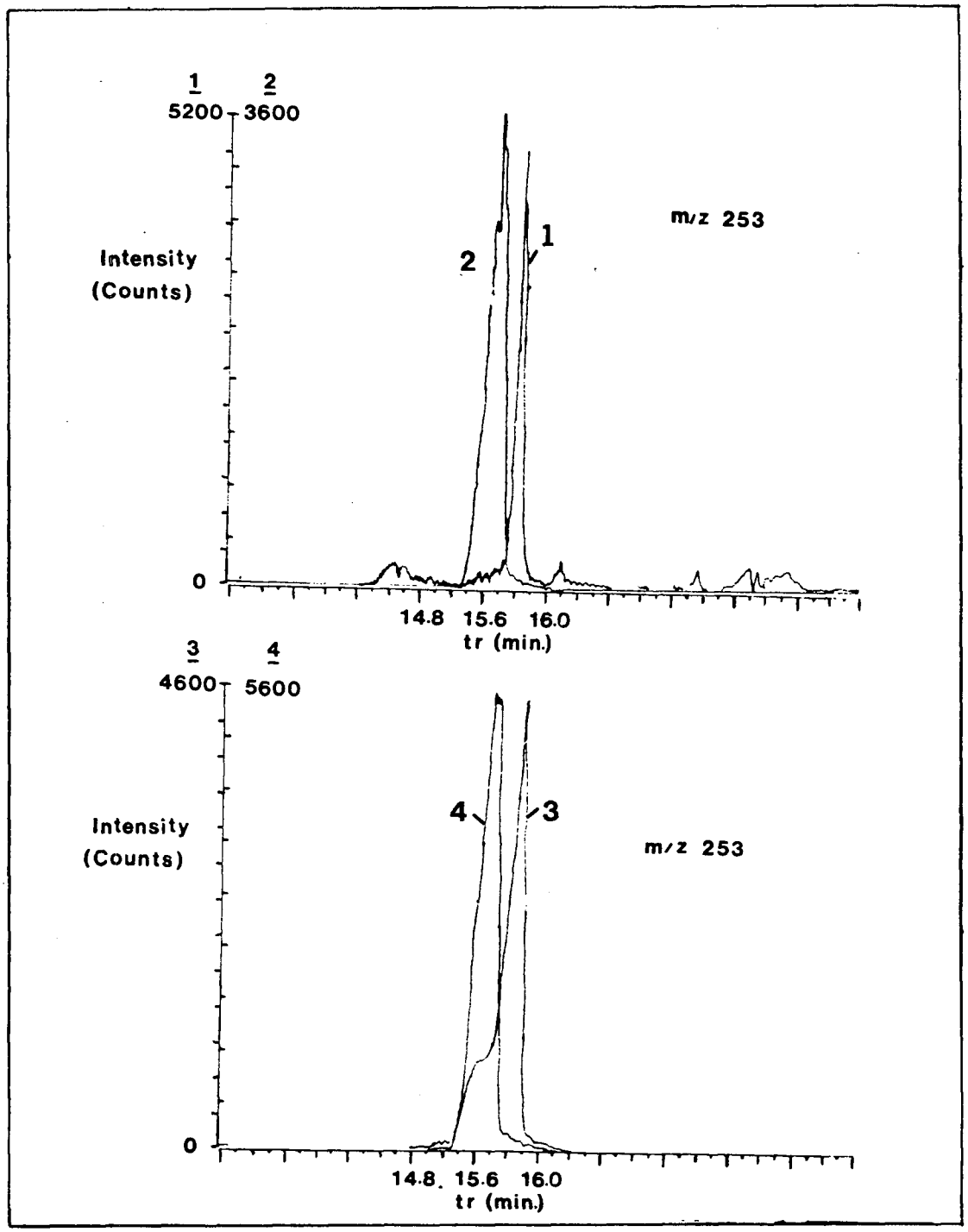


Figure 72.(top): Superimposed mass chromatograms of m/z 253 in the synthetic formamide (12)(sample 2, 30 ng, tr=15.68), and bile extract formamide (sample 1, tr=15.84). Bottom: Mass chromatograms of the m/z 253 ion in spiked samples 3 and 4 showing that a 5 fold decrease in bile concentration brings the retention time of the formamide peak almost back to the standard value (sample 3, tr=15.85, sample 4, tr=15.62).

The sharpness of the recipavrin phenol peak was decreased and retention time was increased (from 15.7 min.) by the addition of co-eluted secondary formamide.

B. Attempted detection of chloroform generated artifacts

The isocyanide (65) and carbamates (66-69) were synthesized as reference compounds for the detection of formyl chloride or phosgene mediated artifacts arising from chloroform extraction of recipavrin metabolites. This was prompted by the observation of both carbamate and formamide artifacts arising from the use of chloroform to extract metabolites of pethidine (Stillwell *et al*. 1978) and other compounds (Cone *et al*. 1981, Wester *et al*, 1981). It was reasoned that since the formamide artifacts were less abundant than the carbamates in these studies, the carbamates should be apparent when chloroform was used for metabolite extraction.

The conditions which favor formamide artifact generation are chloroform, alkaline conditions plus a primary (or secondary) amine precursor (20 or 15). The formamide could arise by a direct formylation of the amine with formyl chloride or indirectly arise from the isocyanide (65) (Smith, 1964).

The conditions favoring carbamate artifact generation are chloroform, alkaline conditions, an amine precursor and methanol or ethanol. These conditions were all satisfied during bile sample preparation, but no evidence of carbamates was seen in chloroform extracts of recipavrin metabolites (figure 73).

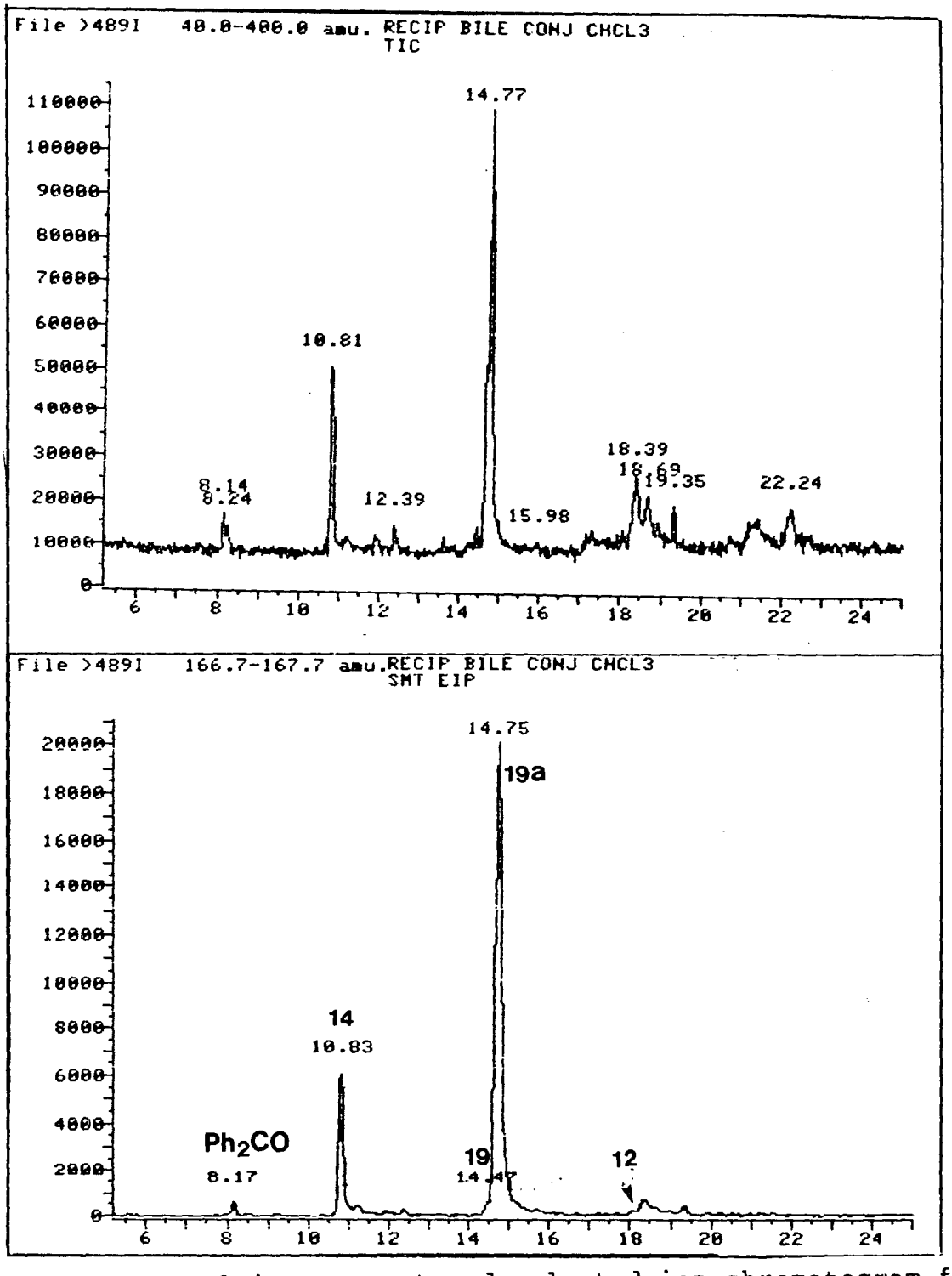


Figure 73. Total ion current and selected ion chromatogram for β-glucuronidase-hydrolyzed chloroform extract of biliary recipavrin metabolites. (top) Total ion current for all metabolites. (bottom) Ion chromatogram m/z 167 showing phenyl ring intact metabolites. The formamide is present at 18.2 minutes.

C. Experiments to establish that the secondary formamide metabolite of recipavrin arises from a glucuronide precursor

i. Sulfatase hydrolysis of the conjugated metabolites

Bile from a rat dosed with recipavrin-D_3 was treated with sulfatase in place of β-glucuronidase, to determine whether sulfatase contaminants in the β-glucuronidase preparation Glucurase^R contributed to the observation of the secondary formamide metabolite. The ion chromatograms shown in figure 74 show that diphenylbutanone (14)(tr=13.37 min.) was present as a major metabolite in the sulfate conjugated fraction. As with the phenylpropanone metabolite of amphetamines (Caldwell, 1976) this was the only sulfate metabolite of consequence. Traces of diphenylbutene (55, tr=10.23), dinorrecipavrin (20, tr=13.78), recipavrin (9, tr=15.08) and diphenylbutanone oxime (19, tr=17.61) and diphenylbutanone O-methylcatechol (91, tr=18.4, m/z 213) were also detected. No secondary formamide (12, tr=21.15) was detected, implying that it arises exclusively from a glucuronide precursor and not from a sulfate.

ii. Control incubation of the recipavrin metabolite conjugated fraction without β-glucuronidase enzyme.

Bile from a rat dosed with recipavrin was extracted free of nonconjugated metabolites and treated with sodium acetate buffer (0.1 M, pH 5) at 38^o overnight to determine whether non enzymatic hydrolysis contributed to the observation of the secondary formamide metabolite.

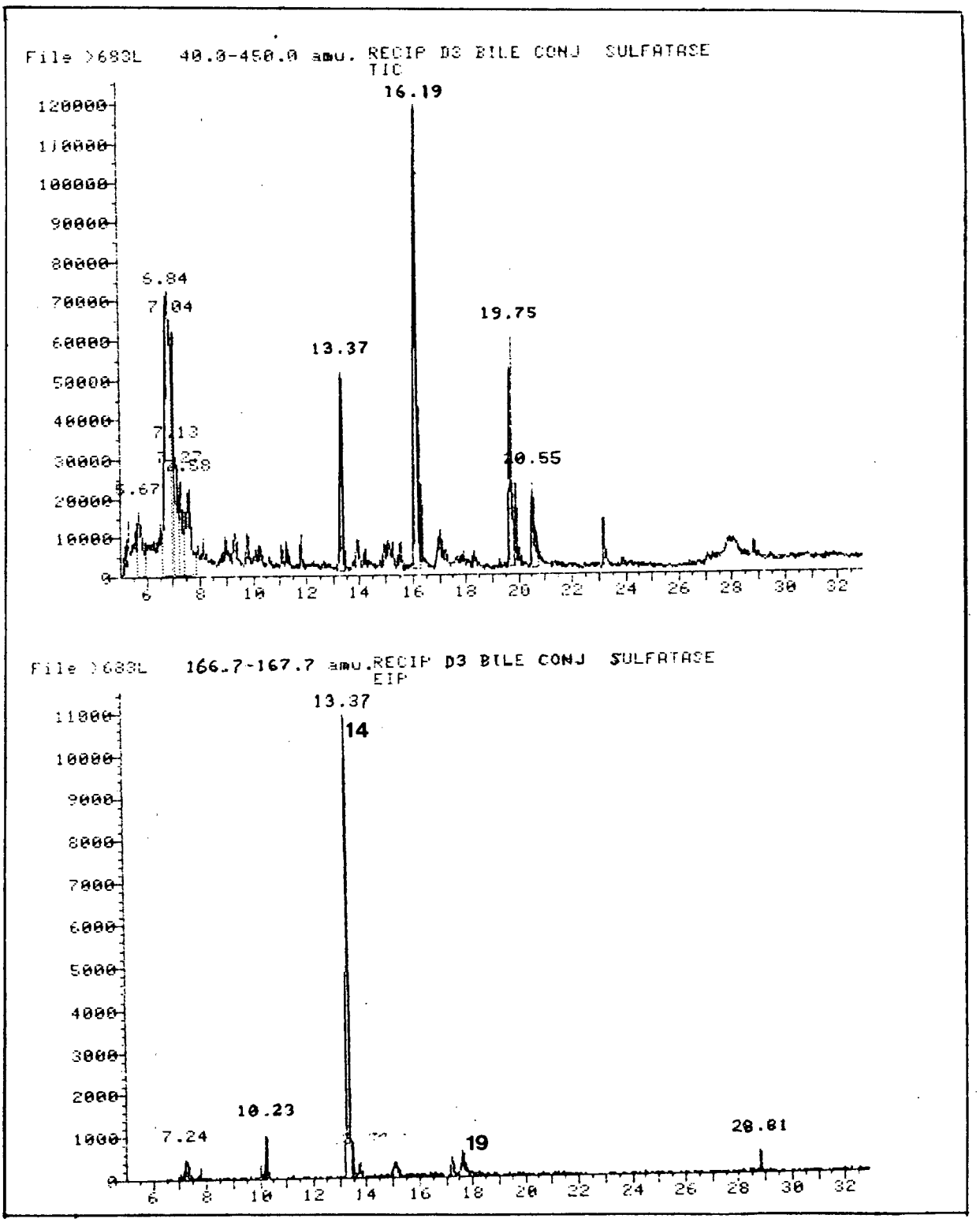


Figure. 74. Total ion current (top) and m/z 167 ion chromatogram (bottom) for the sulfatase hydrolyzed extract of bile from rats dosed with recipavrin-D_3. Diphenylbutanone (14) is present at 13.37 min. GCMS condition B.

Traces of diphenylbutanone (14) and diphenylbutanone oxime (19) were observed at approximately one quarter of the concentration of those observed in a parallel incubation with β-glucuronidase. No secondary formamide metabolite was seen in the non enzymatic incubation thus indicating that the secondary formamide arises from a glucuronide precursor.

D. Free radical oxidation of recipavrin (9) and norrecipavrin (15) as a source of the formamide (12)

The absence of formamides in samples of recipavrin and norrecipavrin used for metabolism studies was confirmed by GCMS prior to administration of the drug. Recipavrin was considered as a potential precursor of the secondary formamide in the conjugated fraction because tertiary arylaliphatic amines are known to undergo N-glucuronidation (Caldwell,1982) and because tertiary amines autoxidize to amides (Henbest and Stratford, 1962). Basic conditions and non polar solvents favor the hydroperoxide mediated oxidation (Beckwith, *et al.* 1983). If recipavrin (9) were the source of the secondary formamide (12), it is likely that the initial oxidation product, the tertiary formamide (26) would also be observed.

i. Incubation of recipavrin with control bile under simulated workup conditions

Recipavrin added to control bile and extracted at alkaline pH after standing at room temperature for one week at

pH 8 did not afford any detectable secondary formamide (12). Recipavrin added to control bile and worked up by the standard protocol did not afford any secondary formamide or tertiary formamide (26) in the nonconjugated or conjugated fractions. This indicates that alkaline pH or oxidation of recipavrin catalyzed by bile constituents is not responsible for the observation of the secondary formamide metabolite. When bile from a recipavrin dosed rat was left standing at room temperature for several weeks before workup the tertiary formamide (26) was apparent by GCMS of the nonconjugated metabolite extract. This shows that a free radical oxidation of recipavrin is possible, but is an unlikely source of the secondary formamide metabolite.

ii. Incubation of norrecipavrin with blank bile under simulated workup conditions

Norrecipavrin (15, $Ph_2CHCH_2CH(CH_3)N(H)CH_3$), which could be oxidized in alkaline solution to the secondary formamide (12, $Ph_2CHCH_2CH(CH_3)N(H)CHO$) was observed as a trace component of the conjugated fraction and therefore was a possible precursor for post enzymatic oxidation to the secondary formamide observed in rat bile extracts.

When blank bile was spiked with norrecipavrin and carried through a normal isolation procedure no secondary formamide was detected by GCMS. This argues against norrecipavrin as a substrate for free radical oxidation to the secondary formamide observed in the conjugated fraction of bile from recipavrin dosed rats.

iii. Effect of extraction pH on the observation by GCMS of the recipavrin secondary formamide metabolite.

The secondary formamide metabolite of recipavrin is a non basic compound and should be extracted from bile under neutral to mildly acidic conditions, along with the other non basic metabolites derived from diphenylbutanone (14) and diphenylbutanone oxime (19). When bile from recipavrin dosed rats was hydrolyzed with β-glucuronidase and extracted at pH 5 with ethyl acetate, the ketone (14) and oxime (19) were apparent, but the secondary formamide (12) was not detected. This implies that alkali may be involved in a transformation of an aglycone precursor to the secondary formamide.

E. Solid phase extraction of recipavrin metabolites

SPE experiments were undertaken to demonstrate the recipavrin formamide metabolite under less rigorous isolation conditions, and to develop a faster isolation scheme. The cartridges were chosen based on literature methods for metabolite group separations (Sjovall, 1983, Sjovall and Axelson, 1982, J.T. Baker Ltd, 1982)

i. Preliminary experiments

a. Elution of synthetic reference compounds from RPC_{18} columns

Aqueous solutions of the synthetic standards diphenylbutanone (14) (a non polar neutral metabolite), dinorrecipavrin free base (20)(a medium polarity basic metabolite) and the secondary formamide (12)(a medium polarity

neutral metabolite and target compound) were separated on 1 ml RPC_{18} columns. The samples were eluted with successive one column volumes of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% aqueous methanol. Ultraviolet measurement of the cartridge eluent revealed that elution of the three standards began with the 30% methanol aliquot and was maximal at 60% methanol in water. Forty percent methanol was chosen as an appropriate minimum concentration for the elution of nonconjugated metabolites on reversed phase cartridges.

b. RPC_{18} test elutions using UV and TLC detection of eluates

Solutions of d-glucuronic acid, methadone HCl, phenolphthalein glucuronide and aliquots of bile from control and recipavrin dosed rats were applied to separate RPC_{18} columns. The columns were washed with water and then eluted with successive aliquots of 20, 40, 60, 80, and 100% aqueous ethanol. Eluates were checked for components of interest by spotting on thin layer chromatography plates and visualizing under UV light or with a variety of spray reagents.

Methadone, a representative basic compound was eluted with 80% ethanol. Glucuronic acid was eluted with the water wash. Phenolphthalein glucuronide was eluted with 20% ethanol, and all bile samples afforded naphthoresorcinol positive spots that were most intense in the 40% ethanol fraction. The conclusions were that 80% ethanol is required to rapidly elute

nonconjugated basic metabolites, and that conjugates are most likely driven from the column with 40% ethanol, along with most other visible (yellow) bile components.

ii. Fractionation of bile components by SPE methods

a. Attempted fractionation of biliary recipavrin metabolites using RPC_{18} columns.

RPC_{18} columns have been used for a preliminary cleanup of glucuronide conjugates for HPLC analysis. The glucuronides were eluted with 100% methanol (Liberato, *et al.*, 1983).

Bile from a recipavrin dosed rat was passed through a RPC_{18} column, the cartridge was eluted first with water and then with methanol. β-glucuronidase hydrolysis, solvent extraction and GCMS analysis revealed no metabolites in the aqueous eluate. The methanol fraction contained a low recovery of metabolites, primarily the oxime (19) and diphenylbutanone (14), plus small amounts of other nonconjugated metabolites. The column was stripped with a variety of less polar solvents and with methanolic pH 5, pH 10 and acidic solutions yet no other recipavrin related compounds were recovered. The common endogenous bile constituents were co-eluted with the metabolites. Thus on the basis of sensitivity, purity, and metabolite group separation the fractionation of recipavrin metabolites on a single RP cartridge was unsuccessful.

b. Preliminary cleanup and deionization of bile using a XAD-2 column

Amberlite XAD-2, a weak lipophilic resin, has been used for preliminary cleanup and deionization of biological samples containing glucuronides. Bile from a recipavrin dosed rat was passed through a XAD-2 column. The aqueous eluate was discarded since glucuronidase hydrolysis and extraction failed to reveal significant amounts of recipavrin metabolites. The ethanol eluent was further purified by SPE to separate nonconjugated metabolites from conjugates, and to demonstrate the formamide metabolite.

c. Fractionation on FPSiO2 columns

The XAD-2 ethanol eluate was passed through a FPSiO2 cartridge. The cartridge was eluted with ethanol, 60% ethanol in water, and then with water. The metabolites were detected in the ethanol wash by TLC with naphthoresorcinol detection. This fraction was blown free of ethanol, hydrolyzed with B-glucuronidase, adjusted to pH 10 and passed through a RPC_{18} cartridge. The cartridge was washed with water, 40% ethanol and 100% ethanol. Several β-glucuronidase-hydrolyzed metabolites, including the secondary formamide (12, tr=17.59 min.), dinorrecipavrin (20, tr=10.16 min.), diphenylbutanone

(14, tr=10.44 min.), norrecipavrin (15, tr=11.56 min.), diphenylbutanone oxime (19, tr=14.24 min.), diphenylbutanone phenol (87, tr=16.5 min.) and diphenylbutanone O-methylcatechol (91, tr=17.8 min.) were found by GCMS in the 40% ethanol fraction (figure 75). The secondary formamide was also carried over into the ethanol fraction (figure 76). The extracts also contained endogenous components which dominated the total ion current. It could be concluded that the ethanol solution applied to the FPSiO2 column was too polar for metabolite group separation or purification, thus a less polar eluting solvent was required.

d. Separation of nonconjugated metabolites on the $FPSiO_2$ column

To separate the nonconjugated metabolites on the $FPSiO_2$ column, the XAD-2 ethanol extract was evaporated and reconstituted in 92:8 benzene:ethanol, which was sufficiently polar to just move the N-methylnitrone reference compound on a silica gel TLC plate (this was the most polar synthetic compound available). The solution was passed through the $FPSiO_2$ cartridge and the cartridge was eluted with an aliquot of ethanol to recover conjugated metabolites. Evaporation of the 92:8 fraction revealed the nonconjugated metabolites diphenylbutanone (14, tr=10.42 min.), recipavrin (9, tr=11.91 min.) and diphenylbutanone oxime (19, tr=14.29) by GCMS (figure 77).

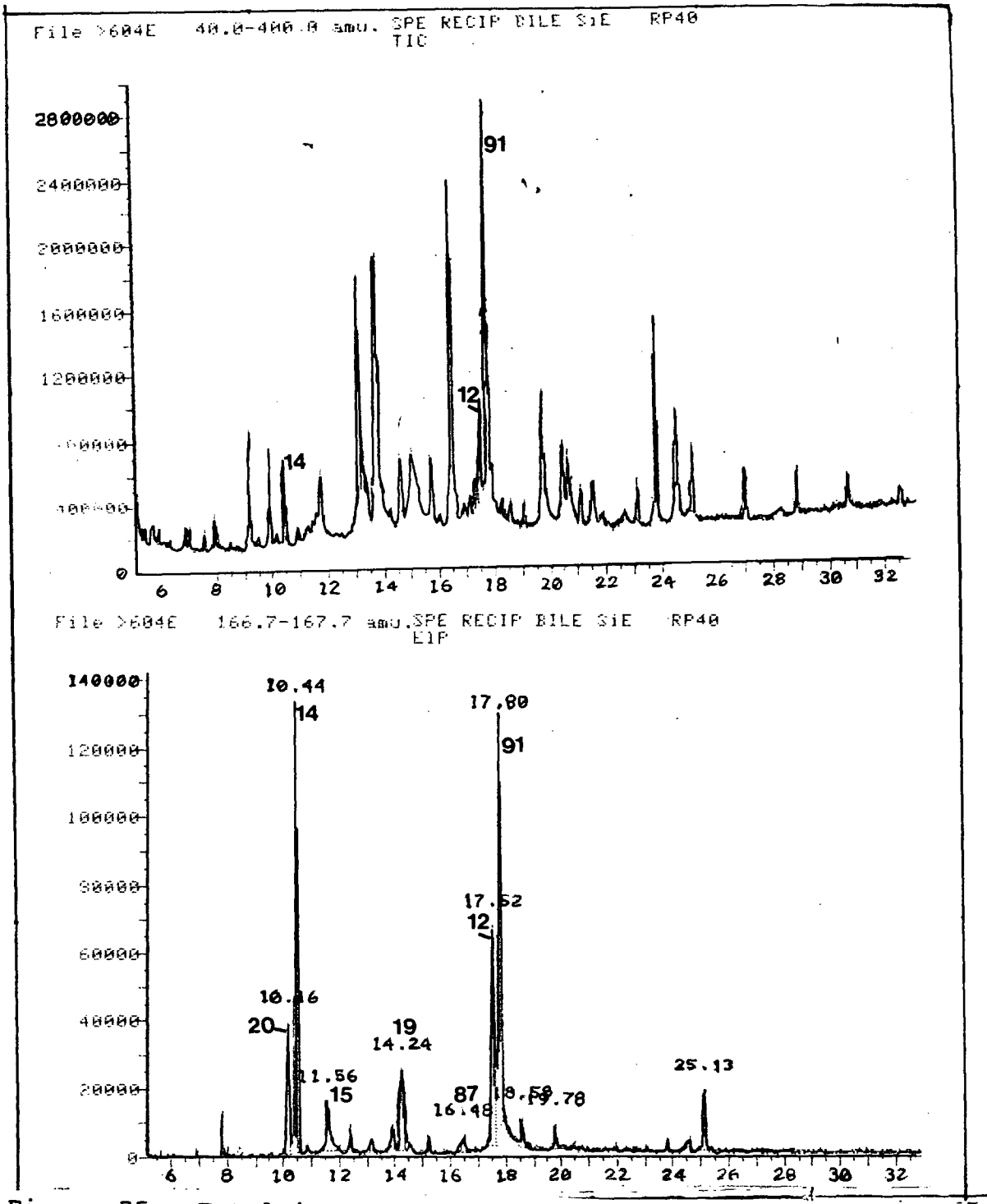


Figure 75. Total ion current (top) and mass chromatogram m/z 167 (bottom) for a β-glucuronidase-hydrolyzed biliary recipavrin metabolites eluted from the $FPSiO_2$ column with ethanol, hydrolyzed with β-glucuronidase and eluted from a RPC_{18} column with 40% ethanol. The electron multiplier was failing when this sample was run.

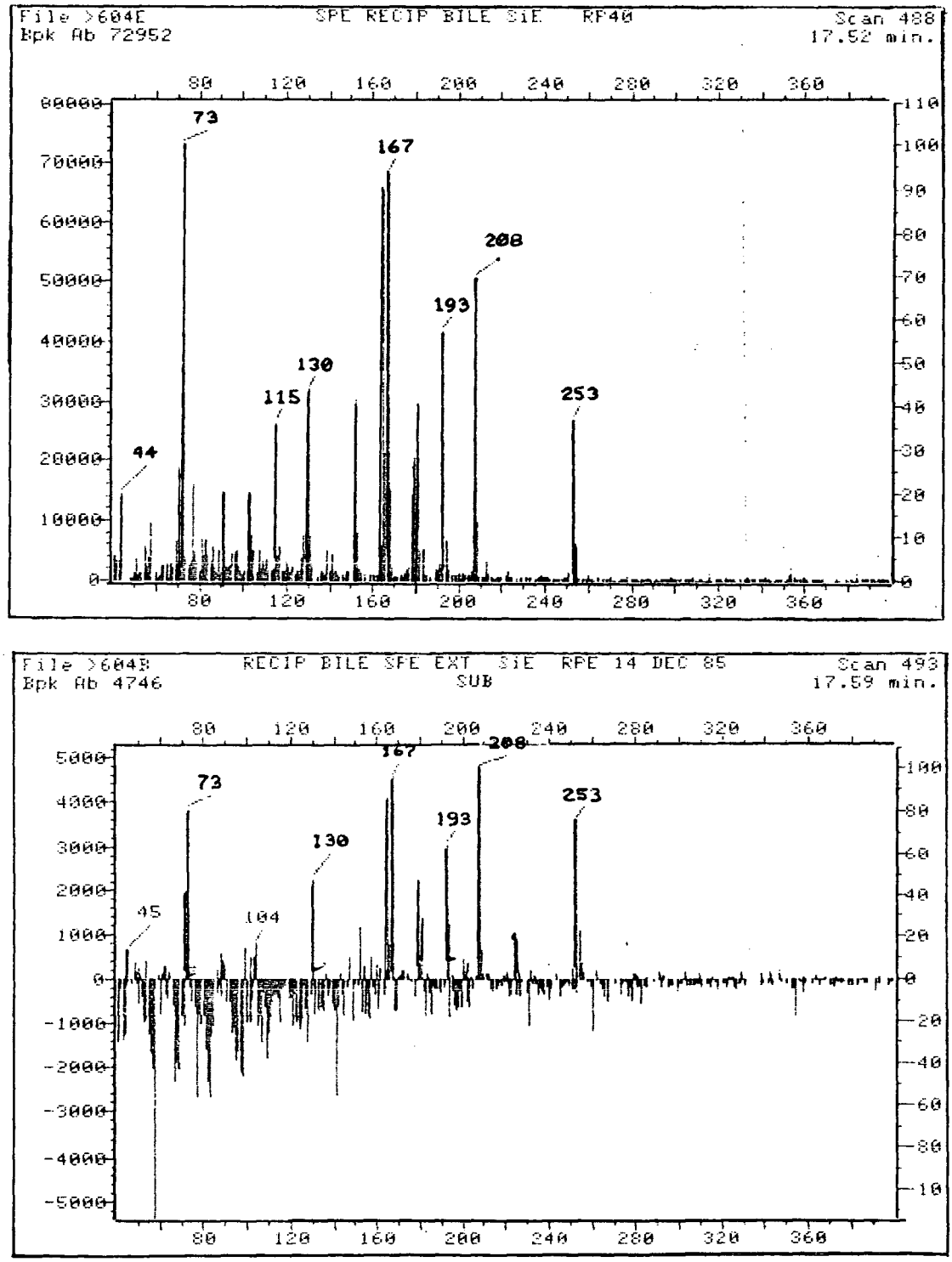


Figure 76. (top) Mass spectrum of the secondary formamide. metabolite of recipavrin eluted from the $FPSiO_2$ column with ethanol, hydrolyzed with β-glucuronidase and eluted from a RPC_{18} column with 40% ethanol.(bottom) Mass spectrum of the secondary formamide metabolite of recipavrin eluted from the RPC_{18} column with 100% ethanol.

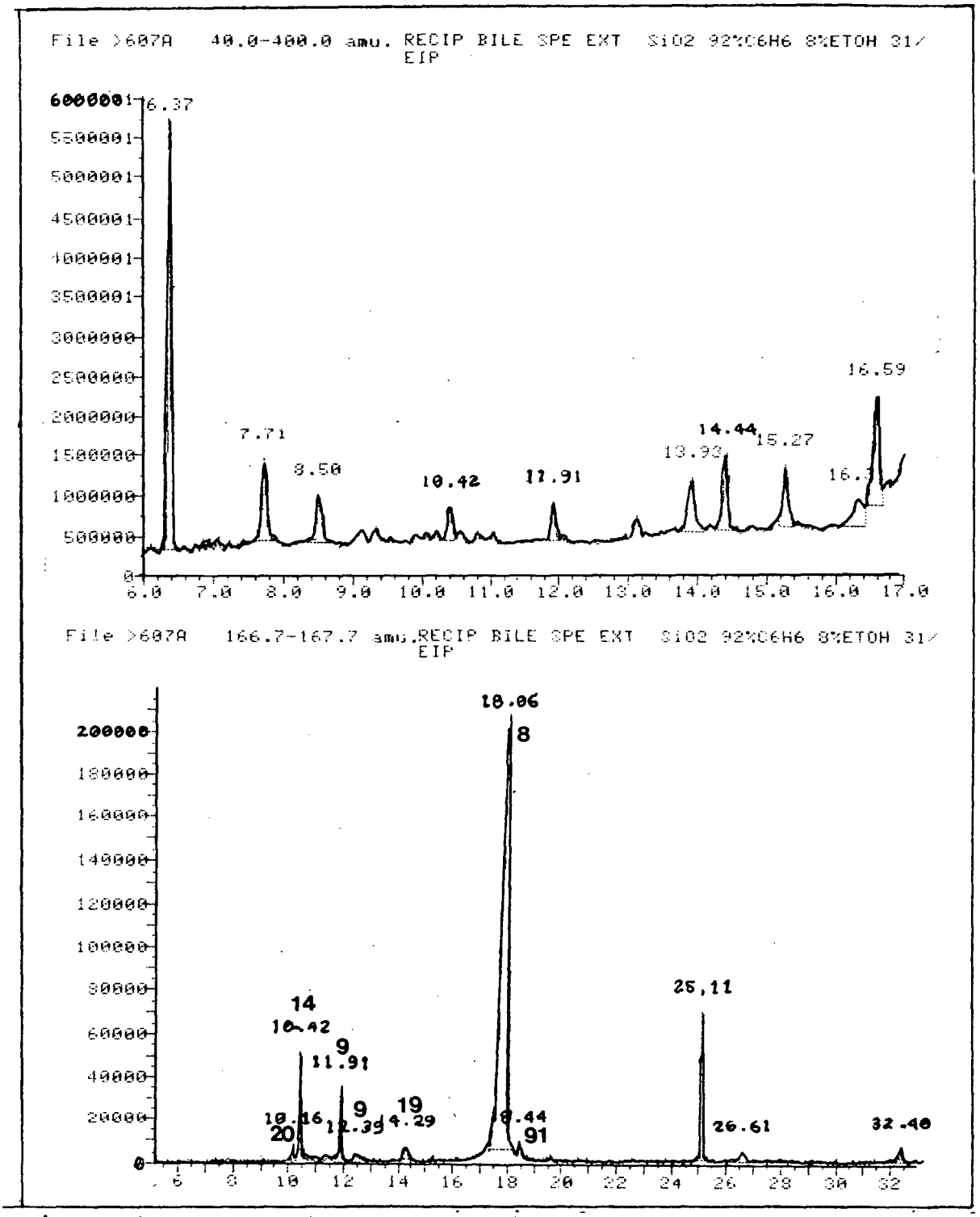


Figure 77. Total ion current (top) and mass chromatogram m/z 167 (bottom) for nonconjugated biliary recipavrin metabolites eluted from the $FPSiO_2$ column with 92/8 benzene/ethanol. Methadone (8) is present as an reference compound at 18.06 min. The electron multiplier was failing when this sample was run.

e. Conclusions regarding the use of SPE cartridges

When sample isolation is complicated by the requirements of metabolite group separation, enzymatic hydrolysis, as well as purification, the use of a series of SPE columns resulted in no savings in time, afforded lower yields, and required large washout volumes for complete elution without carryover. While it was possible to observe the secondary formamide metabolite by GCMS of solid phase extracts, the time required for isolation and the exposure to multiple, (albeit different) solvents negated the utility of SPE in a qualitative investigation of this type. SPE is more useful in automated assays for one or two well characterized components and large numbers of samples. The SPE technique would be much easier to apply to a search for drug metabolites if a radiolabelled drug was used, and cartridge eluates were monitored for radioactivity.

F. Attempts to increase secondary formamide (12) production by the addition of formic acid, formaldehyde, or formaldehyde and hydrogen peroxide during sample preparation.

To investigate possible formic acid mediated formylation of dinorrecipavrin during sample preparation, bile samples were prepared in the presence of a large excess of formic acid. To determine whether formaldehyde is involved in formamide

generation by reaction with primary hydroxylamine (22, $Ph_2CHCH_2CH(CH_3)N(H)OH$) (this would give rise to a nitrone precursor (24, $Ph_2CHCH_2CH(CH_3)N^+(O^-)=CH_2$) of the secondary formamide (12) during sample preparation), bile samples were prepared in the presence of a large excess of formaldehyde. To determine whether oxidation of norrecipavrin (15) to the formamide (12) or peroxidation of Schiff base (23, $Ph_2CHCH_2CH(CH_3)N=CH_2$) to the labile oxaziridine (25) was occurring, bile samples were prepared in the presence of excess hydrogen peroxide. To mimic the synthesis of the oxaziridine (25) from dinorrecipavrin (20, $Ph_2CHCH_2CH(CH_3)NH_2$), the reagents formaldehyde and hydrogen peroxide were added together to the bile sample prior to isolation and analysis.

i. Nonconjugated fraction

The m/z 167 mass chromatograms for the nonconjugated fractions were integrated and the area representing each metabolite was compared to that of the internal standard N-ethyl recipavrin (85). This comparison was only possible in samples not treated with hydrogen peroxide, because H_2O_2 oxidized the internal standard and recipavrin to the corresponding N-oxides (detected by GCMS as the cis and trans diphenylbutenes (54 and 55)) and dinorrecipavrin (20) and norrecipavrin (15) to diphenylbutanone (14) and diphenylbutanone oxime (19). Common degradation products for the recipavrin metabolites and internal standard precluded inter sample comparison of effects of bile treatments.

The secondary formamide (12) was not detected in the control, formate-, formaldehyde-, or peroxide-treated samples. It was however readily apparent in the sample treated with formaldehyde and peroxide, (figure 78, remaining ion chromatograms in appendix). This could represent a Schiff base/peroxidation mediated formation of the thermolabile oxaziridine (25) from dinorrecipavrin (20) with subsequent isomerization to the formamide (12). This is a direct analogy to the oxaziridine synthesis discussed earlier. Since the hydrogen peroxide oxidized all the amines, the generation of the formamide (12) could not be correlated with the disappearance of dinorrecipavrin (20). The absence of the tertiary formamide (26, $Ph_2CHCH_2CH(CH_3)N(CH_3)CHO$) in any of the samples (tr=22.3 min.) and the absence of the secondary formamide (12) in the peroxide treated fraction are evidence against a free radical peroxidation of the secondary amine norrecipavrin (15) to the secondary formamide (12) during sample preparation. Besides proving that the oxidation was possible, this observation was of little value since the formamide metabolite was not present in the nonconjugated fraction.

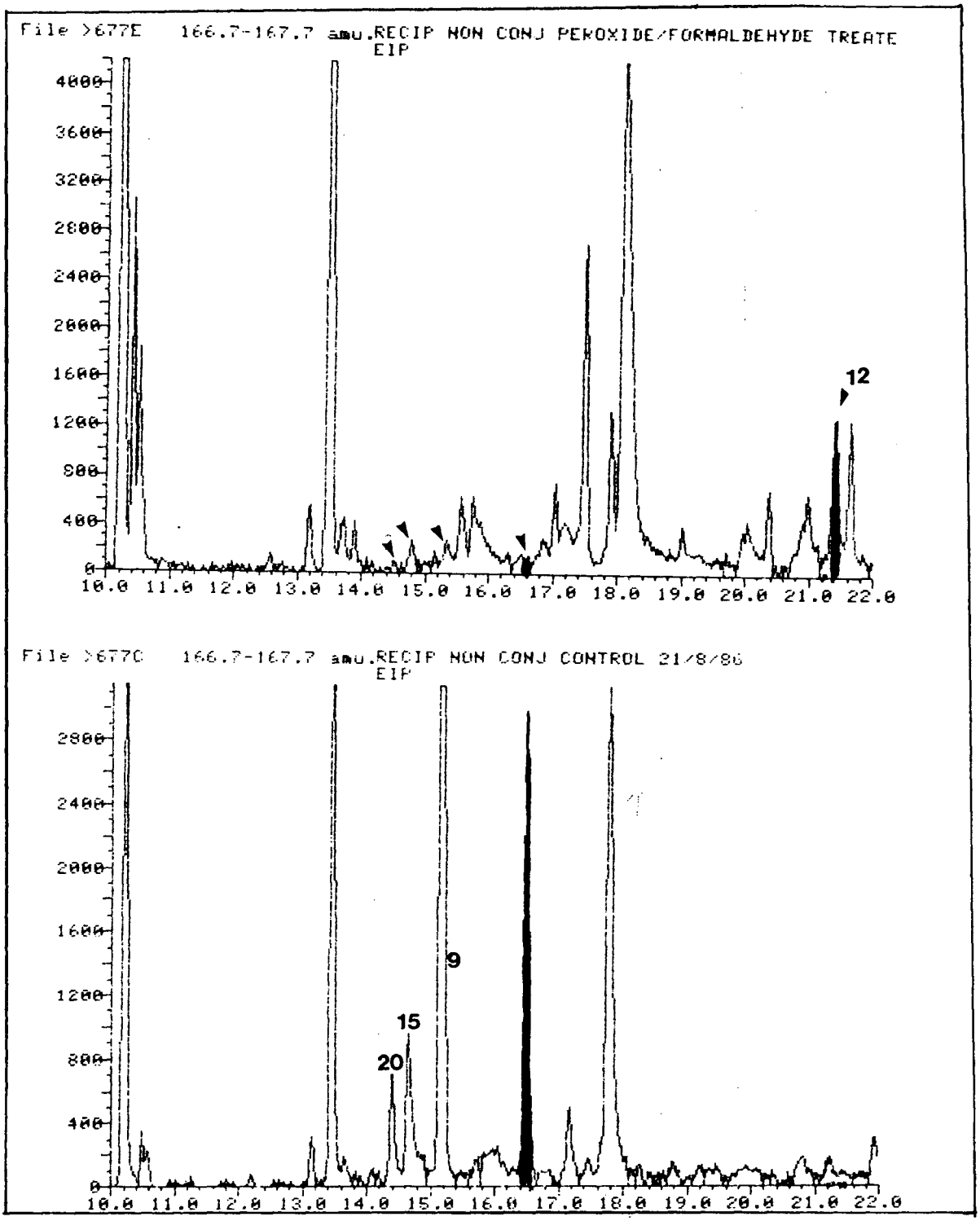


Figure 78. Ion chromatogram m/z 167 of the control (bottom) and formaldehyde/peroxide treated (top) nonconjugated fraction of bile from recipavrin dosed rats. Shaded peaks are internal standard (tr=16.6 min.) and secondary formamide (21.4 min.). Note the destruction of recipavrin (tr=15.2), norrecipavrin (tr=14.7), internal standard (tr=16.6 min.) and dinorrecipavrin (tr=14.4) by treatment with peroxide.

ii. Conjugated fraction

The same problems encountered with the oxidation of the internal standard N-ethylrecipavrin (85, $Ph_2CHCH_2CH(CH_3)N(CH_3)Et$) occurred with the use of terodiline (99, $Ph_2CHCH_2CH(CH_3)N(H)t\text{-}Bu$) to estimate the relative amounts of conjugated metabolites from sample to sample.

The amount of formamide metabolite in each sample was estimated by integrating the area of the m/z 167 peak of the secondary formamide (12), and dividing it by the integrated area of the m/z 100 base peak of the internal standard terodiline (99). The m/z 167 peak of terodiline could not be used because of peak overlap with the oxime metabolite (19). The ratios 0.279, 0.447, and 0.433 for the control, formate, and formaldehyde treated samples respectively indicated a small increase (approximately 1.6 fold) for both treated samples. Visual inspection indicated no relative increase in the peroxide treated sample. The combination of peroxide and formaldehyde increased the amount of secondary formamide (12), and resulted in the appearance of the tertiary formamide (26)(figure 79). The peak intensities were much lower in both peroxide treated samples, possibly due to deactivation of the β-glucuronidase by peroxide, as these samples evolved gas upon addition of the enzyme.

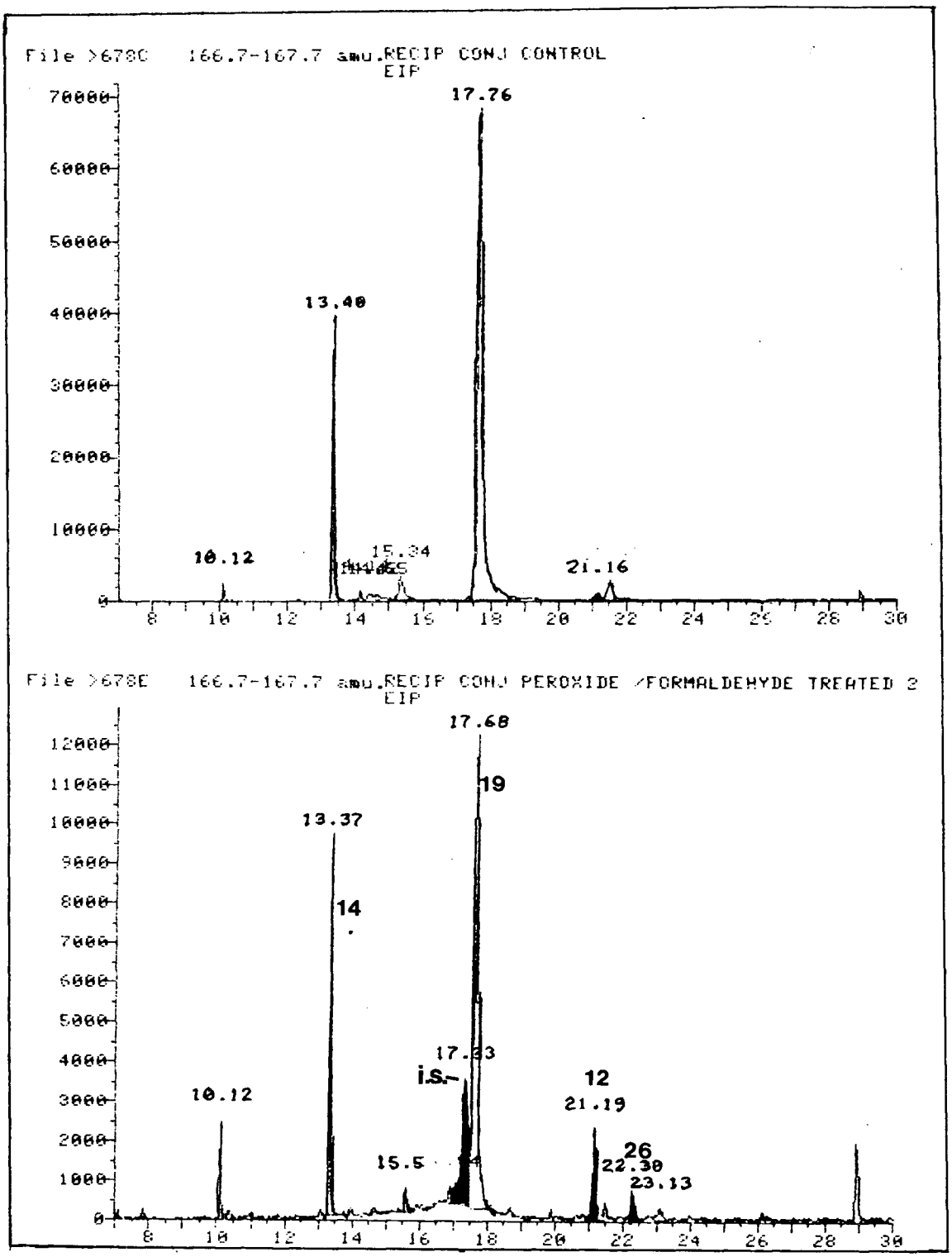


Figure 79. Figure showing m/z 167 mass chromatograms for the control (top) and peroxide/ formaldehyde treated (bottom) conjugated fraction of bile from recipavrin dosed rats. Shaded peaks are the secondary formamide (12, tr=21.2) and tertiary formamide (26, tr=23.13). Other major peaks are the oxime (19, tr=17.7) and diphenylbutanone (14, tr=13.40).GC condition B.

The area of the secondary formamide peak was measured relative to all the integrated m/z 167 peaks in each injection and compared to the same ratio obtained in the control sample. Values of 1.29 (formate), 2.00 (formaldehyde), 0.92 (peroxide) and 12.7 (peroxide/ formaldehyde) bear out that the peroxide/ formaldehyde combination can artificially increase levels of the secondary formamide metabolite.

A possible source of the secondary formamide (12, RNHCHO), dinorrecipavrin (20, RNH_2), was only detected in the formate treated sample. The relative amount of norrecipavrin (15, $RNHCH_3$) stayed close to control values (97% and 87% of control) in formate and formaldehyde treated samples respectively, decreased to 22% of control in the peroxide-treated sample and was not detected in the peroxide/formaldehyde-treated sample. Norrecipavrin is a possible substrate for oxidative production of the formamide in these cases, since peroxidation of N-methylamines is known to afford formamides (Sayigh and Ulrich, 1964). The mechanism whereby formaldehyde enhances the oxidation is not clear.

G. Attempts to decrease formamide production with the use of antioxidants and a formaldehyde complexing reagent

In several of the proposed pathways for the post enzymatic generation of the formamide in the conjugated fraction of bile from recipavrin dosed rats, there is a requirement for an oxidation step.

In one pathway (A in figure 5), the generation of the labile oxaziridine (25) from the imine (23, $RN=CH_2$), requires a peroxidation step. In another, the free radical oxidation of norrecipavrin (15, $RNHCH_3$) by oxygen or hydrogen peroxide would afford the formamide (12, RNHCHO). Lastly, the oxidation of the secondary hydroxylamine (17, $RN(OH)CH_3$) to an alkali or heat labile nitrone (24, $R-N^+(O^-)=CH_2$) could also be a pathway leading to the secondary formamide (B in figure 5). To this end, conjugated fractions of bile extracts from recipavrin dosed rats were worked up in the presence of two antioxidants, ascorbic acid and butylated hydroxytoluene.

In another pathway (B in figure 5), condensation of the primary hydroxylamine (22, RNHOH) with formaldehyde affords the labile methylene nitrone (24, $RN^+(O^-)=CH_2$). To prevent this step in the workup, a formaldehyde complexing agent, dimedone (5,5-dimethyl-1,3-cyclohexanedione, 0.1 M) was added to the bile prior to the hydrolysis of conjugates with glucurase.

Treatment with the antioxidants BHT and ascorbic acid resulted in slight decreases (60% and 80% of control values respectively) in the amount of the secondary formamide (12) present in the conjugated fraction of bile from recipavrin dosed rats. The fact that the formamide was not abolished is a point in favor of a facile oxidation, while the decrease in the amount of the formamide suggests that chemical oxidation is a contributing factor in formamide production. The

formamide was present at levels close to its limit of detection, and these decreases may not be significant. The use of ascorbic acid increased the amount of norrecipavrin (15, $RNHCH_3$) to five times that of the control and decreased the amount of oxime (19, $Ph_2CHCH_2C(CH_3)=NOH$) to one third of control levels. Dinorrecipavrin (20, RNH_2) was not detected at the concentrations of biliary metabolites employed in this experiment.

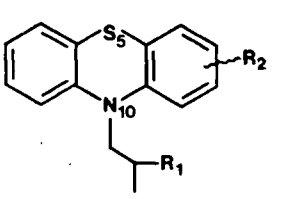
The addition of dimedone to the bile in an attempt to scavenge formaldehyde before it could condense with enzymatically released dinorrecipavrin (20, RNH_2) or primary hydroxylamine (22, RNHOH) resulted in a slight increase in the amount of formamide recovered (136% relative to the control sample). This is the opposite result expected if formaldehyde was involved in formamide production from dinorrecipavrin or the primary hydroxylamine. The increase is difficult to rationalize and its significance is limited without a validated assay and replicated results. The result infers that formaldehyde availability was not a limiting factor and that condensation mediated mechanisms were not major contributors to the generation of the formamide metabolite.

8. METABOLISM OF PROMETHAZINE (10): SEARCH FOR FORMAMIDE METABOLITES

Two factors prompted a detour into promethazine metabolism with the hope of demonstrating yet another formamide metabolite.

The first factor was the structural similarity of promethazine (10) to the other arylaliphatic amines under investigation, i.e. promethazine contains the same isopropyl N,N,-dimethylamine side chain as recipavrin (9, $Ph_2CHCH_2CH(CH_3)N(CH_3)_2$).

The second factor was the publication of the mass spectra of an unidentified *in vitro* metabolite of promethazine (Clement and Beckett, 1981b). This metabolite (X) was thought (probably correctly) to be a ring hydroxylated (phenol) metabolite of promethazine. The metabolite X had a mass spectrum as follows: m/z 300 (M^+,20%); 229 (29); 228 (15); 215 (17); 214 (40); 196 (25); 170 (12); 73 (80); 72 (100); 58 (40); 56 (40); 44 (80); 42 (60). Since the secondary formamide metabolites and the parent tertiary amines are isobaric (M^+=284), the molecular ion m/z 300 for this compound would correspond to a phenolic metabolite of either compound. The m/z 72/73 combination together with enhanced intensity of the higher mass ions (unlike all the parent tertiary amines in this study which have very low abundance ions above the base peak m/z 72) suggested the compound may be the secondary formamide phenol (100), a secondary formamide sulfoxide (101) or N_{10}-oxide (75) (figure 80).

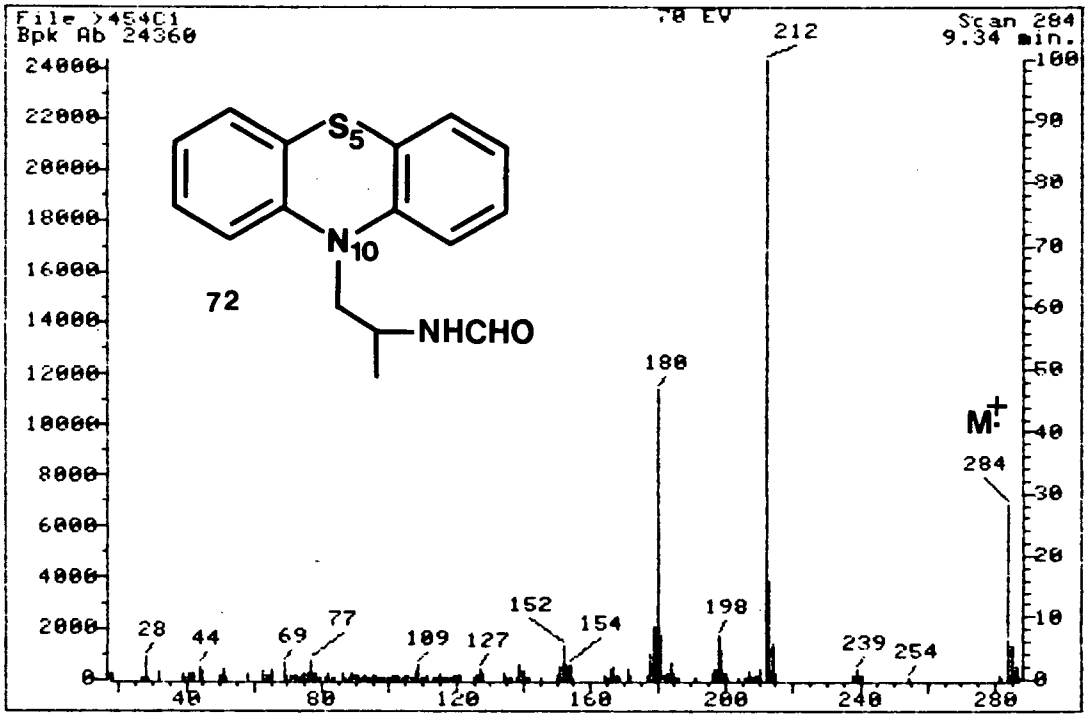


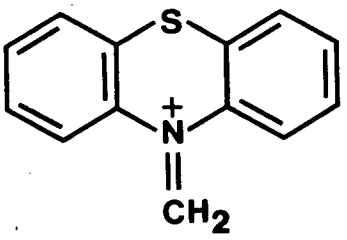
10	R_1= $N(CH_3)_2$,	R_2=H
76	R_1= $N^+(O^-)(CH_3)_2$,	R_2=H,
72	R_1= NHCHO,	R_2=H
100	R_1= NHCHO,	R_2=OH
101	R_1= NHCHO,	R_2=H, $^+S_5$-O^-
75	R_1= NHCHO,	R_2=H, $^+N_{10}$-O^-

Figure 80. Structures of promethazine (10), promethazine N-oxide (76), promethazine secondary formamide (72) and its phenol (100) and sulfoxide (101) and N_{10}-oxide (75).

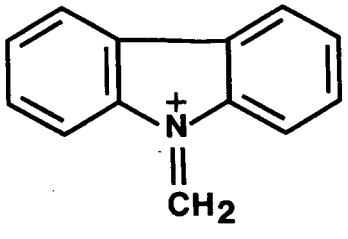
Another metabolite (IX) (Clement and Beckett, 1981b) was assigned the promethazine N-oxide structure (76) based on the following mass spectral data: (20 eV): 300 (M^+, 3); 284 (6, M^+-16); 239 (9); 213 (32); 212 (8); 199 (11); 198 (4); 72 (100). This mass spectrum was similar to that which would be expected of a secondary formamide analogue of promethazine (72, M^+=284, base peak m/z 72). The weak ion at m/z 300 was, however, a point against this possibility. The possibility of identifying a formamide metabolite of promethazine prompted the Leuckart synthesis of the secondary formamide (72) for comparison to the literature compounds IX and X.

The mass spectrum of the secondary formamide analogue of promethazine (Figure 81. (top)) was very different from the literature metabolites. There was no detectable m/z 72, unlike any of the other formamides that had been studied to date. The m/z 212 base peak and the intense m/z 180 ion corresponded to the alpha cleavage products shown in figure 81 (bottom). Low eV and DIP mass spectra failed to enhance the m/z 72 ion (appendix).





m/z 212



m/z 180

Figure 81. (top) Mass spectrum of the synthetic phenothiazine secondary formamide. (bottom) Methylene phenothiazine (m/z 212) and β-carboline (m/z 180) resonance stabilized cations arising from ring directed heterolytic cleavage.

With perfect hindsight, it was apparent that the alpha heterolytic cleavage reaction that affords the dominant m/z 72 base peak in basic compounds like promethazine was not likely to be as dominant in the less basic formamides. In the recipavrin secondary formamide this resulted in an increase in the intensity of higher mass ions, due to energetically feasible alternative fragmentations. In the promethazine formamide, alpha cleavage still occurred, but with charge localization on the resonance stabilized m/z 212 methenyl-phenothiazine cation instead.

Two additional syntheses of promethazine analogues were done. The first involved the peroxidation of the secondary formamide to the N_{10}-oxide (75), possibly similar to metabolite X. The formamido N_{10}-oxide (75) was thermolabile and decomposed in the GC inlet to the formamide (72). The direct inlet mass spectrum was similar to the formamide (72) but had a small molecular ion at m/z 300 (figure 82). It was clearly not similar to the metabolite X described by Clement and Beckett.

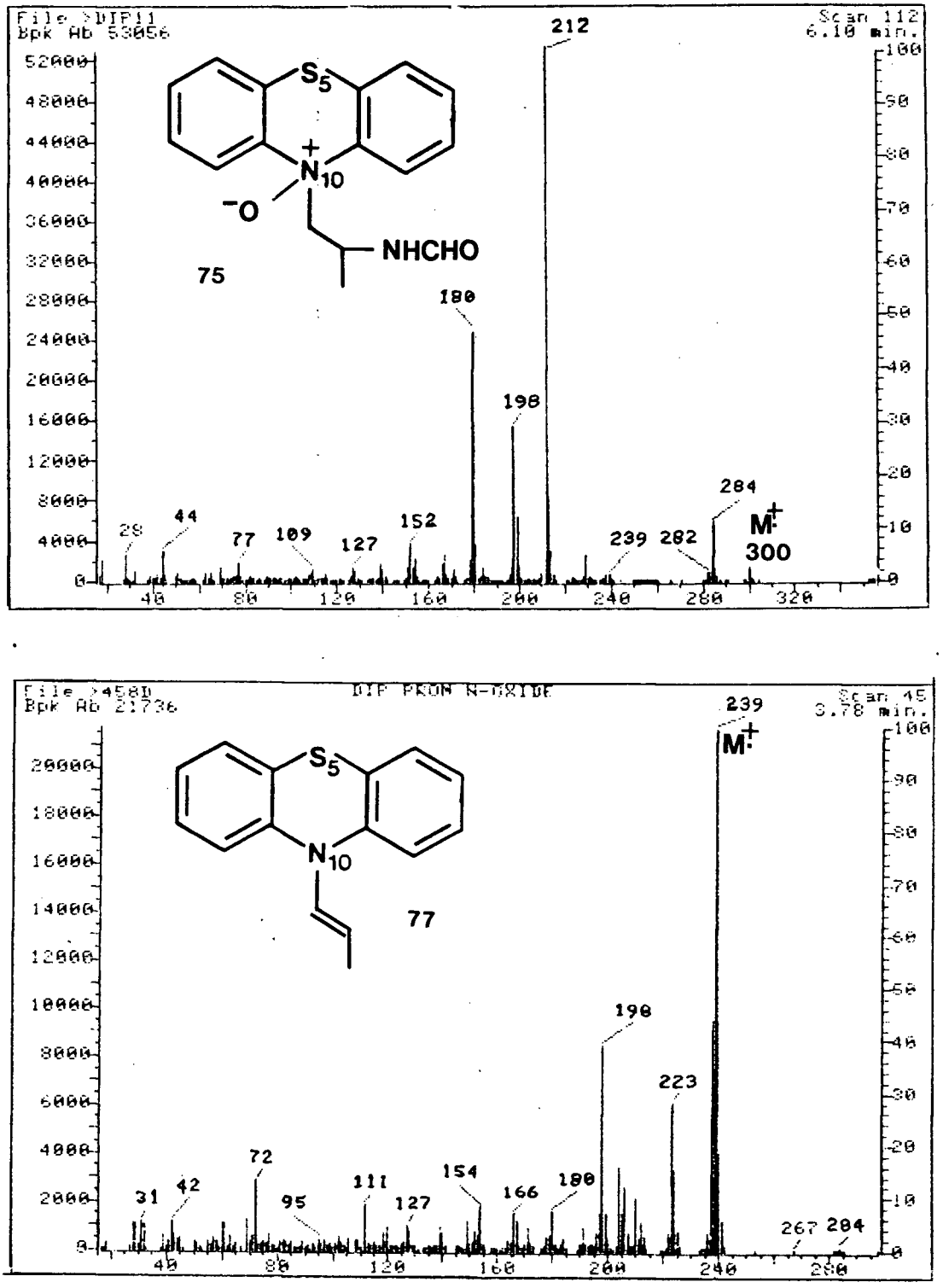


Figure 82. (top) Direct insertion probe mass spectrum of the secondary formamide N_{10}-oxide (75). (bottom) Direct insertion probe mass spectrum of the Cope elimination product 10-(2-propenyl)phenothiazine (77) which arises from the GCMS analysis of the promethazine N-oxide (76).

The last synthesis was of the promethazine N-oxide (76), which was synthesized by Clement and Beckett (1981a), but only characterized by direct inlet low eV mass spectrometry. The N-oxide decomposes on the GC column to the Cope elimination product 10-(2-propenyl)phenothiazine. In this study the direct inlet 70 eV mass spectrum afforded only the Cope elimination product (figure 82 (bottom). Our probe temperature conditions must have been higher than the inlet temperature used by Clement and Beckett since their mass spectrum (metabolite IX summarized above) had abundant m/z 72 (like promethazine) and a molecular ion (M^+=300) corresponding to the N-oxide.

It appeared that the spectral assignments made by Clement and Beckett were correct. Nonetheless, we pursued the study of the *in vivo* metabolism of promethazine in the rat with the hope of identifying the secondary formamide (72) in β-glucuronidase-hydrolyzed rat bile.

Metabolites with intact tertiary amine and secondary amine side chains were detected using the m/z 72 and 58 ions respectively Figure 83. The low intensity of other ions in these basic compounds made assignment of structure to the phenothiazine portion impossible in underivatized samples analyzed by EI GCMS. No peaks were detected in the m/z 212 mass chromatogram corresponding to the secondary formamide analogue of promethazine.

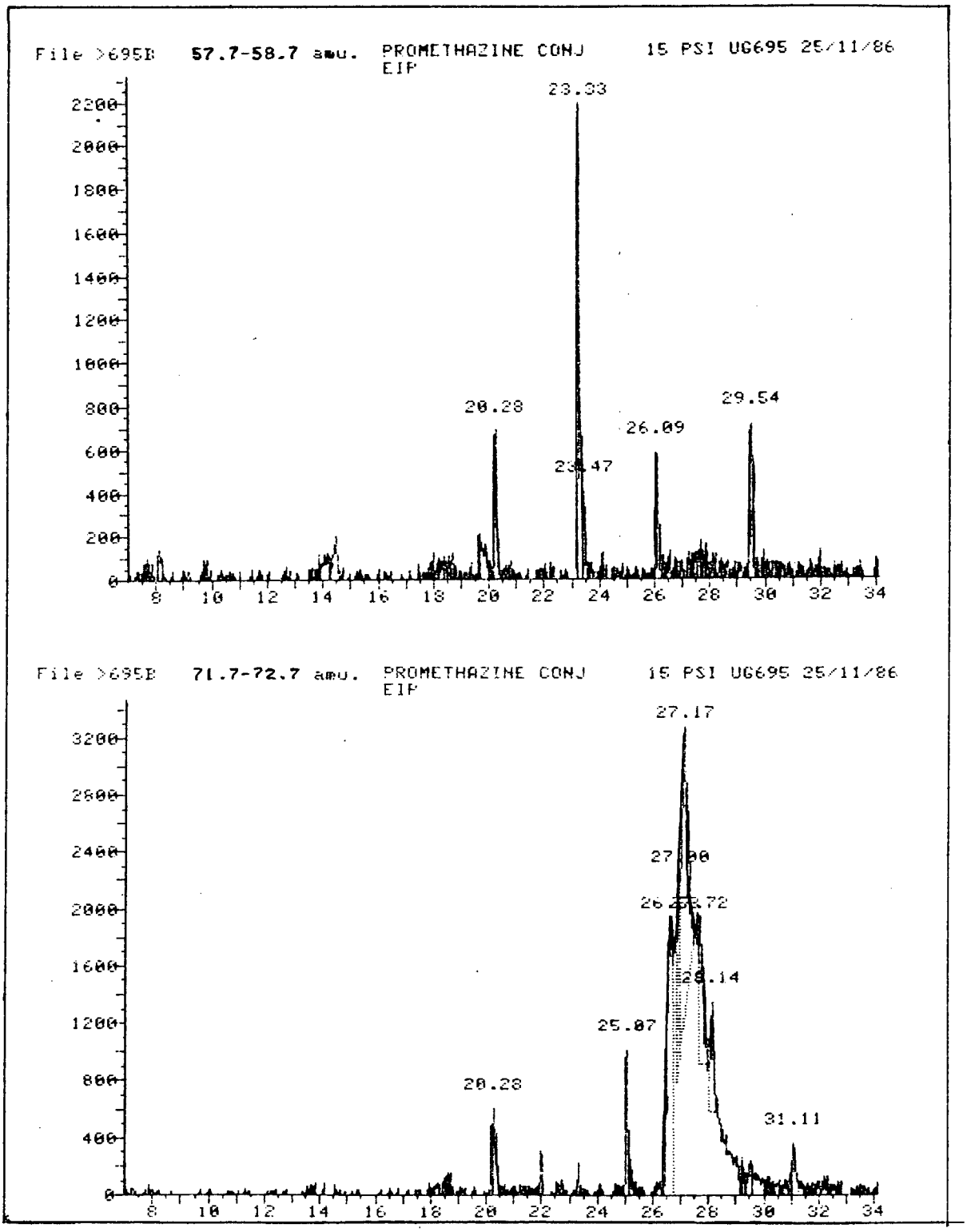


Figure 83. Mass chromatograms for an extract of β-glucuronidase-hydrolyzed conjugated promethazine metabolites (above) Ion chromatogram m/z 58 showing intact secondary amine metabolites (below) Ion chromatogram m/z 72 showing intact tertiary amine metabolites

9. DECOMPOSITION OF N-OXIDIZED METABOLITES AS A SOURCE OF THE SECONDARY FORMAMIDE (12) ISOLATED FROM RAT BILE

A. Decomposition of recipavrin N-oxide under simulated sample workup conditions

N-oxides are thermolabile metabolites of a number of basic tertiary amines (Rose and Castagnoli, 1983). There are several thermal and chemical rearrangements common to N-oxides which are known to complicate the isolation and analysis of this compound class (Oae and Ogino, 1977).

Before HPLC became a routine analytical method, it was common to extract a sample free of parent amine, then reduce any N-oxide metabolites to the parent amine with titanous chloride and extract and quantitate the product tertiary amine as a measure of N-oxide content (Beckett *et al*. 1972). It was the chemical lability of N-oxides and the ease with which the parent amine was oxidized to the N-oxide that resulted in early controversy as to whether N-oxides were really metabolites or artifacts (Anggard *et al*. 1975).

The questions of early investigators regarding the source of N-oxide metabolites of methadone and related compounds were similar to those being posed for the secondary formamide metabolite in this study. In the case of the secondary formamide, the issue was further complicated by the lack of a readily identifiable precursor. In this experiment the N-oxide (53, $Ph_2CHCH_2CH(CH_3)N^+(O^-)(CH_3)_2$) was considered as a possible precursor to the formamide because it was a thermo- and chemo-labile metabolite of recipavrin that was not easily extracted from aqueous solution.

Although none of the rearrangements of N-oxides reported in the literature would afford the secondary formamide metabolite, the N-oxide was considered as a possible precursor to the formamide because of chemical lability and the similarity in chemical conditions required to generate the N-oxide and formamide functional groups by the oxidation of amines (Sayigh and Ulrich, 1964).

The N-oxide (53) was exposed to a variety of mildly acidic, basic and oxidizing conditions, and then checked by GCMS to see if any secondary formamide was produced, and to identify and roughly quantitate the degradation products. Results are summarized in table 13.

Table 13. GCMS peak areas of compounds arising from the chemical and/or thermal degradation of recipavrin N-oxide under various simulated workup conditions.

		Treatment*							
		A	B	C	D	E	F	G	H
		cont	NaAc	NaAc β-glu	H_2O	borate	NaOH	$FeCl_3$	$FeCl_3$ H_2O_2
Cpd**	tr(min.)	Total ion current integrated peak areas***							
1	7.56	36.2	1.3	1.7	1.5	7.47	12.1	0.9	1.3
2	7.82	30.6	1.8	2.5	2.7	10.9	17.0	1.75	1.1
3	10.30	0.2	1.5	0.8	0.1	0.3	1.6	6.8	6.6
4	10.68	0.2	0.3	0.2	0.2	0.6	0.7	1.6	0.0
5	11.49	5.0	32.8	33.4	28.7	16.1	25.6	20.4	44.1
6	11.91	0.0	11.9	7.4	26.0	21.6	3.4	0.0	19.3
7	11.98	22.1	36.2	44.0	25.3	34.1	20.0	48.5	0.0
8	14.20	0.0	5.9	2.0	5.9	1.0	0.8	0.0	1.3
9	18.47	0.2	2.7	2.8	4.2	4.6	18.7	5.4	2.0

*Treatment: A. Control (purified N-oxide in MeOH). B. Sodium acetate buffer 0.1 M, pH 5. C. Sodium acetate buffer 0.1 M, pH 5 plus 250 ul glucurase. D. Water. E. Sodium borate 0.12 M pH 10. F. 3 M Sodium hydroxide. G. Ferric chloride 5 mg/ml. H. Ferric chloride 5 mg/ ml plus 300 ul of 30% hydrogen peroxide.

**Cpd.= decomposition product: 1. Cis 1,1-diphenyl-but-2-ene (54). 2. Trans 1,1-diphenyl-but-2-ene (55). 3. Diphenylbutanone (14). 4. dinorrecipavrin (20). 5. Norrecipavrin (15). 6. Unidentified. 7. Recipavrin (9). 8. Oxime (19). 9. Tertiary formamide (26).

*** Peak area from total ion current X 100 / total area of all integrated peaks in that sample.

Figure 84 shows the total ion current chromatogram for the control injection of purified recipavrin N-oxide (53). The total ion currents for the other samples are in the appendix. The major peaks in figure 84 correspond to the two Cope elimination products, cis 1,1-diphenyl-but-2-ene (54, tr=7.92 min.) and trans 1,1-diphenyl-but-2-ene (55, tr=8.26 min.)(compounds 1 and 2 in table 13). The thermal Cope elimination of N-oxides is well known (Cope *et al.*, 1949) and along with the thermal desoxygenation product, recipavrin (9), the isomeric butenes (54 and 55) were expected to be the sole decomposition products. A trace of the tertiary formamide (26, tr=18.47 min.) survived the isolation of the N-oxide and is a byproduct of the synthesis (Sayigh and Ulrich, 1963). A small amount of norrecipavrin (15) was also detected.

The major observation was that none of the sample isolation methods afforded any secondary formamide (12, tr=21.1 min). The Cope elimination products were the safest measure of how much N-oxide survived the simulated isolation. Acidic conditions decomposed most of the N-oxide and resulted in the formation of norrecipavrin (15) and diphenylbutanone oxime (19). β-glucuronidase had no effect on the decomposition induced by pH 5 sodium acetate. One other product which had a base peak m/z 140, and lacks m/z 165 or m/z 167 (denoting a reaction of the diphenyl portion) was also produced.

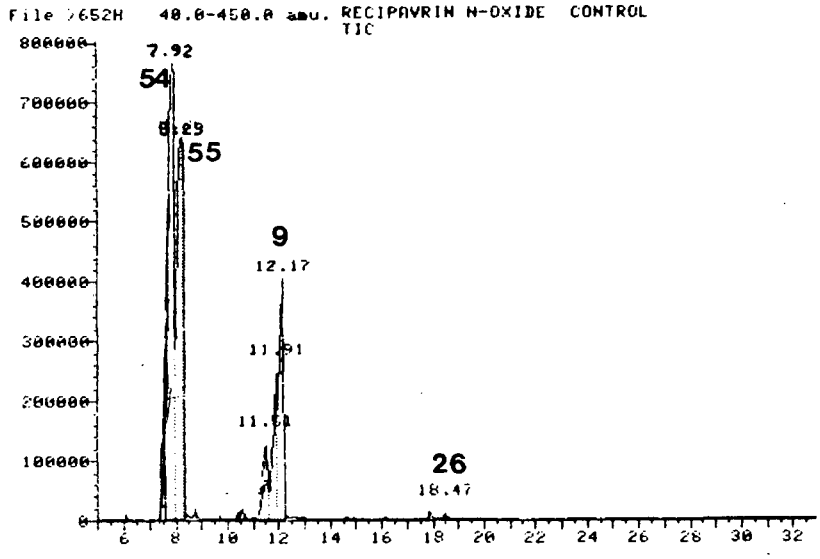


Figure 84. Total ion current for the control GCMS analysis of recipavrin N-oxide.

Alkaline conditions E and F did not completely decompose the N-oxide (53) and produced a similar amount of norrecipavrin (15) when compared to the acidic treatments.

The acidic oxidizing agent ferric chloride decomposed the N-oxide almost totally, and partially deaminated the norrecipavrin to diphenylbutanone. Addition of hydrogen peroxide to ferric chloride almost completely destroyed the N-oxide and recipavrin. Norrecipavrin was the major product observed by GCMS.

It can be concluded that the secondary formamide metabolite does not arise by exposure of the recipavrin N-oxide to sample isolation procedures. It was also evident that quantitation of intact N-oxides precludes even mildly acidic or basic conditions during sample workup and that thermolability precludes using GLC for quantitation of the N-oxide.

B. GCMS analysis and thermal decomposition of the methylene nitrone

GCMS results for the methylene nitrone (24, $Ph_2CHCH_2CH(CH_3)N^+(O^-)=CH_2$) were inconsistent from sample to sample. GCMS analysis resulted in the observation of diphenylbutanone oxime (19), the methylene imine monomer (23, $Ph_2CHCH_2CH(CH_3)N=CH_2$), a compound which direct probe GCMS analysis showed was the nitrone (24), and at slightly longer retention time an overlapping peak with the retention time and mass spectrum of the secondary formamide (12) (Slatter, 1983).

Analysis of the purified nitrone at low concentration using a freshly silanized injection port liner and GC column showed that it was possible to analyze the nitrone (24) by GCMS without decomposition.(figure 85). The high resolution mass spectrum showed that the single peak had peaks at m/z 56 and 236 diagnostic of the nitrone structure (Coutts, *et al.*, 1978) (figure 86 (top)).

The decomposition of the nitrone (24) in the GC inlet was demonstrated to be dependent on sample loading, contamination in the injection port liner, and the presence of co-injected blank bile extract. Inter run comparisons of low (10 ug) and high (100 ug) sample loads were run on clean or dirty injection port liners (figures 87-89). The synthetic nitrone was also mixed with control rat bile and then extracted and analyzed by GCMS (figure 86 (bottom), figure 90).

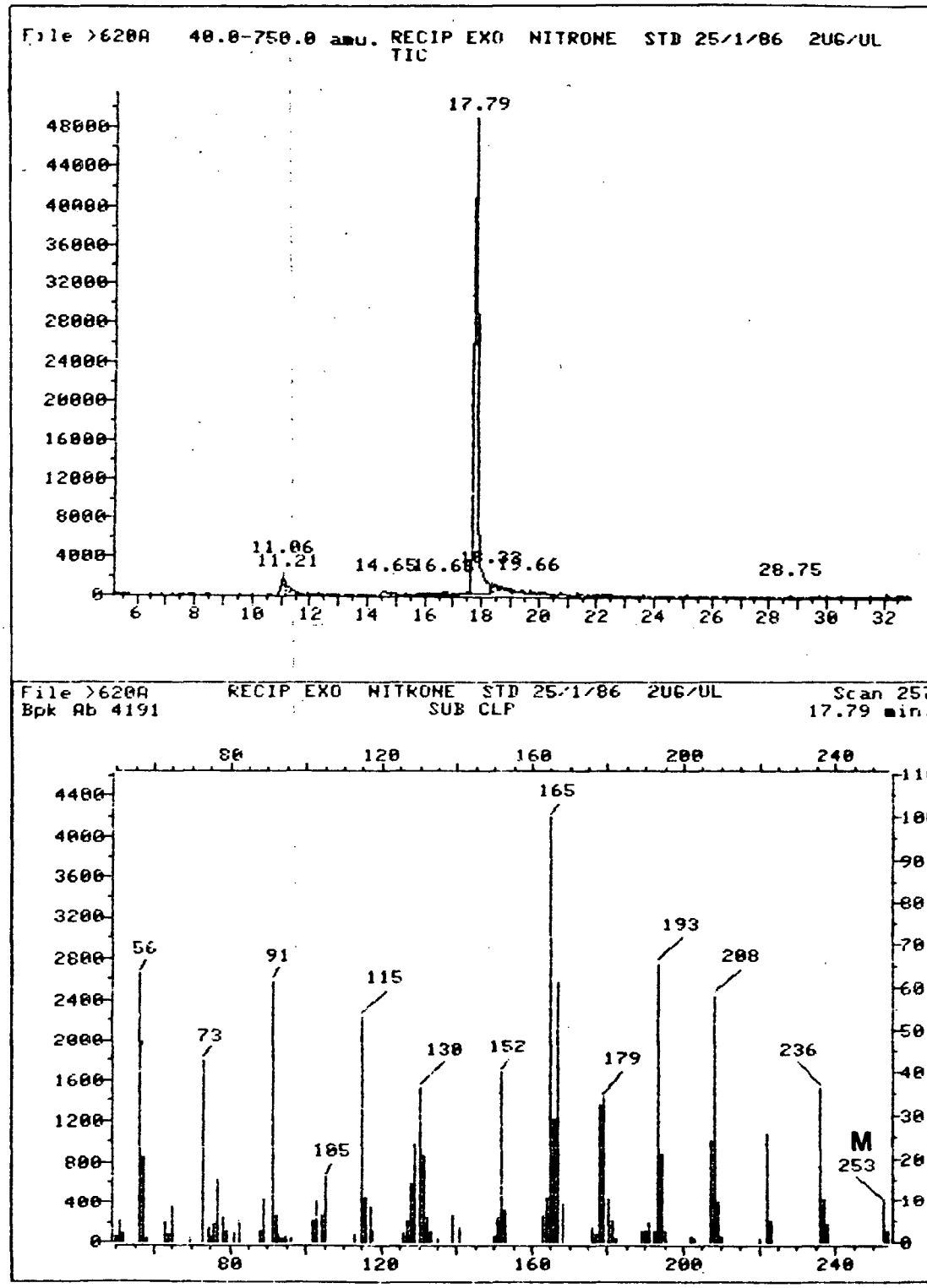


Figure 85. (top) Capillary GCMS total ion current of freshly synthesized and purified recipavrin methylene nitrone injected at low concentration onto a freshly silanized injection port liner and GC column. (bottom) Mass spectrum of the single peak in 2a.

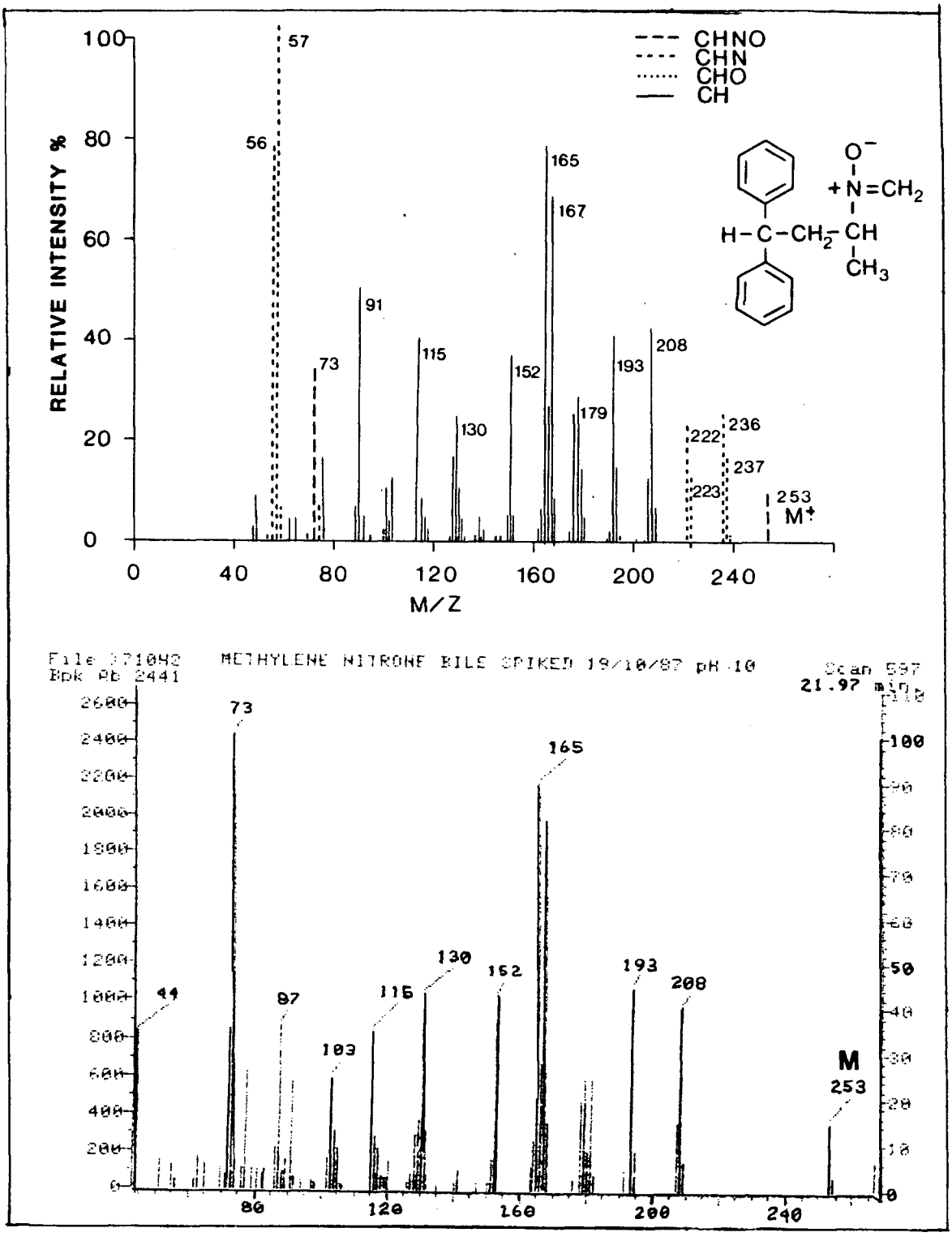


Figure 86. (top) Summary of high resolution mass spectrum of the nitrone (24) (Source temperature 120°) Some decomposition to the methylene imine (m/z 57, 237) and formamide (m/z 73) is apparent. (bottom) Mass spectrum of the formamide arising from decomposition of the nitrone extracted from control bile (see figure 90).

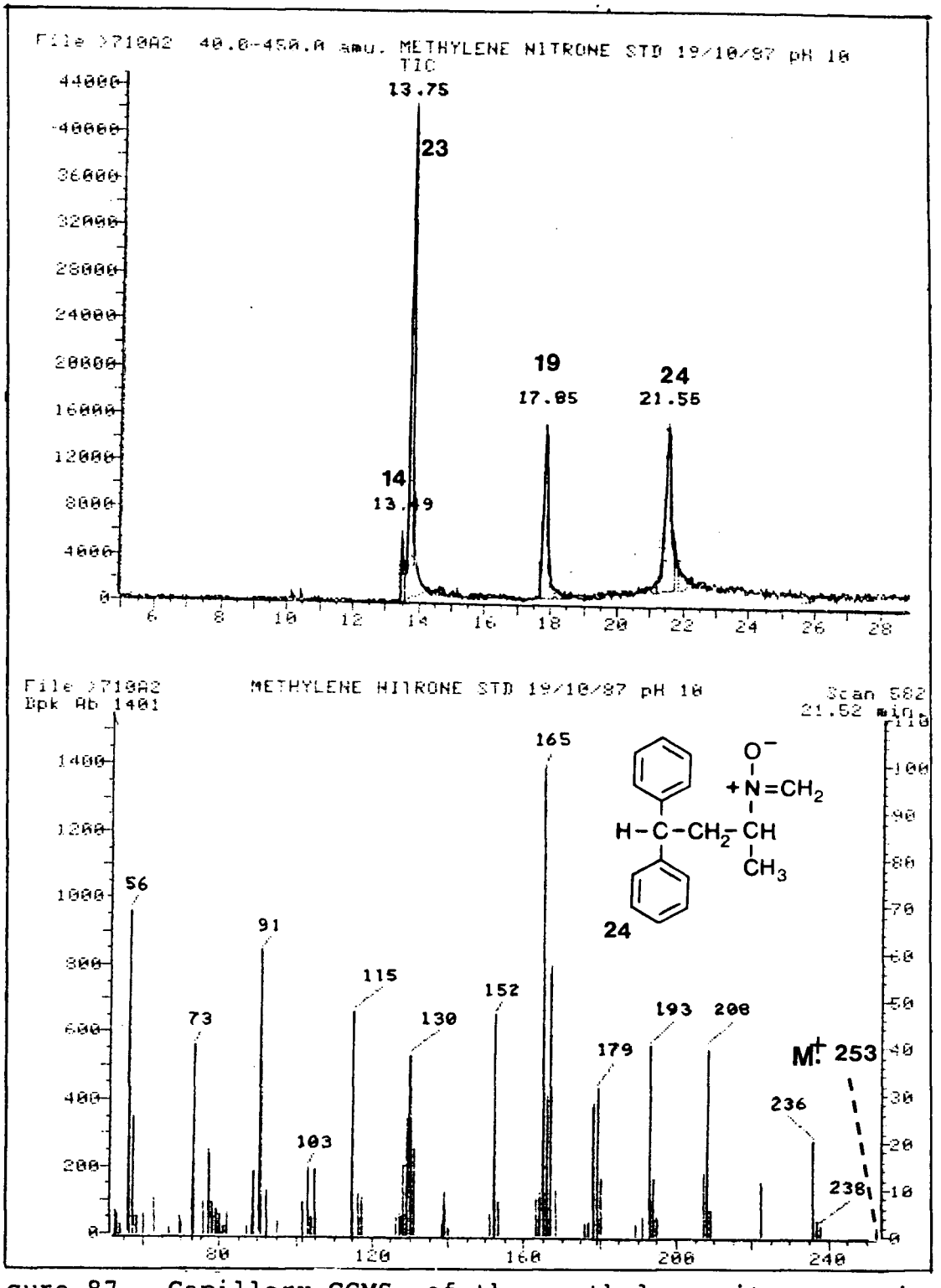


Figure 87. Capillary GCMS of the methylene nitrone using a clean injection port liner. (top) Total ion current showing decomposition of 10 ug of freshly purified recipavrin methylene nitrone (24) to the oxime (19, tr=17.85 min.), imine (23, tr=13.75 min.), diphenylbutanone (14, tr=13.49 min.) and the nitrone (24, tr=21.55) min. (Bottom) Mass spectrum of the nitrone (24). GC condition B.

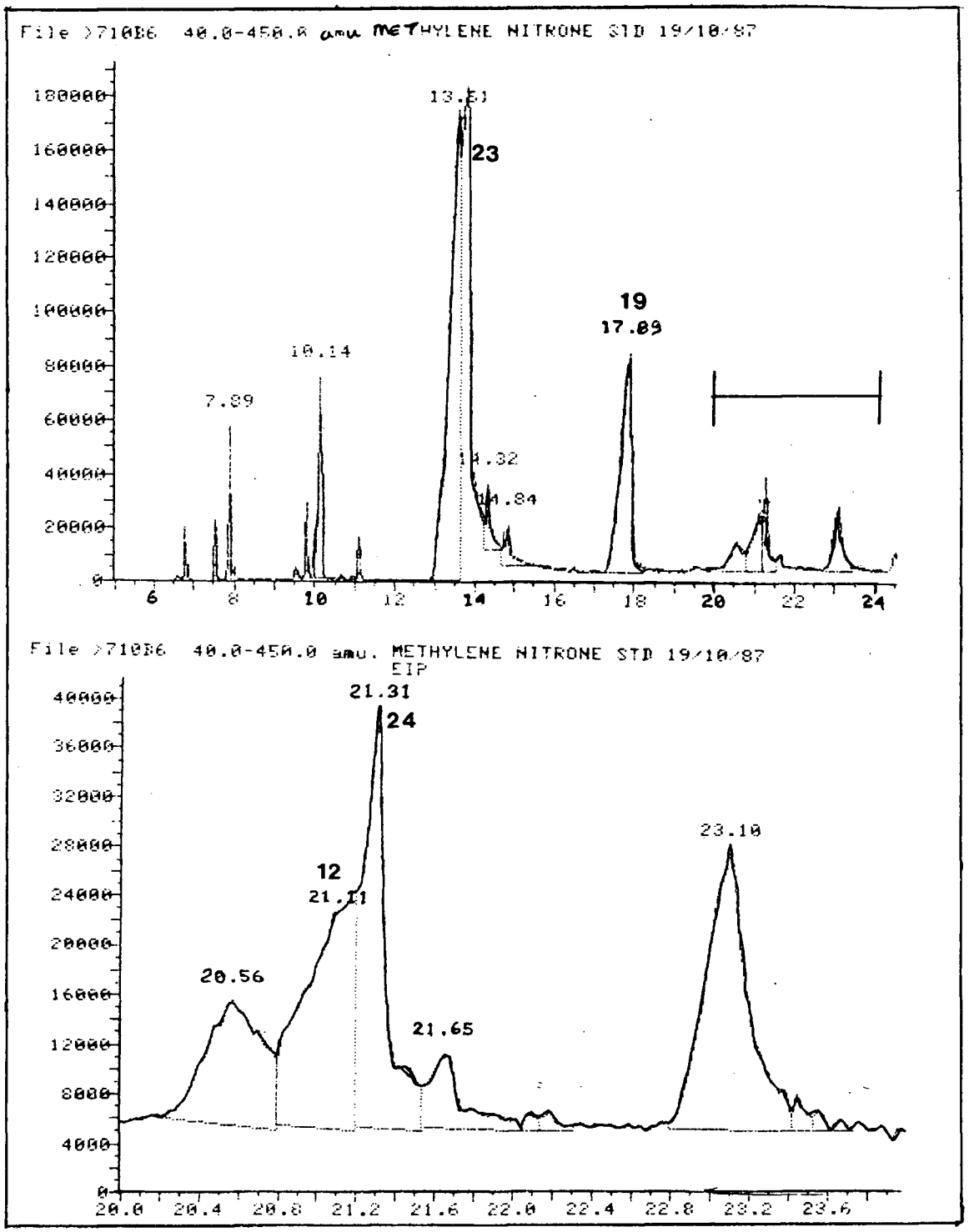


Figure 88. GCMS analysis of the methylene nitrone using a clean injection port liner. (top) Total ion current of a sample overload (100 ug). (bottom) Close up of the total ion current showing the formamide (12, tr=21.31) and nitrone (24, shoulder at tr=21.11 min.). GC condition B.

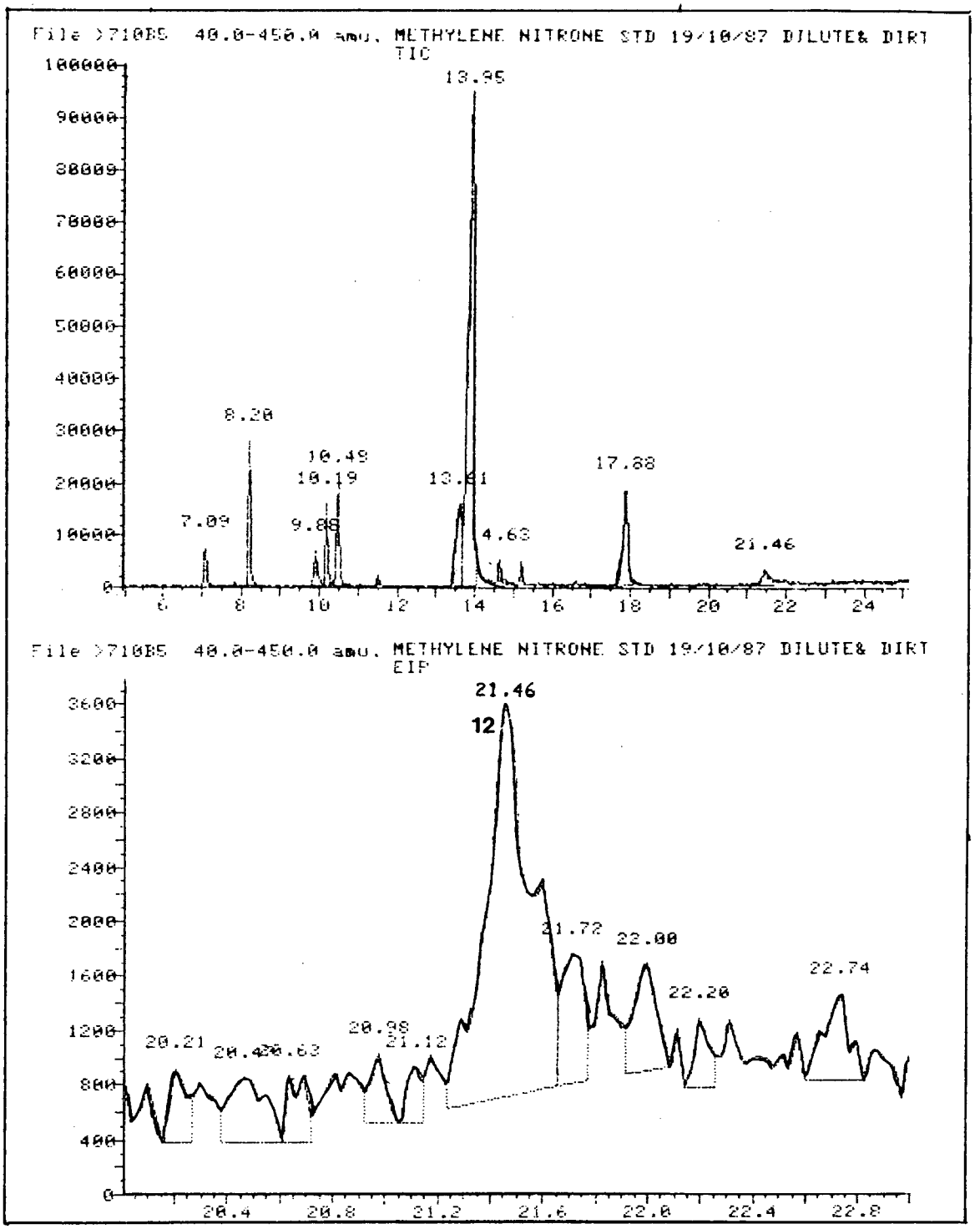


Figure 89. Capillary GCMS of the methylene nitrone (24) using a dirty injection port liner. (top) Total ion current showing decomposition of 10 ug of freshly purified recipavrin methylene nitrone. Close up of the total ion current showing the formamide (12, tr=21.46) and no nitrone. GC condition B.

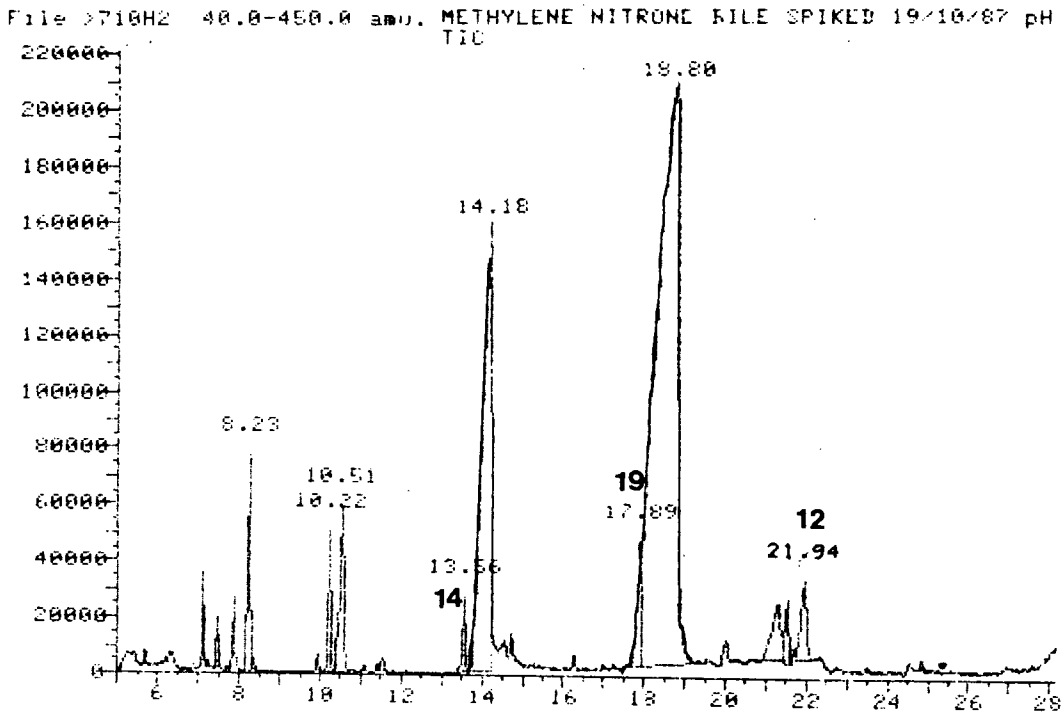


Figure 90. Total ion current chromatogram form GCMS analysis using a clean injection port liner of an extract of control bile spiked with 100 ug methylene nitrone (24). GC condition B.

Figure 85 shows that a silanized injection port liner can completely abolish thermal decomposition of the nitrone to the oxime and imine, giving a peak with a similar mass spectrum to the direct insertion probe analysis of the same sample (figure 3). In figure 87, no formamide (12) was detected when 10 ug of nitrone was loaded on a GC column equipped with a clean injection port liner. In figure 88 the sample load was increased to 100 ug and the formamide began to arise by decomposition in the injection port liner. Injection of the lower concentration on a column equipped with an injection port liner used previously for the analysis of bile samples resulted in complete conversion to the formamide (figure 89). Co-injection of the nitrone (24) and bile extract on a clean liner also resulted in complete disappearance of the nitrone (24) and appearance of the formamide (12)(figure 90).

The thermal decomposition of nitrones commonly gives rise to isomeric oxime ethers (Oae and Ogino, 1977, Lamchen, 1968). In the case of the methylene nitrone this is diphenylbutanone O-methyloxime (16, $Ph_2CHCH_2C(CH_3)=NOCH_3$). The oxime ether (16) was synthesized for this study but was never detected by GCMS in any of the nitrone decomposition studies. The other common thermal degradation undergone by nitrones is desoxygenation (Lamchen, 1968). This was born out by the formation of the major decomposition product, the methylene imine (23, $Ph_2CHCH_2CH(CH_3)-N=CH_2$) which exists as a monomer only in the gas phase (Emmons, 1957). The other major degradation product was the oxime (19, $Ph_2CHCH_2C(CH_3)=NOH$). Beckett *et al*. (1973)

have observed an oxime and an unidentified degradation product for an amphetamine methylene nitrone. The unidentified product may have been the imine monomer (78, $PhCH_2CH(CH_3)N=CH_2$) based on the decomposition of the recipavrin nitrone (24). Coutts *et al*. (1978) have prevented the decomposition of the methylene nitrone of amphetamine in their metabolism studies by using a different GCMS system.

These results have shown that methylene nitrone (24) in a bile extract decomposes to the formamide (12), imine (23) and oxime (19) during GC analysis. If a nitrone to formamide conversion was responsible for the observation of the secondary formamide metabolite of recipavrin, it was the absence of methylene imine (23) in the GCMS results for recipavrin metabolic extracts that suggested an alternative chemical genesis of the formamide prior to GC analysis, rather than the thermal degradation demonstrated here.

C. Alkali catalyzed rearrangement of nitrones to amides.

There are several rearrangements described in the literature which make the methylene nitrone an attractive precursor if the secondary formamide observed by GCMS in bile extracts were a chemically generated artifact. These were shown in figures 7-10. The methyl nitrone (a keto-nitrone, $R=N^+(O^-)CH_3$) could be converted to the methylene isomer (an aldo-nitrone, $RN^+(O^-)=CH_2$) by base catalysed Behrend rearrangement (Hamer and Macaluso, 1964, Lamchen, 1968, Smith and Gloyer, 1975, Heistand, 1978). The same basic conditions have converted other nitrones to their isomeric amides

(Bigiavi and Marri, 1934, Umezawa, 1960, Hamer and Macaluso, 1964, Zinner, 1978). This rearrangement applied to the methylene nitrone (24) would give rise to the formamide (12).

The potential for rearrangement of the methylidene nitrone (24) to the isomeric secondary formamide (12) is strong in the light of four observations: 1. The documented Beckmann rearrangement of nitrones to amides in various chemical systems (Hamer and Macaluso, 1964, Lamchen, 1968, Zinner, 1978); 2. The alkaline workup conditions that would favor this isomerization; 3. The generation of nitrone and easily oxidized hydroxylamine metabolites from structurally related amphetamines and phenothiazines (Coutts and Beckett, 1977, Clement and Beckett, 1981b); 4. The demonstration of the formamide as a decomposition product of the methylene nitrone by LCMS (Slatter, 1983); 5. The demonstration that control bile spiked with the synthetic secondary hydroxylamine and isolated from mildly alkaline solution affords the secondary formamide when analyzed by GCMS (Slatter, 1983).

10. METABOLISM OF THE RECIPAVRIN METHYLENE NITRONE

The recipavrin methylene nitrone was administered to a rat and bile was collected for 24 hours. Sample preparation involved immediate adjustment to pH 5, 24 hour β-glucuronidase hydrolysis, followed by extraction of all metabolites from pH 10 solution. Ion monitoring of the m/z 167 diphenylmethyl cation (figure 91) revealed diphenylbutanone (14, tr=13.69 min.), diphenylbutanol (97, tr=14.12 min., $Ph_2CH-CH_2-CH(CH_3)OH$), dinorrecipavrin (20, tr=14.63 min.), the oxime

(19, tr=18.42 min.) and the secondary formamide (12, tr=22.16 min.) (figure 91). No nitrone was observed by GCMS, in accord with the results in section 9 showing that the nitrone decomposes in the GC inlet to the formamide when co-injected with bile extract. It would take a LC study to determine whether the nitrone decomposed during sample isolation or GC analysis. This was not performed.

Diphenylbutanone oxime phenol (89) was detected at tr=25.04 min. using the m/z 183 ion (figure 91). Compounds tentatively identified as O-methylcatechols of diphenylbutanol (92, tr=22.72 min.), diphenylbutanone oxime (93, tr=26.24 min.) and 1,1-diphenyl-3-nitrobutane (98, tr=25.87 min.,) were detected by monitoring the m/z 213 ion (figure 92).

Derivatization with BSTFA left the formamide (12) intact (17.47 min.) and revealed a compound with similar mass spectrum to the primary hydroxylamine TMS ether (102, tr=19.98 min), the TMS oxime (103, tr=14.82 min.) and several phenyl ring intact unidentified compounds (figure 93). It is likely that the primary amine (dinorrecipavrin) in the underivatized sample arose from decomposition of the hydroxylamine since no primary amine TMS derivative (104) was detected.

A logical extension of this experiment would be to study the conjugated metabolites of i.v. infused secondary hydroxylamine. As with the recipavrin metabolism studies, LCMS or the trapping of the nitrone in a stable form (Coutts *et al.*, 1978) would be required to demonstrate the intact nitrone as a metabolite.

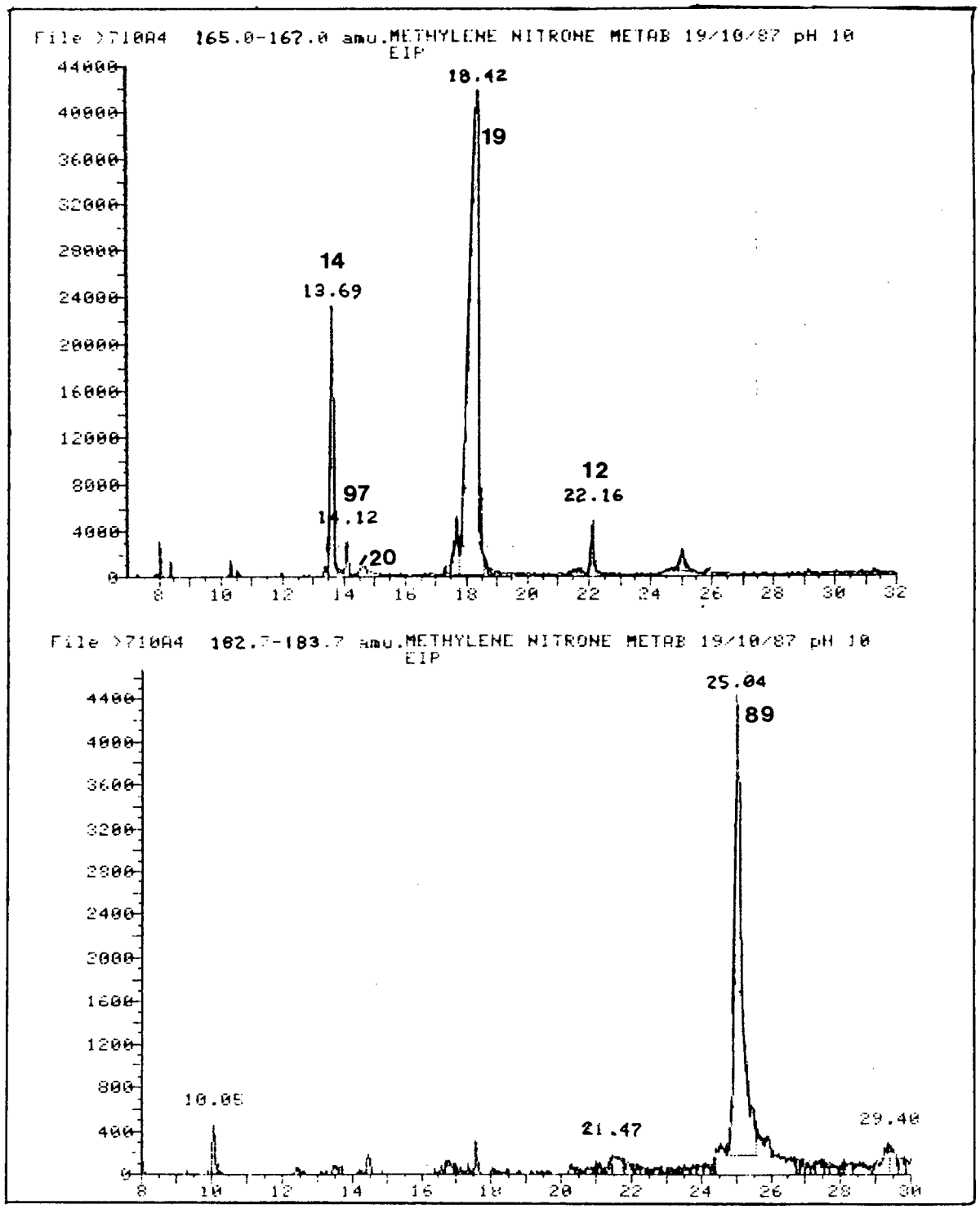


Figure 91. (top) Mass chromatogram m/z 167 showing phenyl ring intact metabolites of the methylene nitrone (24). (bottom) Mass chromatogram m/z 183 showing phenol metabolites of the recipavrin methylene nitrone. GC condition B.

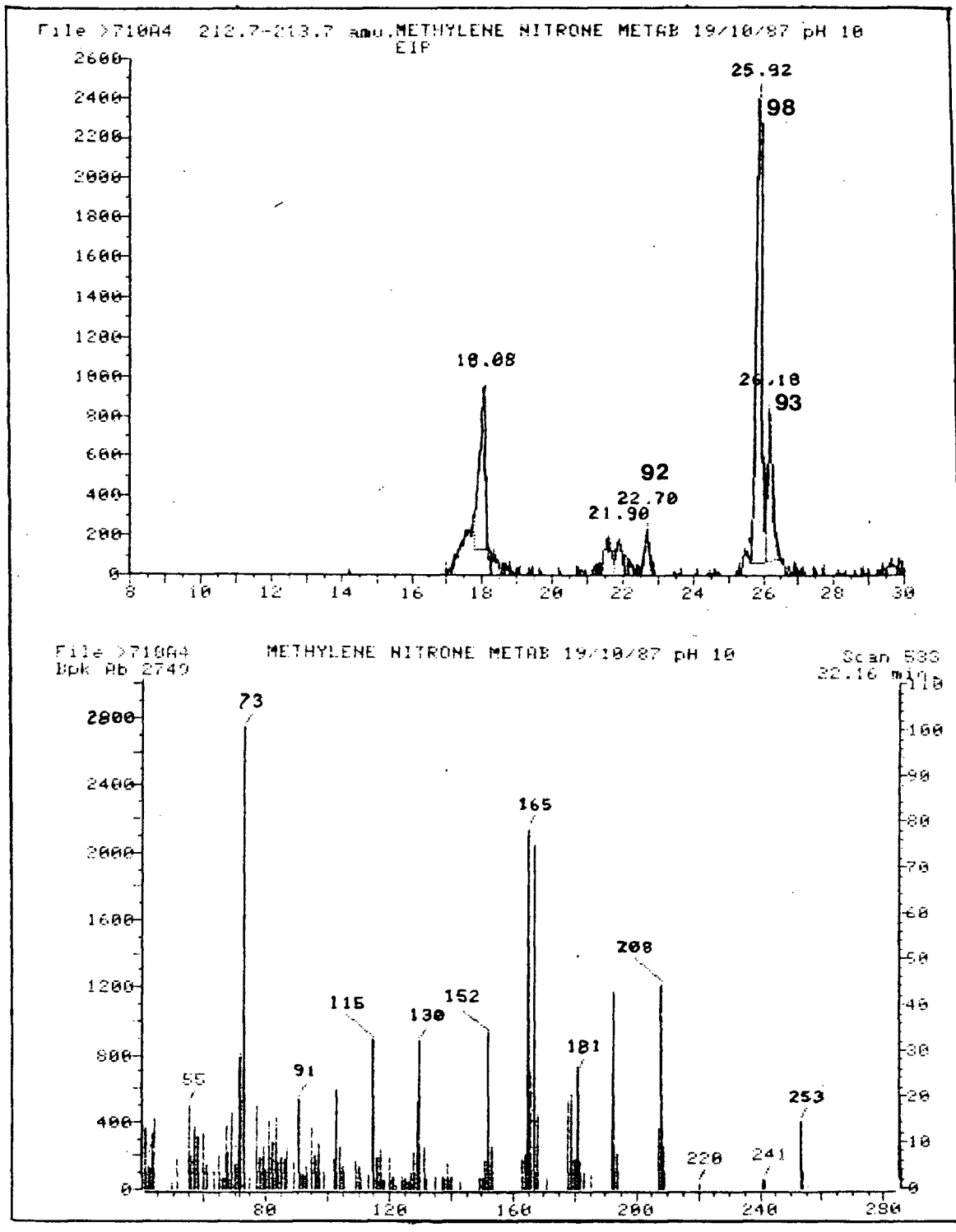


Figure 92. (top) Mass chromatogram m/z 213 showing O-methylcatechol metabolites of the methylene nitrone (24). (bottom) Mass spectrum of the secondary formamide (12) arising from GCMS analysis of the nitrone metabolites. GC condition B.

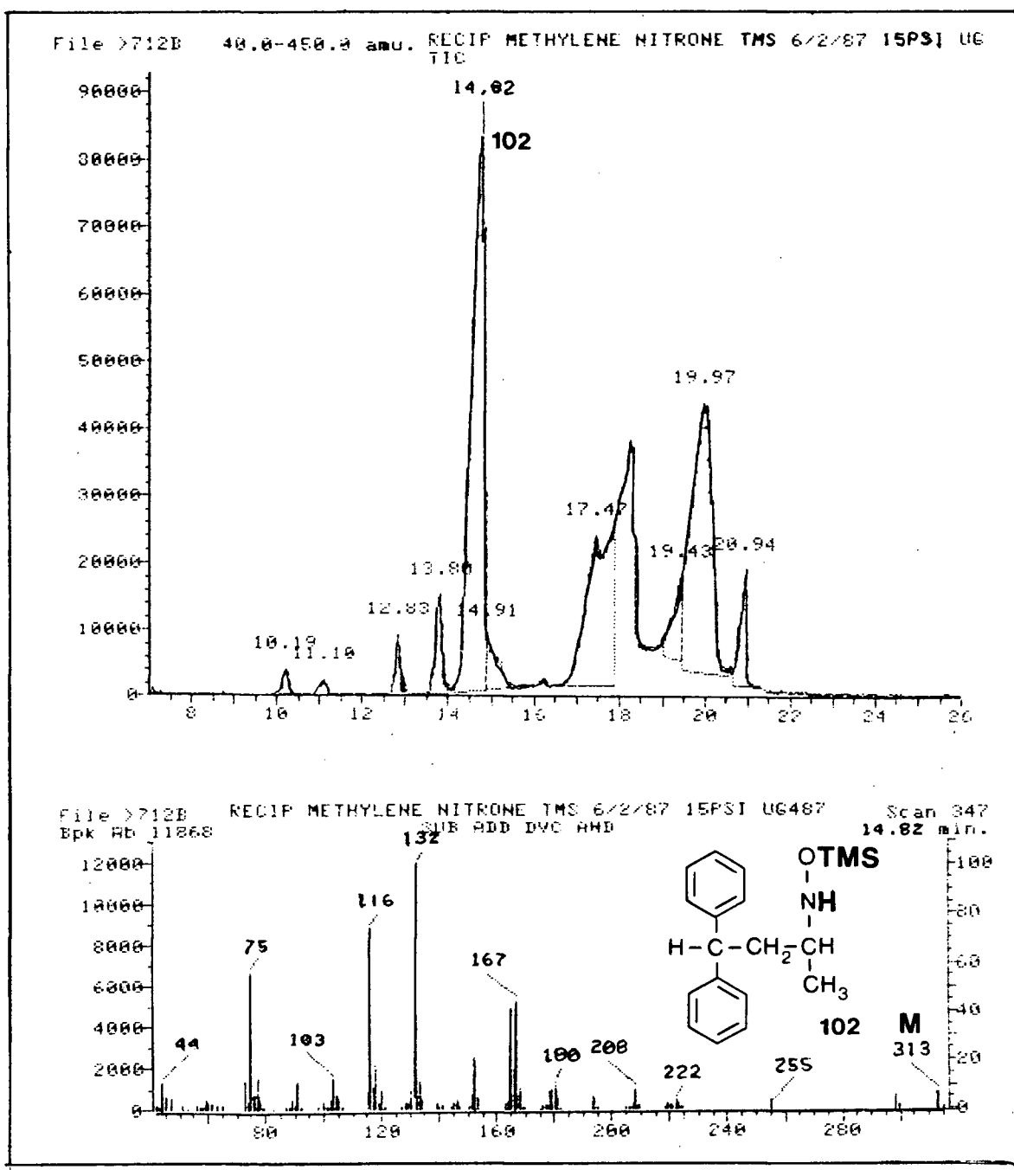


Figure 93 (top) Total ion current for the BSTFA derivatized bile extract of nitrone (24) metabolites. (bottom) Mass spectrum of the suspected primary hydroxylamine TMS derivative (102). Standard GC condition.

11. METABOLISM OF THE RECIPAVRIN TERTIARY FORMAMIDE (26)

The metabolism of the tertiary N-methylformamide (±) N-methyl-N-(1-methyl-3,3-diphenylpropyl)formamide (26, $Ph_2CHCH_2CH(CH_3)N(CH_3)CHO$) was investigated to determine whether the carbinolamide metabolite (47, figure 12, $Ph_2CHCH_2CH(CH_3)N(CH_2OH)CHO$) was a possible precursor of the secondary formamide observed in the recipavrin study. Because the metabolism of higher aliphatic formamides was a virtually unknown area, we have characterized the metabolites of the tertiary formamide.

The systematic and trivial names, substituents and formulae of compounds of general structure A and B (figure 51, page 153) are summarized in Table 14. GCMS data for all metabolites, TMS derivatives and reference compounds are tabulated as follows: Table 15. Metabolites with intact phenyl rings; Table 16. Phenolic metabolites; Table 17. O-methylcatechol metabolites. Figure 94 and 95 show selected ion chromatograms used to locate metabolites.

Table 14. Names structures and formulae for the tertiary formamide (26) and its metabolites.

COMPOUND NUMBER	SYSTEMATIC NAME	TRIVIAL NAME	R_1	R_2	R_3	EMPIRICAL FORMULA
26	(±) N-formyl-N,α-dimethyl-γ-phenylbenzenepropanamine	Tertiary formamide (3^0-formamide)	$-N(CH_3)CHO$	H	H	$C_{18}H_{21}NO$
12	(±) N-formyl-α-methyl-γ-phenyl benzenepropanamine	Secondary formamide (2^0-formamide)	-N(H)CHO	H	H	$C_{17}H_{19}NO$
47	(±) N-formyl-N-hydroxymethyl -α- methyl-γ-phenylbenzene-propanamine	Carbinolamide	CHO | $-N(CH_2OH)$	H	H	$C_{18}H_{21}NO_2$
14	1,1-diphenyl-3-butanone	Diphenylbutanone (DPB)	=O	H	H	$C_{16}H_{16}O$
97	(±) 1,1-diphenyl-3-butanol	Diphenylbutanol (DPB-OH)	-OH	H	H	$C_{16}H_{18}O$
19	1,1-diphenyl-3-butanone oxime	Diphenylbutanone oxime (DPB-oxime)	=NOH	H	H	$C_{16}H_{17}NO$
15	(±)N,α- dimethyl-γ-phenyl-benzenepropanamine	Norrecipavrin	$NHCH_3$	H	H	$C_{17}H_{21}N$
105	(±)N-formyl-N,α-dimethyl -γ- (4-hydroxyphenyl) -benzene propanamine	3^0-formamide phenol	$-N(CH_3)CHO$	H	OH	$C_{18}H_{21}NO_2$
106	(±)N-formyl-α-methyl-γ-(4-hydroxyphenyl) -benzenepropanamine	2^0-formamide phenol	-N(H)CHO	H	OH	$C_{17}H_{19}NO_2$

Table 14 (Continued). Names structures and formulae for the tertiary formamide (26) and its metabolites.

NUMBER	SYSTEMATIC NAME	TRIVIAL NAME	R_1	R_2	R_3	EMPRICAL FORMULA
107	(±)N-formyl-N-hydroxymethyl-α-methyl-γ-(4-hydroxyphenyl)-benzenepropanamine	Carbinolamide phenol	$N(CH_2OH)CHO$	H	OH	$C_{18}H_{21}NO_3$
87	(±)1-(4-hydroxyphenyl)-1-phenyl-3-butanone	DPB-phenol	=O	H	OH	$C_{16}H_{16}O_2$
89	(±)1-(4-hydroxyphenyl)-1-phenyl-3-butanone oxime	DPB-oxime phenol	=NOH	H	OH	$C_{16}H_{17}NO_2$
108	(±)N-formyl-N,α-dimethyl-γ-(4-hydroxy-3-methoxyphenyl)-benzenepropanamine	3^0-formamide O-methylcatechol	$-N(CH_3)CHO$	OCH_3	OH	$C_{19}H_{23}NO_3$
109	(±)N-formyl-α-methyl-γ-(4-hydroxy-3-methoxyphenyl)-benzenepropanamine	2^0-formamide O-methylcatechol	-N(H)CHO	OCH_3	OH	$C_{18}H_{21}NO_3$
110	(±)N-formyl-N-hydroxymethyl-α-methyl-γ-(4-hydroxy-3-methoxyphenyl)-benzenepropanamine.	Carbinolamide O-methylcatechol	$-N(CH_2OH)CHO$	OCH_3	OH	$C_{19}H_{23}NO_4$
91	(±)1-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-butanone	Diphenylbutanone O-methylcatechol	=O	OCH_3	OH	$C_{17}H_{18}O_3$
92	(±)1-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-butanol	Diphenylbutanol O-methylcatechol	-OH	OCH_3	OH	$C_{17}H_{20}O_3$
93	(±)1-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-butanone oxime	DPB-oxime O-methyl-catechol	=NOH	OCH_3	OH	$C_{17}H_{19}NO_3$

Table 15. GCMS data for phenyl ring intact metabolites of the tertiary formamide (26) and their derivatives. Legend: A. Synthetic reference compound. B. TMS derivative. C. TMAH derivative.

COMPOUND NUMBER	TRIVIAL NAME	RETENTION TIME (minutes)	M‡ (INTENSITY)	BASE PEAK	OTHER DIAGNOSTIC IONS ((m/z) (% of base peak))					
26	3° Formamide	19.32	267(19)	87	86(80)	165(34)	167(32)	158(30)	208(23)	193(23)
12	2° Formamide	18.20	253(44)	73	167(95)	165(86)	208(79)	193(61)	72(31)	44(29)
12a	2° Formamide N-TMS[A]	15.42	325(6)	145	73(70)	165(42)	167(40)	221(36)	152(24)	130(23)
47	Carbinolamide	20.88	283(0)	85	167(81)	265(75)	45(70)	165(67)	193(53)	207(34)
47a	Carbinolamide O-TMS[B]	22.92	355(3)	85	167(97)	265(76)	165(70)	144(64)	73(60)	103(48)
14	Diphenylbutanone	10.84	224(35)	167	103(32)	165(32)	181(28)	152(18)	77(14)	43(10)
97	Diphenylbutanol	11.27	226(2)	167	165(50)	208(48)	193(32)	152(28)	130(22)	115(20)
19	DPB-oxime (syn)	14.48	239(0)	167	165(30)	209(32)	165(30)	152(28)	222(18)	77(14)
19a	DPB-oxime (anti)	14.81	239(8)	167	165(34)	152(18)	118(12)	103(10)	77(8)	222(6)
103	DPB-oxime TMS[B] (syn)	13.64	311(1)	167	180(62)	165(36)	152(16)	116(12)	73(6)	220(6)
103a	DPB-oxime TMS[B] (anti)	14.57	311(11)	167	166(26)	152(16)	181(10)	220(9)	103(8)	77(6)
16	DPB-O-methyl-oxime[C] (anti)	12.03	253(8)	167	165(32)	152(18)	118(16)	103(12)	181(8)	77(5)
15	Norrecipavrin	12.02	239(7)	58	167(18)	165(12)	152(5)	208(4)	103(4)	193(4)
15a	Norrecipavrin[B] N-TMS	15.17	311(5)	130	167(19)	193(31)	73(25)	105(20)	270(13)	165(7)
9	Recipavrin[C]	12.42	253(3)	72	167(12)	165(6)	152(3)	115(3)	91(3)	73(3)
111	Unidentified	12.91	251(12)	167	165(48)	208(32)	152(28)	130(22)	115(15)	70(6)

Table 16. GCMS data for phenolic metabolites of the tertiary formamide (26) and their derivatives. Legend: A. Synthetic reference compound. B. TMS derivative. C. TMAH derivative.

COMPOUND NUMBER	TRIVIAL NAME	RETENTION TIME (minutes)	M+ (INTENSITY)	BASE PEAK	OTHER DIAGNOSTIC IONS ((m/z) (% of base peak))					
105	3^{o} Formamide phenol	25.94	283(20)	87	183(56)	58(33)	224(27)	209(22)	130(28)	181(14)
105a	3^{o} Formamide Phenol-O-TMS	25.90	355(24)	255	296(73)	73(60)	87(58)	295(48)	269(40)	165(20)
105b	3^{o} Formamide O-methyl phenol[C]	24.72	297(32)	197	87(82)	238(74)	211(38)	165(30)	86(36)	58(22)
106	2^{o} Formamide phenol	25.70	-	-	Buried under 8	-	-	-	-	
106a	2^{o} Formamide phenol O-TMS[B]	24.71	341(15)	255	73(58)	296(22)	295(18)	281(13)	103(11)	45(9)
106b	2^{o} Formamide O-methylphenol[C]	22.79	283(17)	197	238(22)	165(17)	211(11)	73(11)	115(6)	44(4)
107	Carbinolamide phenol	-	-	-	Decomposes to 9	-	-	-	-	
107a	Carbinolamide Phenol Di-O-TMS[B]	28.84	443	73	255(82)	295(72)	267(54)	85(50)	353(42)	296(36)
87	DPB-Phenol	17.19	240(18)	183	43(12)	185(8)	103(6)	119(5)	153(5)	225(2)
87a	DPB-phenol[B] O-TMS	17.71	312(13)	255	257(22)	73(14)	165(8)	179(4)	103(4)	43(4)
87b	DPB-O-methyl phenol	15.90	254(18)	197	199(12)	165(11)	153(10)	103(6)	77(3)	43(3)
89	DPB-oxime phenol	21.2	255(2)	183	165(26)	196(16)	184(12)	199(10)	152(10)	121(10)
89a	DPB-oxime phenol Di-O-TMS[B]	21.3	399(6)	255	73(30)	256(20)	269(10)	103(6)	268(6)	165(5)
89b	DPB-oxime phenol Di-O-methyl ether[C]	17.3	283(3)	197	198(12)	211(10)	165(9)	103(8)	153(8)	133(6)

Table 17. GCMS data for O-methylcatechol metabolites of the tertiary formamide (26) and their derivatives. Legend: A. Synthetic reference compound. B. TMS derivative. C. TMAH derivative.

COMPOUND NUMBER	TRIVIAL NAME	RETENTION TIME (minutes)	M_i (INTENSITY)	BASE PEAK	OTHER DIAGNOSTIC IONS ((m/z) (% of base peak))					
108	3^0 Formamide -O-methylcatechol	26.70	313(46)	87	213(76)	86(39)	223(36)	58(32)	152(30)	254(29)
108a	3^0 Formamide O-methylcatechol TMS ether	27.67	385(53)	285	295(54)	87(42)	73(41)	286(29)	299(25)	326(16)
108b	3^0 Formamide dimethylcatechol	27.31	327(68)	227	237(80)	87(76)	242(32)	86(26)	165(26)	58(20)
109	2^0 Formamide O-methylcatechol	25.71	299(30)	213	44(47)	123(36)	73(25)	161(20)	239(19)	103(11)
109a	2^0 Formamide O-methylcatechol TMS ether[B]	26.61	371(33)	285	73(85)	192(58)	75(20)	286(28)	295(25)	177(20)
109b	2^0 Formamide dimethylcatechol[C]	26.51	313(36)	227	237(27)	228(25)	165(18)	103(12)	73(12)	152(10)
110	Carbinolamide O-methylcatechol	-	-	-	Decomposes to 14	-	-	-	-	
110a	Carbinolamide O-methylcatechol Di-TMS ether[B]	30.38	473	73	285(72)	297(46)	383(40)	103(36)	85(35)	129(32)

Table 17 (continued). GCMS data for O-methylcatechol metabolites of the tertiary formamide (26) and their derivatives.

Compound Number	Trivial Name	Retention Time (Minutes)	M^+ (% Intensity)	Base Peak 100%	Other Diagnostic Ions ((m/z)(% of base peak))					
91	Diphenylbutanone O-methylcatechol	18.55	270(26)	213	43(38)	103(20)	152(18)	153(16)	181(12)	72(10)
91a	Diphenylbutanone O-methylcatechol TMS ether[B]	20.01	342(23)	285	286(24)	144(18)	73(14)	167(10)	255(9)	103(4)
91b	Diphenylbutanone dimethylcatechol[C]	18.85	284(32)	227	228(18)	165(12)	196(11)	103(10)	181(8)	73(6)
92	Diphenylbutanol O-methylcatechol	18.95	272(26)	213	44(72)	182(15)	151(8)	(poor mass spectrum)		
92a	Diphenylbutanol O-methylcatechol TMS ether[B]	21.65	344(0)	285	73(38)	286(24)	75(18)	255(8)	117(6)	165(4)
92b	Diphenylbutanol dimethylcatechol[C]	19.10	286(30)	227	212(20)	181(9)	57(12)	168(10)	253(10)	103(8)
93	DPB-oxime O-methylcatechol	22.2	285(2)	213	226(52)	152(26)	181(20)	105(16)	115(14)	76(4)
93a	DPB-Oxime-OMC Di-TMS ether[B]	23.24	429(0)	285	73(12)	152(5)	45(5)	299(4)	325(3)	181(2)
93b	DPB-O-methyloxime dimethylcatechol[C]	20.05	313(4)	227	228(14)	196(8)	165(8)	241(8)	282(6)	152(4)

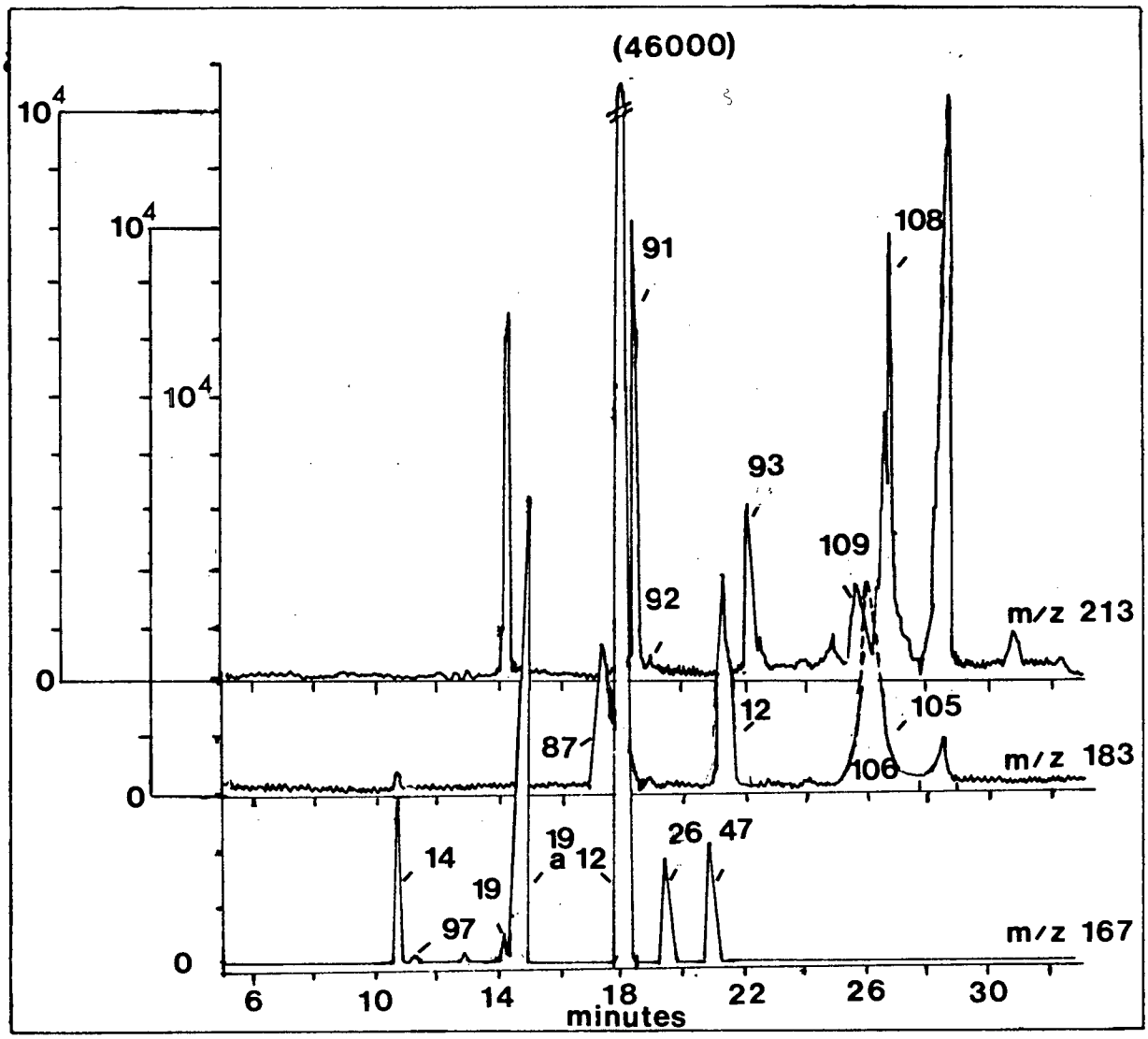


Figure 94. Composite selected ion chromatograms used to locate metabolites in β-glucuronidase-hydrolyzed extracts of bile from tertiary formamide dosed rats. A. Ion chromatogram m/z 167 showing phenyl ring intact metabolites, m/z 183 showing phenol metabolites and m/z 213 showing O-methylcatechol metabolites in the underivatized bile extract. Standard GC conditions.

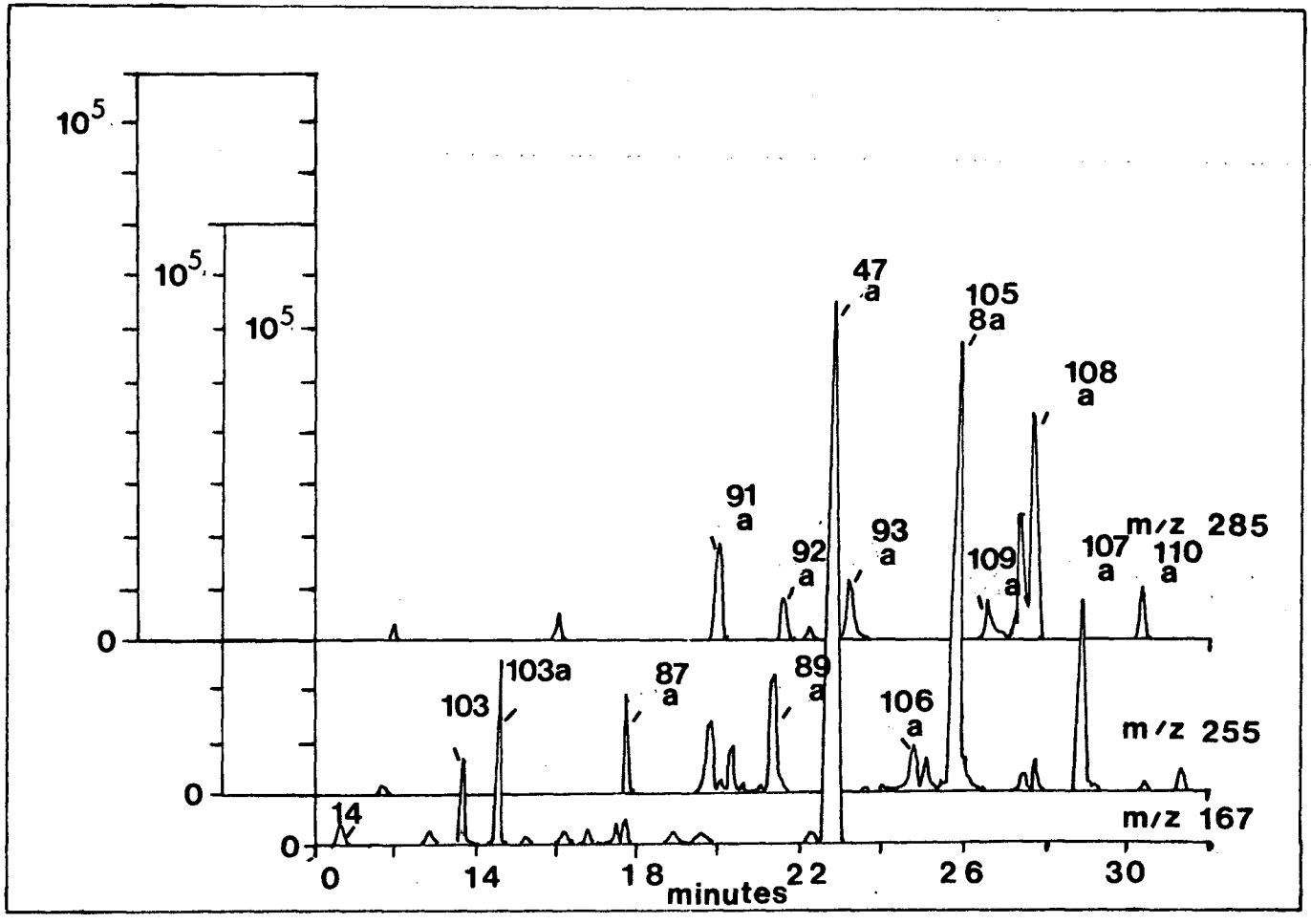


Figure 95. Composite selected ion chromatograms used to locate metabolites in β-glucuronidase hydrolyzed extracts of bile from tertiary formamide dosed rats. B. Ion chromatogram m/z 167 showing phenyl ring intact metabolites, m/z 255 showing TMS derivatized phenols and m/z 285 showing TMS derivatized O-methylcatechols in the BSTFA derivatized bile extract.

A. Mass spectrometry of arylaliphatic formamides and carbinolamides

In the formamides (26) and (12), cleavage adjacent to nitrogen with gamma proton transfer afforded a m/z 208, 193, 179, 167, 165, 152, 130, 105, 91 arylaliphatic cascade (see figure 47 for fragmentation). Phenyl ring substitution resulted in the loss of most ions in this cascade. However, benzylic cleavage, resulting in intense diarylmethyl cations was common to all metabolites and allows the detection of intact phenyl (m/z 167), phenol (m/z 183), TMS phenol (m/z 255), O-methylcatechol (m/z 213), and TMS O-methylcatechol (m/z 285) metabolites by monitoring the appropriate ions. Alpha cleavage was also important, especially in the formamides where base peaks arise from proton transfer to the alpha cleavage product. In formyl derivatives of some sympathomimetic amines, the alpha cleavage product gives rise to the base peak cation (Vilvala, 1979). It is apparently the longer alkyl chain which allows the proton transfer in the diarylformylbutanamine series studied here. This results in base peak cation radicals at m/z 87 and 73 in (26) and (12) respectively. The proton transfer reactions of amides are well known (Pelah, *et al.*, 1963).

The mass spectral fragmentation of the underivatized

carbinolamide (47) (figure 96) has the expected arylaliphatic cascade plus two important carbinol directed fragmentations which result in a M^+-H_2O ion at m/z 265 and a related alpha cleavage ion giving the base peak ion at m/z 85. A possible alcohol directed fragmentation affording a formyl aziridinium ion can account for the m/z 265 ion (figure 97). The TMS ether group directs the same fragmentation giving rise to the m/z 265 ion in the TMS carbinolamide (47a) and the m/z 353 and 383 ions present in the mass spectra of the di-TMS carbinolamide phenol (107a) and O-methyl catechol (110a) respectively (figure 97 and 98). The same mechanism with transfer of the diphenylethyl moiety rather than a proton to the carbinol oxygen is a possible explanation for the m/z 85 ion that is common to all the carbinolamide derived compounds (figure 99). A rearrangement elimination (*re*) mechanism of an odd electron ion (McLafferty, 1980) was used to account for the m/z 85 fragment, although rearrangements of this type commonly involve smaller migrating groups. Diphenylethanol and its TMS ethers lose H_2O or TMSOH to give ions m/z 179 (carbinolamide and carbinolamide TMS), m/z 267 (di-TMS carbinolamide phenol) and m/z 287 (di-TMS carbinolamide O-methylcatechol) (figure 99).

Rearrangement of the carbinol proton or TMS group to the nitrogen accounts for the m/z 72 ion in the mass spectrum of the carbinolamide and m/z 144 in all the carbinolamide TMS derivatives (figure 100). Other ions in the TMS derivatized carbinols can be envisaged by cleavage of bonds with or without proton transfer as summarized in figure 101.

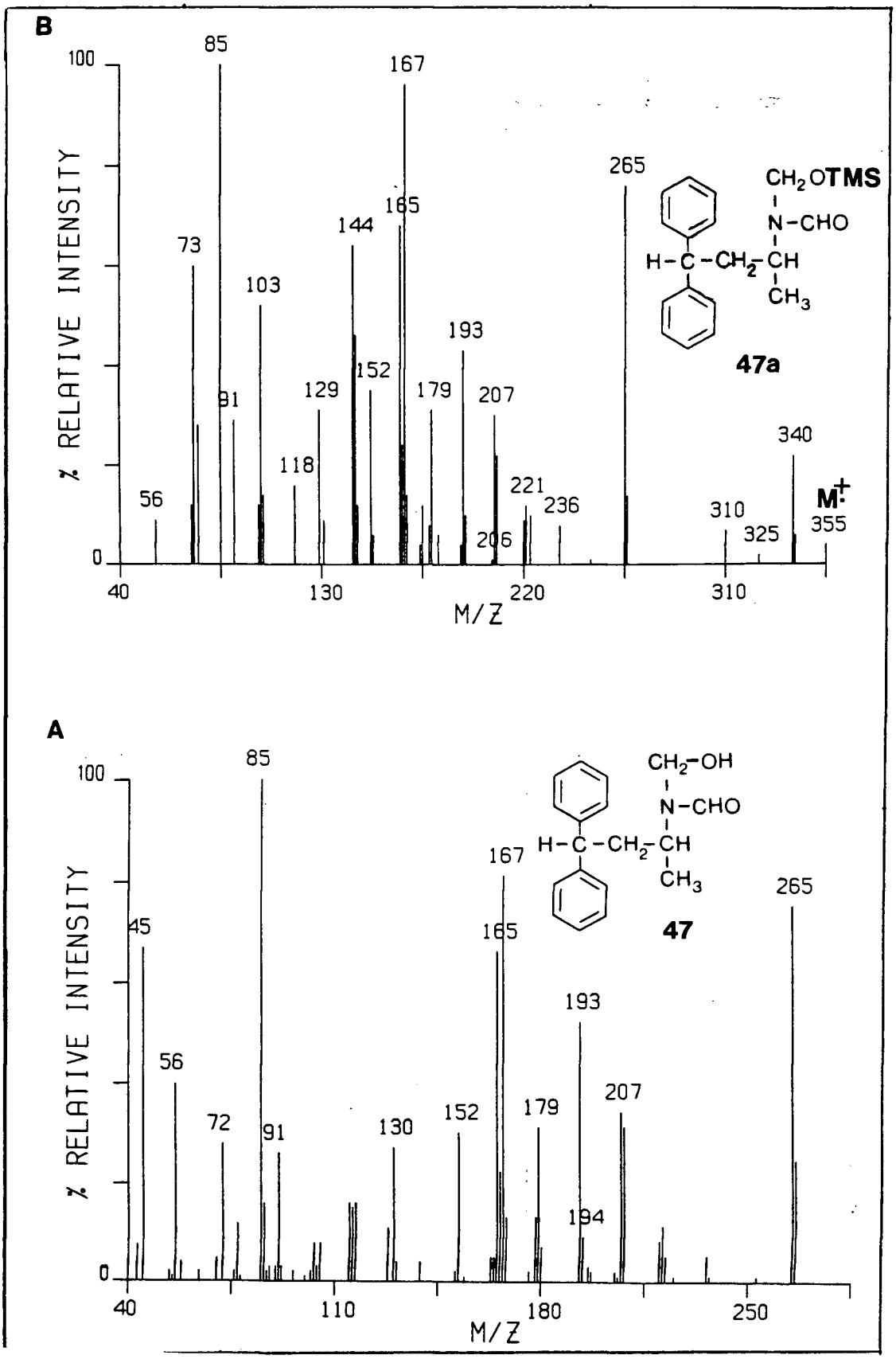


Figure 96. Mass spectra of the carbinolamide metabolites. (a) Carbinolamide (47). (b) Carbinolamide TMS (47a).

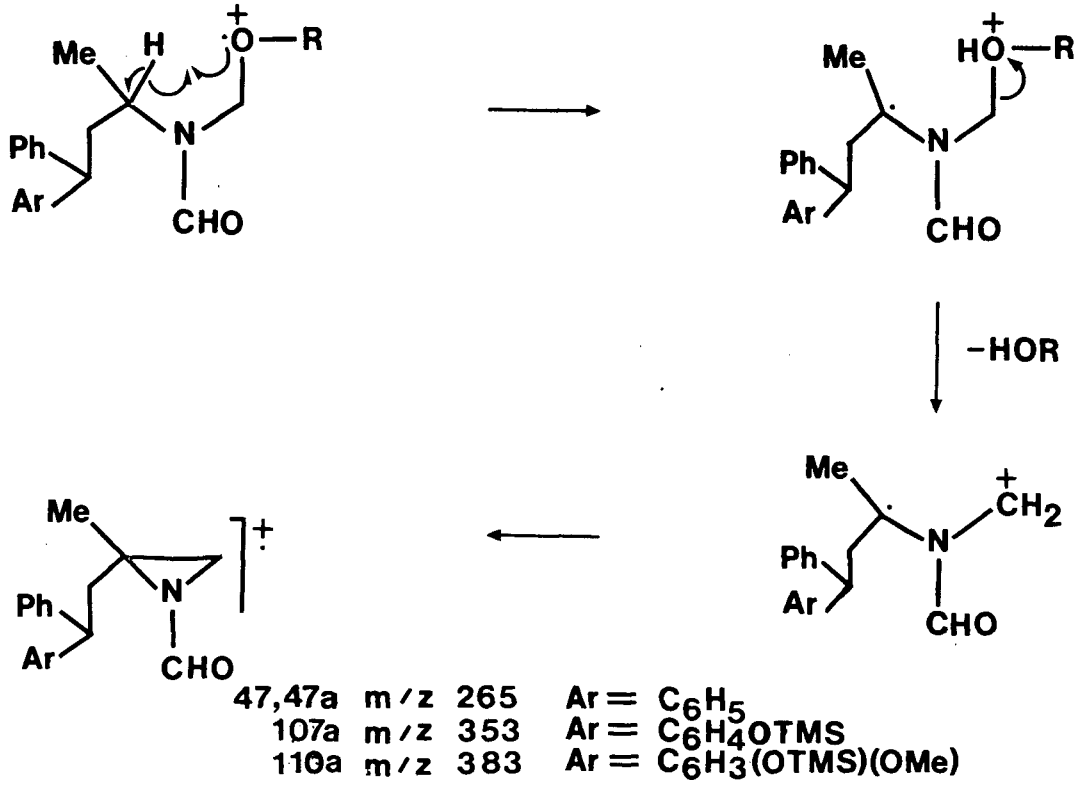


Figure 97. Proposed mass spectral fragmentations of the carbinolamide (47) and TMS derivatives of the carbinolamide (47a), the carbinolamide phenol (107a), and carbinolamide O-methylcatechol (110a). A. Alcohol (R_1=H) or TMS ether (R_1=TMS) directed proton rearrangement/elimination to afford m/z 265 (47, 47a), m/z 353 (107a) and m/z 383 (110a).

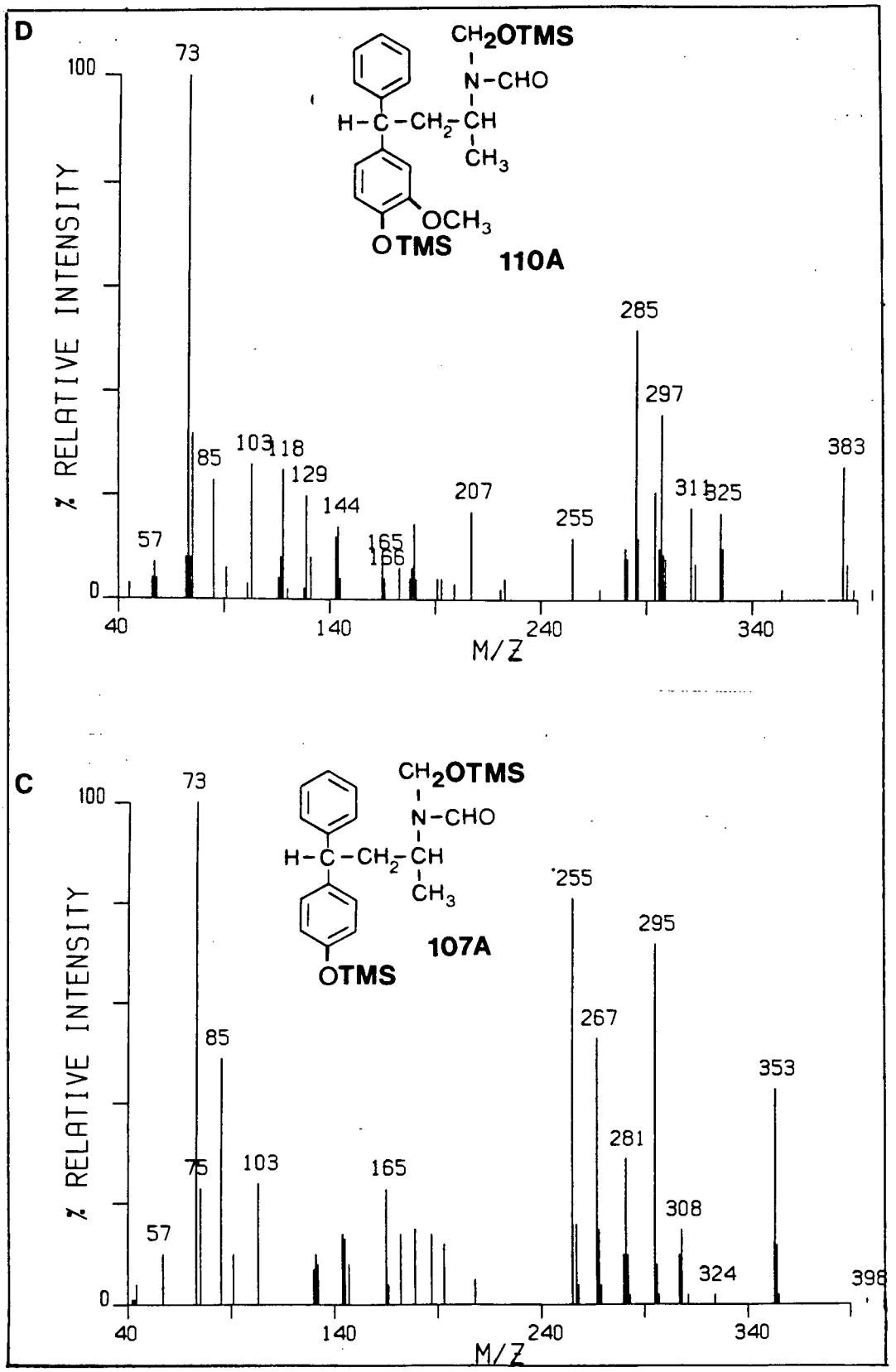


Figure 98. Mass spectra of the carbinolamide metabolites. (c) Carbinolamide phenol di-TMS (107a). (d) Carbinolamide O-methylcatechol di-TMS (110a).

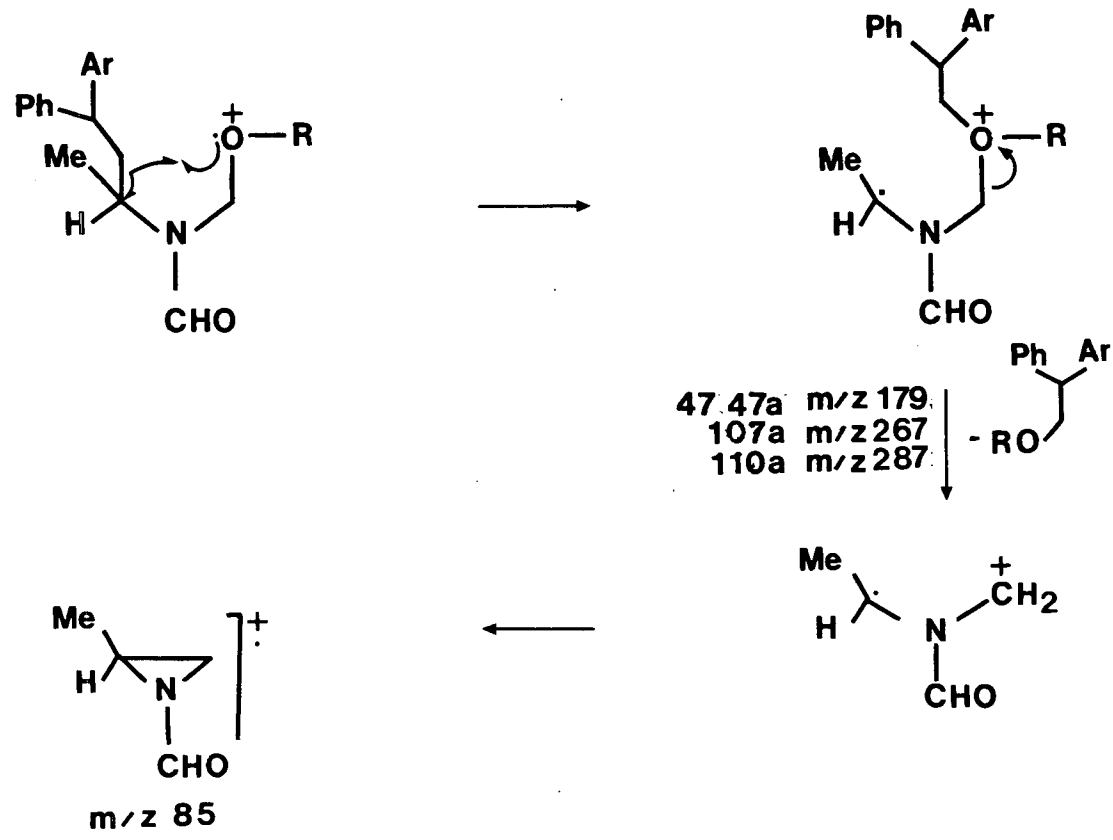


Figure 99. Proposed mass spectral fragmentations of the carbinolamide (47) and TMS derivatives of the carbinolamide (47a), the carbinolamide phenol (107a), and carbinolamide O-methylcatechol (110a). B. Alcohol (R_1=H) or TMS ether (R_1=TMS) directed diphenylethane rearrangement/elimination to afford m/z 85 in all the carbinolamide related compounds.

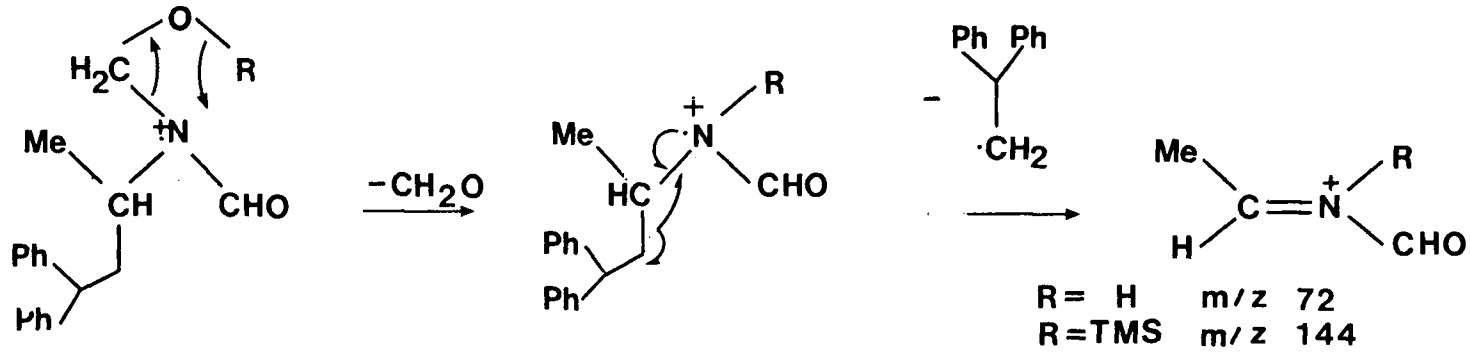


Figure 100. Proposed mass spectral fragmentations of the carbinolamide (47) and TMS derivatives of the carbinolamide (47a), the carbinolamide phenol (107a), and carbinolamide O-methylcatechol (110a). C. Rearrangement of the carbinol proton (R_1=H) or TMS group (R_1=TMS) to nitrogen to afford m/z 72 in the carbinolamide (47) and m/z 144 in the TMS derivatives (47a, 107a and 110a).

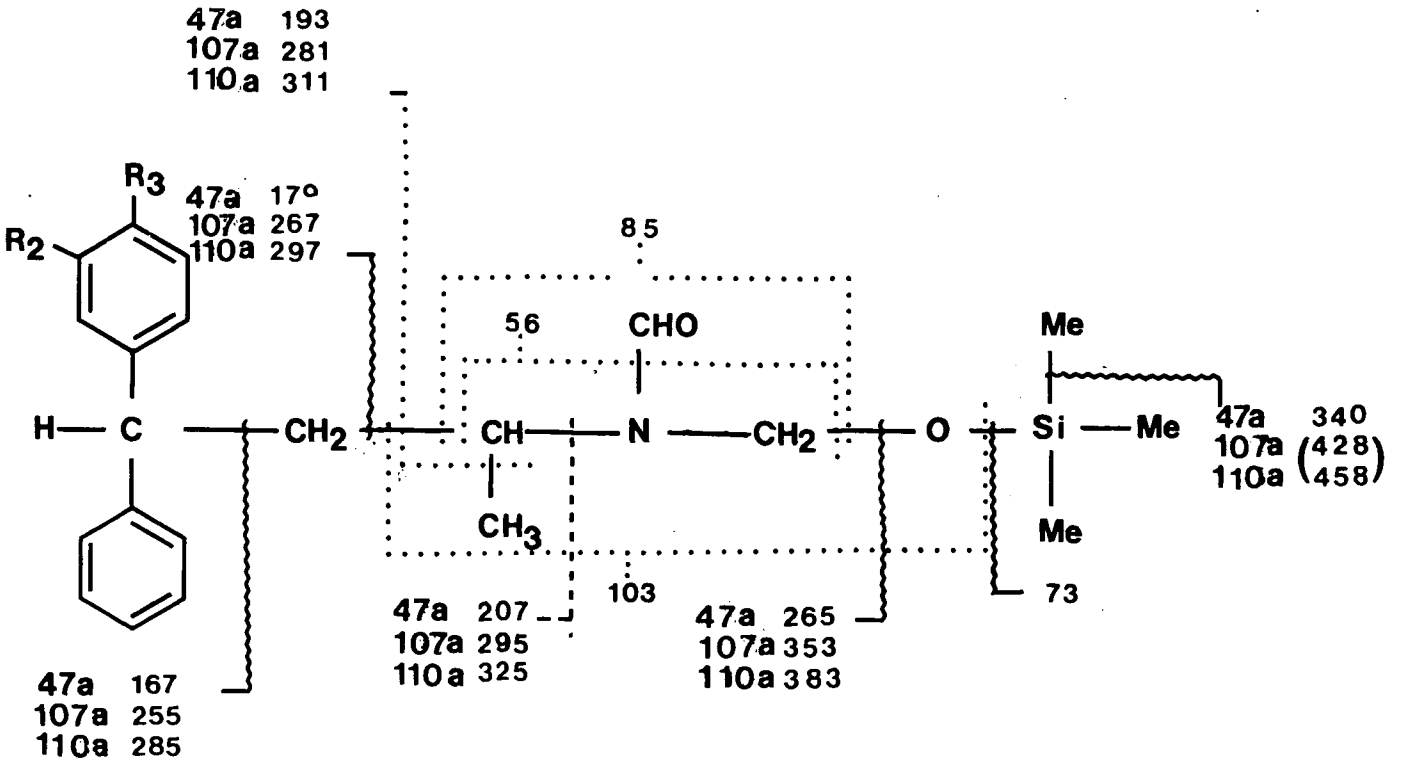


Figure 101. Summary of bond cleavages in the mass spectra of the carbinolamide metabolite TMS derivatives (47a, 107a and 110a). R_1=TMS, R_2=H or OCH_3, R_3= H or OTMS.

B. Metabolism of the tertiary arylaliphatic formamide

Based on the metabolites observed by GCMS, tertiary arylaliphatic formamides follow three general metabolic routes (figure 102). At the nitrogen atom , N-dealkylation via stable intermediate carbinolamides competes with N-deformylation. The phenyl rings are metabolized to the corresponding phenol or O-methylcatechol. The carbinolamide and aromatic hydroxyl groups are conjugated with glucuronic acid.

i. N-deformylation

The small amounts of norrecipavrin (15) detected in the nonconjugated urine fraction partially represents the deformylation pathway. Since norrecipavrin is rapidly metabolized to the oxime (19), diphenylbutanone (14) and diphenylbutanol (97), the extent of deformylation may be underestimated by quantitation of the amount of norrecipavrin alone. It is not certain whether the formamides or carbinolamides also contribute to the pool of oxime, diphenylbutanone, diphenylbutanol and their phenyl ring oxidized analogues by pathways other than deformylation.

Deformylation is a common metabolic pathway for the formamide functional group. N-methylamine accounts for 15% of the metabolites of N-methylformamide (Kestell, *et al.*, 1985), however, dimethylformamide is apparently not deformylated to dimethylamine (Scailteur *et al.*, 1984). The deformylated

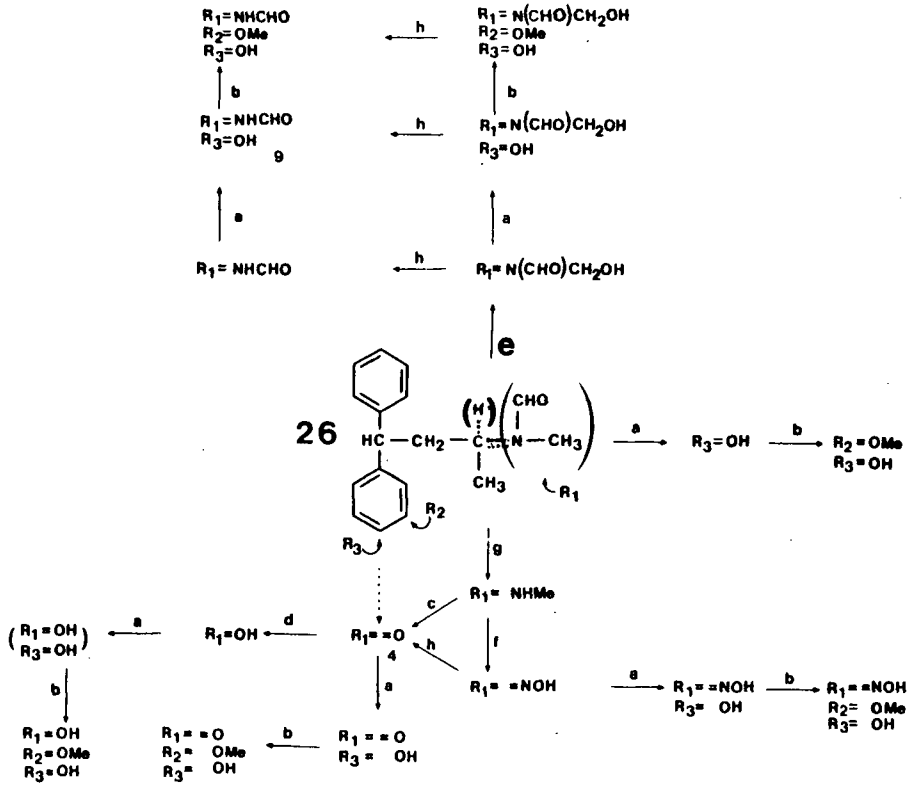


Figure 102. Metabolic pathways for the tertiary formamide (26) based on the metabolites observed by GCMS. a. Phenyl ring oxidation. b. Oxidation to the catechol and 3'-O-methylation. c. Oxidative deamination. d. Ketone reduction. e. α-Carbon oxidation to the carbinolamide. f. N-oxidation. g. N-deformylation. h. Hydrolysis. Dotted arrows = uncertain origin.

metabolite ANFT is rapidly produced by the carcinogen FANFT (Swaminathan and Bryan, 1984), and kynurenine formamidase activates the diformyl antimalarial prodrug 4,4'-diformamidodiphenylsulfone to the corresponding diamine (Chiou *et al*, 1971). Although enzymatic deformylation reactions are well known (Shinohara and Ishiguro, 1970), deformylation could also be a result of metabolic oxidation of the formamide to an unstable carbamic acid with subsequent decarboxylation (Kestell, *et al*., 1985).

ii. Phenyl ring oxidation and conjugation

The phenol and O-methylcatechol metabolites were found by monitoring the appropriate ion for the diarylmethyl moiety (m/z 183, 213 (underivatized), m/z 255, 285 (TMS)). The phenols and O-methylcatechols were expected based on similar biotransformation products of methadone (Kang,*et al*.,1979), recipavrin, terodiline (Noren, *et al*., 1985a,b) and related arylaliphatic amines. Metabolites 12, 14, 19, 15, 87, 89, 91, 92 and 93 were also metabolites of recipavrin. An attempt was made to detect catechol or dihydrodiol metabolites as di-TMS derivatives by monitoring the m/z 343 and 345 ions. Only one compound was detected (tr=31.57 min.) with base peak m/z 343 and m/z 87, 58 suggesting that the metabolite was the tertiary formamide catechol or the 4,4'-diphenol.

iii. N-dealkylation via carbinolamides to desalkylformamides

Analysis of the conjugated fraction of bile by GCMS revealed that the secondary formamide (12) was a major component. However, GCMS analysis of the TMS derivatized bile extract showed that the major metabolite was the carbinolamide (47) which decomposed in the GC inlet to the desalkyl compound (12) during the analysis of the underivatized bile extract. The structure of the carbinolamide was confirmed with a low yield synthesis in which the addition of the secondary formamide to formaldehyde was followed by derivatization with BSTFA.

Analogous di-TMS phenolic and O-methylcatechol carbinolamides (107a, 110a) were observed at long retention time in the BSTFA derivatized extracts of β-glucuronidase-hydrolyzed bile. It was not known whether these are phenyl ring-oxidized metabolites of the carbinolamide or alpha carbon oxidized metabolites of the tertiary formamide phenol and O-methylcatechol.

The phenyl ring-intact compound eluted at 12.91 minutes is also a metabolite of the secondary formamide. It has been tentatively identified as the alpha-methine carbinolamide (111). The mass spectrum is discussed in the section on secondary formamide metabolism.

Metabolic N-dealkylation of tertiary amines proceeds via unstable carbinolamine intermediates (Rose and Castagnoli, (review) 1983). When the electron density at nitrogen is decreased by such factors as amide resonance or aromatic substituents the stability of metabolically-derived carbinolamides or carbinolamines is increased sufficient to allow metabolic conjugation with glucuronic acid. In early studies, the existence of the carbinolamide dealkylation intermediate was inferred from the presence of the desalkyl metabolite in β-glucuronidase-hydrolyzed fractions (McMahon and Sullivan, 1965, Sullivan, *et al.*, 1968, Allen, *et al.*, 1971). Later carbinolamide metabolites were isolated and characterized in intact form by HPLC and as TMS derivatives by GCMS and GLC (Ross, *et al.*, 1983). Carbinolamide metabolites of dimethylformamide (Scailteur, *et al.*, 1984) and N-methylformamide (Kestell, *et al.*, 1987) have been described. Reference metabolites can be synthesized by the addition of the desalkyl compound to formaldehyde (Ross, *et al.*, 1983). Carbinolamides have been proposed to be the active species in the activity of a variety of nonbasic anticancer agents (Soloway, *et al.*, 1983). However, N-hydroxymethylformamide was not found to be the active species in the anticancer action of N-methylformamide (Cooksey, *et al.*, 1983), and interest in toxic or active metabolites of NMF is currently focused on glutathione related adducts (Kestell, *et al.*, 1986).

In conclusion, the tertiary formamide (26) was

metabolized in the rat via three major pathways. N-deformylation with oxidation of the product amine (15) gave rise to oxime (19), diphenylbutanone (14), diphenylbutanol (97) and the phenyl ring oxidized metabolites 87, 89, 91, 92 and 93. N-dealkylation via a stable carbinolamide (47), carbinolamide phenol (107) and carbinolamide O-methylcatechol (110), gave rise to secondary formamide (12) and its phenol and O-methylcatechol analogues (106) and (109). Lastly, the parent formamide (26) gave rise to a phenol and O-methylcatechol. All pathways were common to the N-formyl or arylaliphatic moieties.

The absence of any TMS-carbinolamide (47a) in derivatized recipavrin metabolite extracts suggests that the carbinolamide pathway is not the source of the formamide metabolite of recipavrin.

12. METABOLISM OF THE SECONDARY FORMAMIDE (12)

The metabolism of the secondary formamide (12, $Ph_2CHCH_2CH(CH_3)N(H)CHO$) was investigated to determine whether it was an intermediate in the production of a number of metabolites of the tertiary formamide, notably, those involved in the N-oxidation, N-dealkylation, deformylation and oxidative deamination pathways.

The total ion current and mass chromatograms shown in figure 103-106 show that the secondary formamide (12) is biotransformed to a number of metabolites common to the tertiary formamide (26). The metabolites observed in bile and urine are listed in table 18.

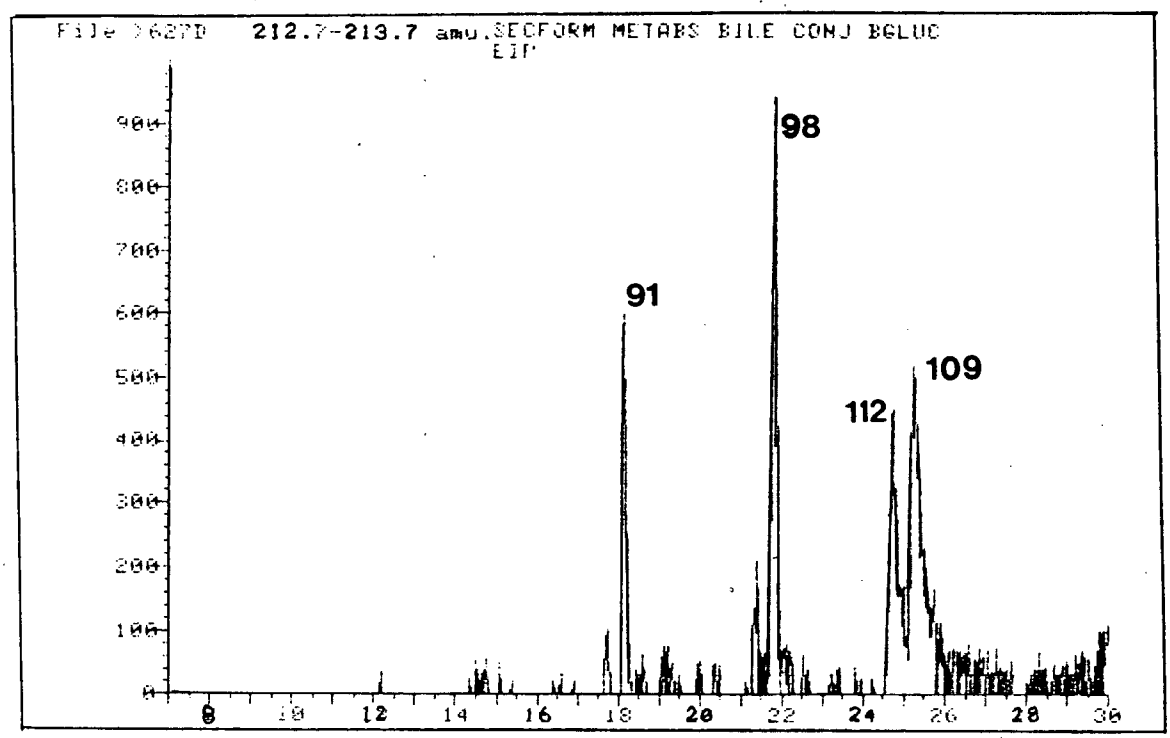


Figure 103. Mass chromatogram m/z 167 for the extract of β-glucuronidase-hydrolyzed fraction of bile from secondary formamide dosed rats. Standard GC conditions.

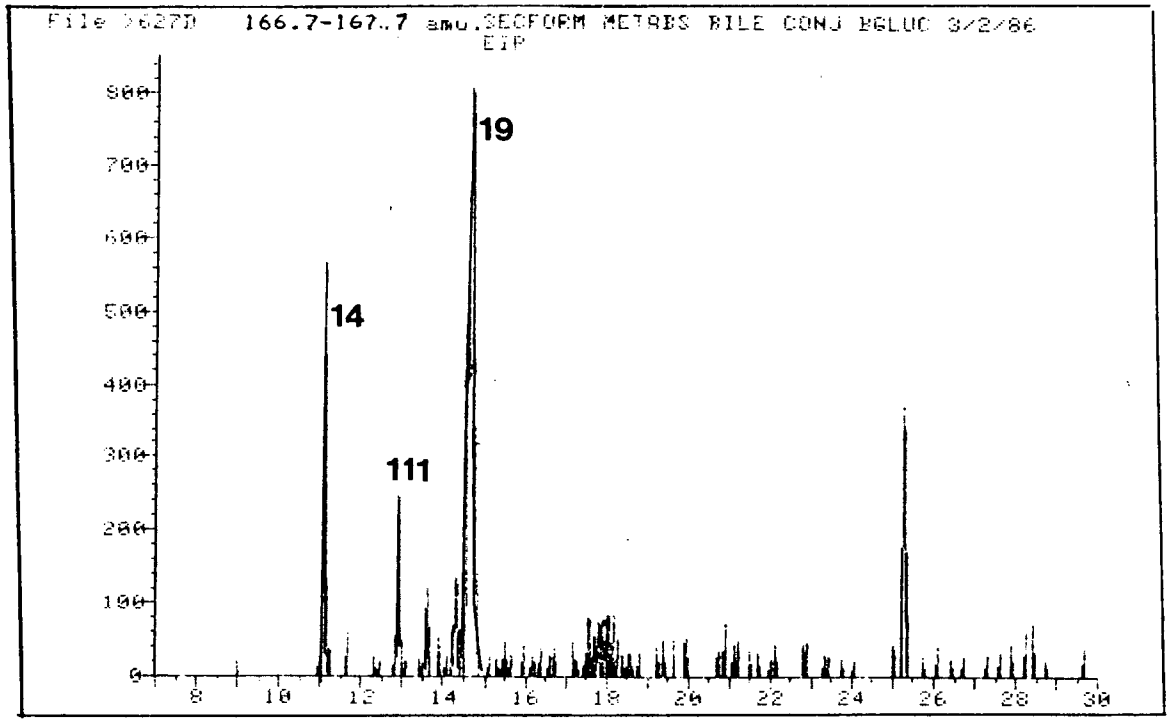


Figure 104. Mass chromatogram m/z 213 for the extract of β-glucuronidase-hydrolyzed fraction of bile from secondary formamide dosed rats. Standard GC conditions.

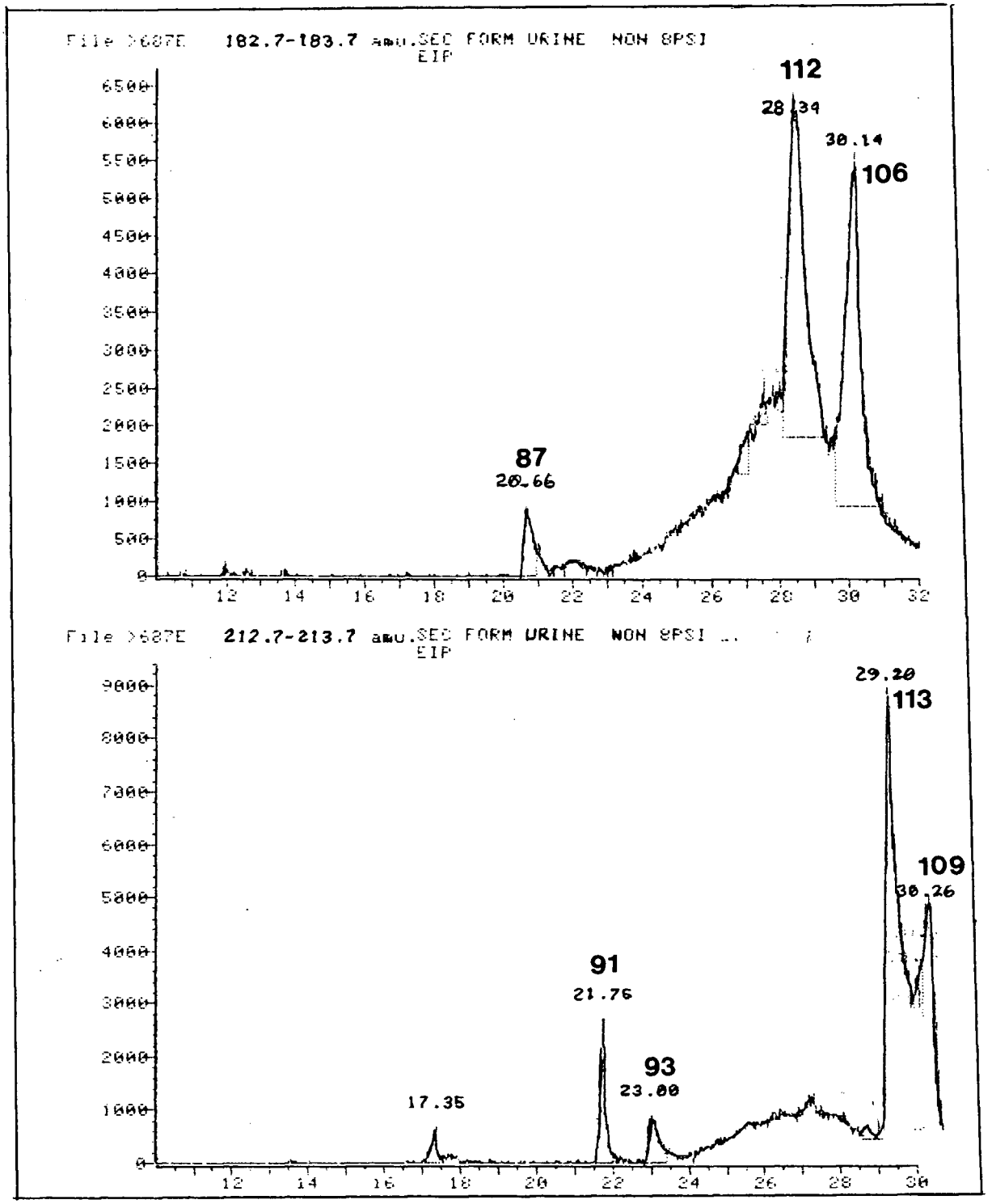


Figure 105. Mass chromatogram m/z 183 (top) and 213 (bottom) for the nonconjugated urine fraction from secondary formamide dosed rats. GC condition B.

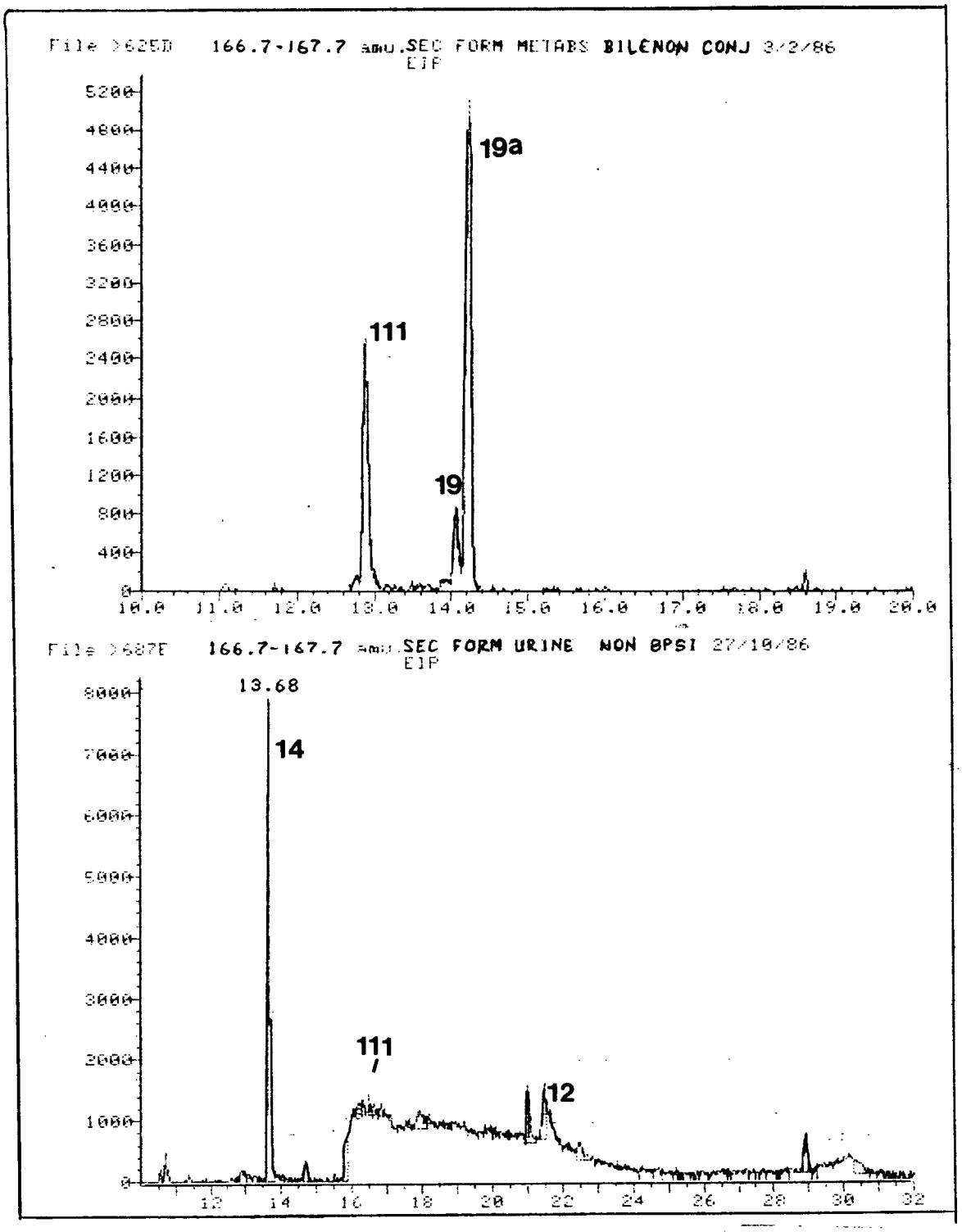


Figure 106. (top) Mass chromatogram m/z 167 for the nonconjugated bile extract of secondary formamide metabolites Standard GC conditions. (bottom) Mass chromatogram m/z 167 for the nonconjugated urine extract of secondary formamide metabolites. GC condition B.

Table 18. Metabolites and retention times for the secondary formamide metabolites.

Phenyl ring Substitution	Retention Time	Metabolite
Intact		
14	11.10 (13.68)	Diphenylbutanone*
111	12.90 (15.84)	Carbinolamide*
19	14.60	Oxime**
12	(21.49)	Sec. formamide***
Phenol		
87	(20.66)	Diphenylbutanone***
112	(28.34)	Carbinolamide***
106	(30.14)	Sec. formamide***
O-methylcatechol		
91	18.30 (21.75)	Diphenylbutanone*
93	(23.00)	Oxime***
98	21.82	Nitro compound****
113	24.80 (29.20)	Carbinolamide*
109	25.40 (30.26)	Sec. formamide*

* Present in nonconjugated urine extract and conjugated bile extract. Retention times from 15 psi injection (retention times in brackets from 8 psi injection).

** Present in both bile fractions.

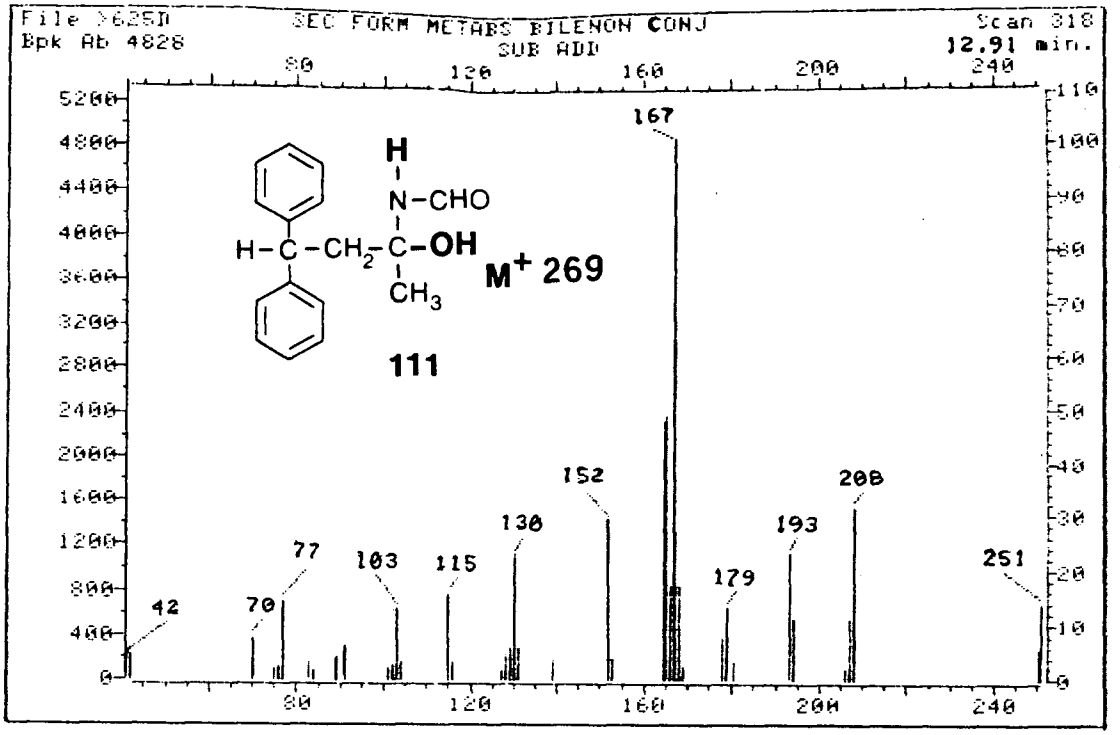
***Observed in the nonconjugated urine extract only, retention time longer due to 8 psi He back pressure.

**** Present in the conjugated bile extract only.

The compound eluting at 12.91 minutes (figure 106) has mass spectral characteristics in accord with the carbinolamide structure (111) shown in figure 108. The carbinolamide (111) is a stable dealkylation intermediate isolated both before and after β-glucuronidase hydrolysis and is probably hydrolyzed to diphenylbutanone and formamide. The high mass ion at m/z 251 suggests that the carbinolamide, (111, 3-formamido-3-hydroxy-1,1-diphenylbutane (M^+=269)) dehydrates on electron impact to afford the M^+-H_2O ion at m/z 251. The mechanism proposed for the tertiary carbinolamide dehydration (figure 99) can be modified to account for the m/z 251 ion as shown in figure 108. The presence of a weak m/z 70 ion cannot be rationalized by the same mechanism as that giving rise to the m/z 85 peak of the tertiary formamide carbinolamide. The carbinolamide (111) was also a metabolite of the tertiary formamide (26). This experiment showed that the secondary formamide is an intermediate in the biotransformation of the tertiary formamide (26) to carbinolamide (111).

The carbinolamide phenol (112) had few diagnostic ions and was identified based on retention time and weak ions at m/z 267 (M^+-18) and m/z 70. The carbinolamide O-methylcatechol (113) had no diagnostic ions other than the m/z 213 base peak and requires confirmation by TMS derivatization.

It is probable that deformylation of 12 to dinorrecipavrin (20) also occurs, with further metabolism to the oxime (19) and diphenylbutanone (14). No dinorrecipavrin was detected.



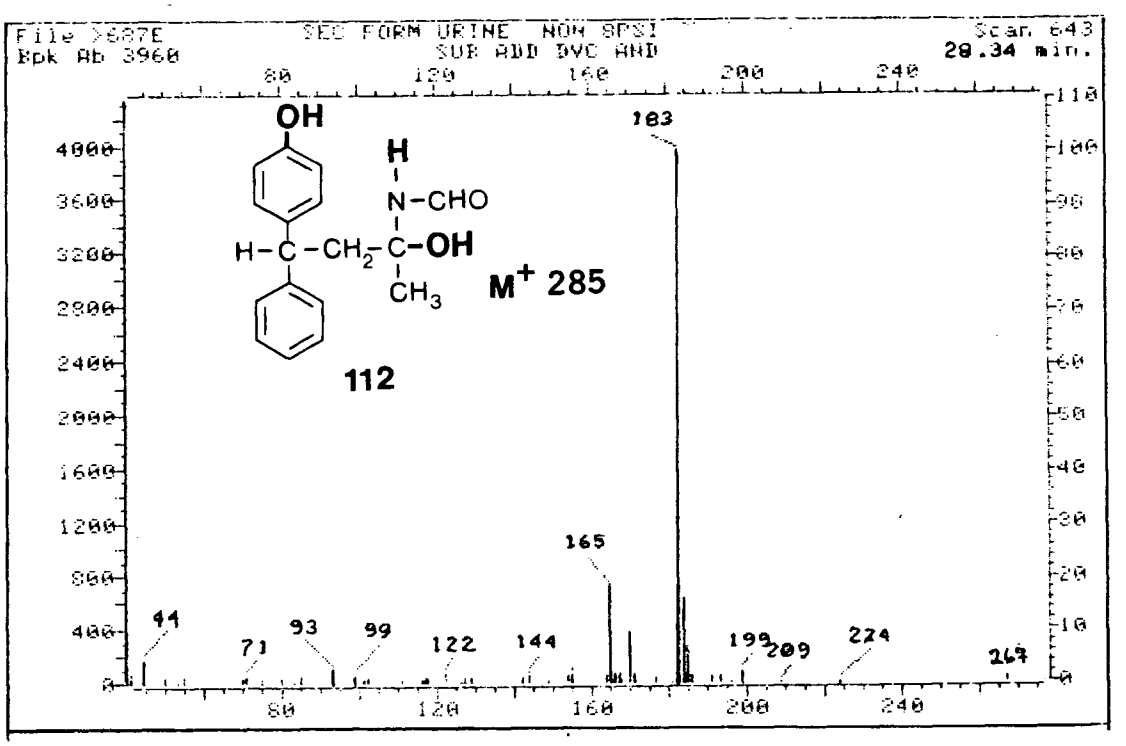
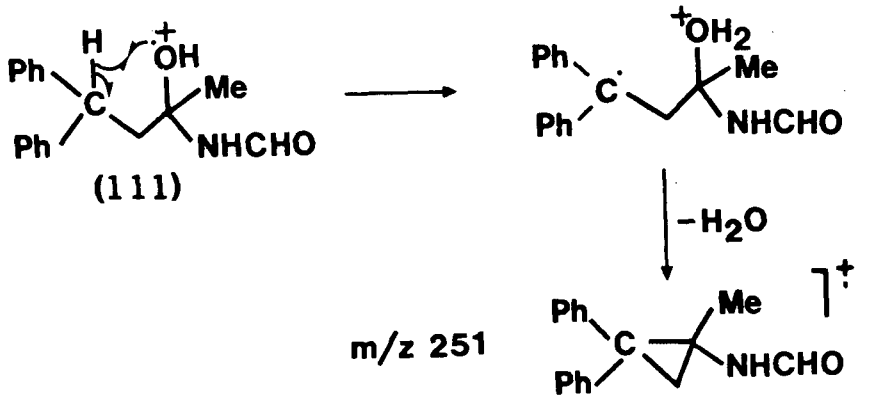


Figure 107.(top) Mass spectrum of the carbinolamide metabolite (111) observed in the conjugated and nonconjugated fraction. (bottom) Mass spectrum of the carbinolamide phenol metabolite (112) observed in the conjugated and nonconjugated fraction.

Figure 108. Fragmentation scheme to account for the M^+-18 peak of the carbinolamide metabolite (111).



It can be concluded that oxidative deamination, N-oxidation, N-dealkylation and phenyl ring oxidation are the major pathways of metabolism of the secondary formamide (12). N-deformylation to dinorrecipavrin (20) may be a requisite step in the generation of ketone and oxime metabolites, but can only be inferred. Diphenylbutanone (14) was also the expected decomposition product of the carbinolamide (111). Conjugation appears to be less important than with recipavrin (9) and tertiary formamide (26) metabolism. Compounds containing the N-methylformamide functional group such as the Leuckart specific byproducts of amphetamine synthesis are probable sources of hepatotoxic N-methylformamide.

As a compound class, N-methylformamide is the only compound available for comparison of metabolic products. N-methylformamide is N-deformylated, N-dealkylated by stable carbinolamide intermediates (Gescher, *et al.*, 1983), and forms glutathione adducts which are currently thought to mediate the hepatotoxic effects of this anticancer agent (Kestell *et al.* 1986, 1987).

IV. SUMMARY AND CONCLUSIONS

1. INVESTIGATION OF POSSIBLE CHEMICAL SOURCES OF A SECONDARY FORMAMIDE (6) METABOLITE OF METHADONE (8)

A. A mechanism was proposed for the oxidation of EDDP (1) with MCPBA to account for products (2,3,4,5,6,7) identified by GCMS.

B. The aldol condensation of 4,4-diphenyl-2,5-heptanedione (3) regiospecifically produced 2,3-dimethyl-5,5-diphenylcyclopent-2-enone (50).

C. Solvent contaminants were not a major source of the secondary formamide metabolite (6).

2.METABOLISM OF THE TERTIARY ARYLALIPHATIC AMINE RECIPAVRIN (9)

A. The *in vivo* biliary metabolites of the tertiary arylaliphatic amine N,N,α-trimethyl-γ-phenylbenzenepropanamine (Recipavrin^{R}) from Wistar rats were characterized by GCMS.

B. Nonconjugated metabolites included recipavrin (9), norrecipavrin (15), diphenylbutanone (14), diphenylbutanone oxime (19), detectable amounts of phenolic diphenylbutanone (87), diphenylbutanone oxime (89), and recipavrin (90), and O-methylcatechol derivatives of diphenylbutanone (91) and diphenylbutanone oxime (18).

C. Following β -glucuronidase hydrolysis and extraction from pH 10 solution, diphenylbutanone (14), diphenylbutanone oxime

(19), an unidentified compound (86), dinorrecipavrin (20), norrecipavrin (15), recipavrin (9), norrecipavrin phenol (88), the phenols 87, 89 and 90, diphenylbutanol O-methylcatechol (92), recipavrin O-methylcatechol (94), the O-methylcatechols 91 and 18 and a secondary formamide (12) were identified by GCMS.

D. To determine whether thermal isomerization of the methylene nitrone in the GC inlet was the source of the formamide, LCMS results for bile extracts were compared to those of synthetic standards 12 and 24 to show that the secondary formamide (12) and not the isomeric nitrone (24) was present in the bile extract prior to GCMS analysis.

E. Various purified extraction solvents were employed in sample workup. The formamide was present regardless of solvent used.

F. Metabolites isolated after β-glucuronidase hydrolysis were characterized by GCMS in their intact form, as trimethylsilyl (TMS) derivatives, or following derivatization with trimethylanilinium hydroxide (TMAH), in an attempt to stabilize and detect labile precursors of the nitrone and formamide such as the hydroxylamines (17 and 22). No hydroxylamines were detected.

3 METABOLISM OF TERTIARY AMINE (9) TO SECONDARY FORMAMIDE (12)

A. Potential chemical precursors of the secondary formamide metabolite (12) of recipavrin (9) that were ruled out:

i. A pathway from recipavrin (9) to tertiary formamide (26) to carbinolamide (47) to carbinolamide glucuronide (48) was ruled out based on the absence of the tertiary formamide in nonconjugated bile extracts and by the absence of carbinolamide (47) in β-glucuronidase-hydrolyzed bile extracts.

ii. Autoxidation of norrecipavrin (15) to formamide (12) was ruled out by adding norrecipavrin to control bile and then following normal extraction procedures. GCMS failed to demonstrate the formamide (12).

iii. Formylation of dinorrecipavrin (20) to formamide (12) was ruled out by studying the effects of added formaldehyde or formic acid on bile extracts from dinorrecipavrin dosed rats. No formamide (12) was detected by GCMS.

iv. Formaldehyde condensation with β-glucuronidase liberated primary hydroxylamine (22) was ruled out by treating the bile extract with excess formaldehyde during hydrolysis and extraction. No change in the amount of formamide (12) was detected by GCMS.

v. Chloroform and solvent related generation of formamide (12) was ruled out by the use of alternative solvents for metabolite extraction, by the use of solid phase extraction methods, and by the absence in bile extracts of carbamate related artifacts which arise by similar mechanisms.

vi. The secondary formamide (12) was not a metabolite of

dinorrecipavrin (20), thus ruling out the primary amine (20) and its metabolites as metabolic intermediates.

B. Potential sources of the formamide:

i. Norrecipavrin was demonstrated to be a metabolic intermediate in the conversion of recipavrin to the formamide (12) by studying the metabolism of norrecipavrin. The secondary formamide (12) was found in the conjugated fraction of bile from norrecipavrin dosed rats, suggesting that recipavrin is dealkylated prior to formamide metabolite generation.

ii. It was possible to increase the relative amount of the secondary formamide observed by GCMS by treating the conjugated fraction of bile from recipavrin dosed rats with a combination of formaldehyde and hydrogen peroxide. Thus peroxidation of an imine intermediate is a possible source of the formamide metabolite (12), but a plausible glucuronide precursor for the imine or amine substrate cannot be logically proposed.

iii. The formamide (12) was detected by GCMS in extracts of bile from rats administered the nitrone (24).

iv. The nitrone (24) was shown to degrade in the GC inlet to the formamide (12), especially when injected at high concentrations or co-injected with bile components.

v. The nitrone (24) had been shown by LCMS to degrade at room temperature to the formamide (12) (Slatter, 1983). The alkali

catalyzed Beckmann rearrangement of nitrones to amides was used to account for this observation.

vi. The nitrone (24) had been shown to arise by oxidation of the secondary hydroxylamine (17) under simulated bile workup conditions (Slatter, 1983). Hydroxylamine metabolites of structurally related arylaliphatic amines are known to be glucuronide conjugated.

C. The available evidence from the study of the recipavrin formamide metabolite favors the metabolic sequence: Recipavrin (9), norrecipavrin (15), secondary hydroxylamine (17), secondary hydroxylamine glucuronide (42). Upon β-glucuronidase hydrolysis, the unstable hydroxylamine (17) could be oxidized to nitrone (24) and then converted to formamide (12) either by Beckmann rearrangement during isolation or by isomerization in the GC inlet.

4. METABOLISM OF ARYLALIPHATIC FORMAMIDES.

A. The *in vivo* biliary and urinary metabolites of the tertiary arylaliphatic formamide (±)-N-methyl-N-(1-methyl-3,3-diphenylpropyl) formamide (26) from male wistar rats have been characterized by GCMS.

i. In urine, nonconjugated metabolites included a ketone (14) and secondary amine (15). β-Glucuronidase treatment liberated the ketone (14), a secondary alcohol (97), a keto-oxime (19), a carbinolamide (47), and its decomposition product, the secondary formamide (12), phenolic analogues of the ketone (87), oxime (89), and tertiary formamide (105) and O-

methylcatechol analogues of the ketone (91), a secondary alcohol (92), the oxime (93), secondary formamide (109) and tertiary formamide (108).

ii. In bile, compounds 12,19,26,47,87,89,91 and 97 were present as nonconjugated metabolites. β-Glucuronidase liberated a phenol analogue of the secondary formamide (106) and a carbinolamide (111). All of the previously listed compounds except the secondary amine (15) were also detected.

iii. Trimethylsilylation of the conjugated bile fraction revealed two additional compounds, 107 and 110 which were derived from phenolic and O-methylcatechol analogues of the carbinolamide (47).

B. The *in vivo* biliary and urinary metabolites of the secondary arylaliphatic formamide (±)-N-(1-methyl-3,3-diphenylpropyl) formamide (12) have been characterized by GCMS in male wistar rats.

i. Secondary formamide (12) was metabolized mainly by deformylation to norrecipavrin (15). The carbinolamide (111) was characterized in β-glucuronidase-hydrolyzed bile extracts.

C. There are very few reports available on formamide metabolism, which is surprising in light of the hepatotoxicity associated with the use of N-methylformamide as an anticancer agent. The tertiary and secondary formamide metabolism studies provide the first report of potentially toxic carbinolamide metabolites of higher molecular weight formamides.

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VI. APPENDIX

1. SPECTRA FOR SYNTHETIC COMPOUNDS

1.1. Mass spectrum of cyclopentenone (50)

1.2.IR spectrum (film) of cyclopentenone (50)

1.3. UV spectrum (in acetonitrile) of cyclopentenone (50)

1.4. Mass spectrum of anti TMS oxime (52a)

1.5. Mass spectrum of syn TMS oxime (52)

1.6. IR spectrum (film) of recipavrin free base

1.7. Mass spectrum of cis diphenylbutene (54)

1.8. Mass spectrum of trans diphenylbutene (55)

1.9. 80 MHz NMR spectrum of recipavrin N-oxide (53) (contaminated with some recipavrin)

1.10. Mass spectrum of dinorrecipavrin (20)

1.11. Mass spectrum of dinorrecipavrin TMS (104)

1.12. Mass spectrum of the TMS primary hydroxylamine (102)

1.13. 300 MHz NMR spectrum of primary hydroxylamine (102) after D_2O exchange

1.14. 300 MHz NMR spectrum of autoxidation product (56)

1.15. IR spectrum (mull) of autoxidation product (56)

1.16. Mass spectrum of recipavrin-D_3

1.17. Mass spectrum of secondary hydroxylamine TMS derivative (18)

1.18. Mass spectrum of secondary hydroxylamine O-methyl ether (58)

1.19. Total ion current and mass spectrum of N-methyl nitrone (44)

1.20. 300 MHz NMR spectrum of N-methyl nitrone (44) showing only trans isomer

1.21. IR spectrum (mull) of N-methyl nitrone (44)

1.22. Mass spectrum of methylene imine (23)

1.23. 300 MHz NMR spectrum of secondary formamide (12) (from ethyl formate reaction) ($CDCl_3$ at 25°)

1.24. 300 MHz NMR spectrum of secondary formamide (12) (Leuckart reaction) Brief D_2O exchange. Arrows denote changes in spectrum

1.25. 300 MHz NMR spectrum of secondary formamide (12) (Leuckart reaction) (DMSO-D_6 at 25°)

1.26. 300 MHz NMR spectrum of secondary formamide (12) (Leuckart reaction) (DMSO-D_6 at 90°)

1.27. 75 MHz ^{13}C NMR spectrum of secondary formamide (12) (Leuckart reaction) (BB decoupled)

1.28. 75 MHz ^{13}C NMR spectrum of secondary formamide (12) (Leuckart reaction) (SFORD)

1.29. 100 MHz ^{13}C NMR spectrum of secondary formamide (12) (ethyl formate reaction) (BB decoupled)

1.30. IR spectrum (film) of secondary formamide (12) (ethyl formate reaction)

1.31. IR spectrum (0.1 mm cell in $CHCl_3$) of secondary formamide (12) (Leuckart reaction)

1.32. Mass spectrum of pyrimidine (59)

1.33. IR spectrum (film) of pyrimidine (59)

1.34. IR spectrum (film) of suspected formohydroxamic acid (61)

1.35. Mass spectrum of the tertiary formamide (26)

1.36. Mass spectrum of the synthetic TMS carbinolamide (62)

1.37. Mass spectrum of the acetamide (63)

1.38. 80 MHz NMR spectrum of the acetamide (63)

1.39. IR spectrum (film) of the acetamide (63)

1.40. Mass spectrum of the synthetic ethanimine (64)

1.41. Mass spectrum of the isocyanide (65) (possibly isomerized to the nitrile)

1.42. 300 MHz NMR spectrum of the isocyanide (65)

1.43. IR spectrum (film) of the isocyanide (65)

1.44. Mass spectrum of the carbamate (66)

1.45. 300 MHz NMR spectrum of the carbamate (66)

1.46. IR spectrum (film) of the carbamate (66)

1.47. Mass spectrum of the carbamate (67)

1.48. 300 MHz NMR spectrum of the carbamate (67)

1.49. IR spectrum (film) of the carbamate (67)

1.50. Mass spectrum of the carbamate (68)

1.51. 300 MHz NMR spectrum of the carbamate (68)

1.52. IR spectrum (film) of the carbamate (68)

1.53. Mass spectrum of the carbamate (69)

1.54. 300 MHz NMR spectrum of the carbamate (69)

1.55. IR spectrum (film) of the carbamate (69)

1.56. 400 MHz NMR spectrum of promethazine secondary formamide (72). NH decoupled.

1.57. Total ion current and mass spectrum of promethazine secondary formamide (72) (30 eV)

1.58. IR spectrum (mull) of promethazine secondary formamide (72)

1.59. IR spectrum ($CHCl_3$ solution) of promethazine secondary formamide (72)

1.60. Mass spectrum of pyrimidine (73)

1.61. IR spectrum (mull) of promethazine secondary formamide N_{10}-oxide (75)

1.62. IR spectrum (film) of promethazine N-oxide (76)

1.63. 80 MHz NMR spectrum of promethazine N-oxide (76)

1.64. Mass spectrum of methylene imine (78)

1.65. IR spectrum (film) of methylene imine (78)

1.66. 80 MHz NMR spectrum of methylene imine (78)(decoupled at arrow)

1.67. 400 MHz NMR spectrum of run at 25° (bottom) and at 60°

1.68. Mass spectrum of secondary formamide (83)

2. RECIPAVRIN METABOLITES

2.1. Mass chromatograms m/z 115 (top) and m/z 130 (bottom) used to locate dinorrecipavrin TMS and norrecipavrin TMS derivatives in the conjugated fraction of bile.

2.2. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the recipavrin conjugated bile chloroform extract.

2.3. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the recipavrin nonconjugated bile ethyl acetate extract.

2.4. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the recipavrin nonconjugated bile chloroform extract.

2.5. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the recipavrin nonconjugated bile chloroform extract.

2.6. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the recipavrin nonconjugated urine ethyl acetate extract.

2.7. Mass chromatogram m/z 183 (top) for the recipavrin nonconjugated urine ethyl acetate extract, GC conditions D. No intensity at m/z 213.

2.8. Mass spectra of recipavrin metabolites and their derivatives: (top) diphenylbutanone (14). (bottom) recipavrin (9).

2.9. Mass spectra of recipavrin metabolites and their derivatives: (top) norrecipavrin (15). (bottom) norrecipavrin TMS (114).

2.10. Mass spectra of recipavrin metabolites and their derivatives: (top) syn-oxime (19). (bottom) syn-oxime TMS (52).

2.11. Mass spectra of recipavrin metabolites and their derivatives: (top) anti-oxime (19a). (bottom) anti-oxime TMS (52a)

2.12. Mass spectra of recipavrin metabolites and their derivatives: (top) Unidentified metabolite (86). (bottom) acetamide (63).

2.13. Mass spectra of recipavrin metabolites and their derivatives: (top) diphenylbutanone phenol TMS (87a). (bottom) diphenylbutanone phenol (87).

2.14. Mass spectra of recipavrin metabolites and their derivatives: (top) norrecipavrin phenol di-TMS (88a). (bottom) .norrecipavrin phenol (88).

2.15. Mass spectra of recipavrin metabolites and their derivatives: (top) recipavrin O-methylcatechol (94). (bottom) recipavrin O-methylcatechol TMS (94a).

2.16. Mass spectra of recipavrin metabolites and their derivatives: (top) syn-oxime phenol di-TMS (89c). (bottom) recipavrin phenol TMS (90a).

2.17. Mass spectra of recipavrin metabolites and their derivatives: (top) anti-oxime phenol di-TMS (89a). (bottom) anti-oxime phenol (89).

2.18. Mass spectra of recipavrin metabolites and their derivatives: (top) diphenylbutanone O-methylcatechol TMS (91a). (bottom) diphenylbutanone O-methylcatechol (91).

2.19. Mass spectra of recipavrin metabolites and their derivatives: (top) suspected norrecipavrin O-methylcatechol (115).(bottom) diphenylbutanol O-methylcatechol (92).

2.20. Mass spectra of recipavrin metabolites and their derivatives: (top) anti-oxime O-methylcatechol di-TMS (93a). (bottom) anti-oxime O-methylcatechol (93)

2.21. Mass spectra of recipavrin metabolites and their derivatives: (top) syn-oxime O-methylcatechol di-TMS (93c). (bottom) syn-oxime O-methylcatechol (93b)

2.22. Mass chromatograms m/z 197 (top) and m/z 227 (bottom) for the recipavrin TMAH derivatized conjugated bile ethyl acetate extract.

2.23. Mass spectra of TMAH derivatized recipavrin metabolites: (top) anti O-methyl oxime (16).(bottom) syn O-methyl oxime (16a).

2.24. Mass spectra of TMAH derivatized recipavrin metabolites : (top) diphenylbutanone O-methyl phenol (87b).(bottom) diphenylbutanone dimethylcatechol (91b).

2.25. Mass spectra of TMAH derivatized recipavrin metabolites: (top) anti O-methyl oxime dimethylcatechol (93e). (bottom) syn O-methyl oxime dimethylcatechol (93d).

2.26. Mass spectra of TMAH derivatized recipavrin metabolites: (top) recipavrin O-methylphenol (90b). (bottom). Norrecipavrin O-methylphenol (88b).

3. TERTIARY FORMAMIDE METABOLITES

3.1. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the tertiary formamide conjugated urine extract.

3.2. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the tertiary formamide conjugated urine extract.

3.3. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the tertiary formamide nonconjugated bile extract.

3.4. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the tertiary formamide nonconjugated bile extract.

3.5. Total ion current (top) for the tertiary formamide nonconjugated urine extract. (bottom) Mass spectrum of norrecipavrin from the nonconjugated urine extract.

3.6. Total ion current (top) for the tertiary formamide conjugated bile extract (positive chemical ionization using methane reagent gas). (bottom) Positive chemical ionization mass spectrum of the TMS carbinolamide (62) from the conjugated bile extract.

3.7. Mass spectrum of a suspected tertiary formamide catechol metabolite. Mass chromatogram m/z 343 used to locate underivatized catechol metabolites

3.8. Mass spectra of tertiary formamide metabolites and their derivatives: (top) diphenylbutanone (14). (bottom) diphenylbutanol (97).

3.9. Mass spectra of tertiary formamide metabolites and their derivatives: (top) carbinolamide (111). (bottom) diphenylbutanol O-methylcatechol (92).

3.10. Mass spectra of tertiary formamide metabolites and their derivatives: (top) syn-oxime (19). (bottom) anti-oxime (19a).

3.11. Mass spectra of tertiary formamide metabolites and their derivatives: (top) syn-oxime TMS (103). (bottom) anti-oxime TMS (103a).

3.12 . Mass spectra of tertiary formamide metabolites and their derivatives: (top) tertiary formamide (26). (bottom) secondary formamide (12).

3.13. Mass spectra of tertiary formamide metabolites and their derivatives: (top) diphenylbutanone phenol (87). (bottom) diphenylbutanone phenol TMS (87a).

3.14. Mass spectra of tertiary formamide metabolites and their derivatives: (top) oxime phenol (89). (bottom) oxime phenol di-TMS (89a).

3.15. Mass spectra of tertiary formamide metabolites and their derivatives: (top) secondary formamide phenol TMS (106a). (bottom) secondary formamide phenol (106).

3.16. Mass spectra of tertiary formamide metabolites and their derivatives: (top) tertiary formamide phenol (105). (bottom) tertiary formamide phenol TMS (105a).

3.17. Mass spectra of tertiary formamide metabolites and their derivatives: (top) diphenylbutanone O-methylcatechol (91) (some peak overlap with the secondary formamide (12)). (bottom) diphenylbutanone O-methylcatechol TMS (91a).

3.18. Mass spectra of tertiary formamide metabolites and their derivatives: (top) oxime O-methylcatechol (93). (bottom) oxime O-methylcatechol TMS (93a).

3.19. Mass spectra of tertiary formamide metabolites and their derivatives: (top) secondary formamide O-methylcatechol (109). (bottom) secondary formamide O-methylcatechol TMS (109a).

3.20. Mass spectra of tertiary formamide metabolites and their derivatives: (top) tertiary formamide O-methylcatechol (108). (bottom) tertiary formamide O-methylcatechol TMS (108a).

3.21. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the tertiary formamide TMAH derivatized conjugated bile extract. (Retention times not reliable, subtract approx 0.5 min.)

3.22. Mass chromatograms m/z 197 (top) and m/z 227 (bottom) for the tertiary formamide TMAH derivatized conjugated bile ethyl acetate extract. (Retention times not reliable, subtract approx 0.5 min.)

3.23. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) Suspected O-methyl derivative of carbinolamide (47). (bottom) Suspected recipavrin O-methyl phenol (116)(a norrecipavrin phenol TMAH derivative).

3.24. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) secondary formamide O-methyl phenol (106b). (bottom) tertiary formamide O-methyl phenol (105b).

3.25. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) secondary formamide dimethylcatechol (109b). (bottom) tertiary formamide dimethylcatechol (108b).

3.26. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) Diphenylbutanone dimethylcatechol (91b). (bottom) Diphenylbutanol dimethylcatechol (92b).

3.27. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) O-methyl oxime O-methyl phenol (89b). (bottom) O-methyl oxime dimethylcatechol (93b).

4. SECONDARY FORMAMIDE (12) METABOLITES

4.1 (top) Total ion current for the nonconjugated biliary metabolites of the secondary formamide (12). (bottom) Mass spectrum of the suspected nitro O-methyl catechol (98)

5. NITRONE (24) METABOLITES

5.1 (top) Total ion current for the biliary metabolites of the methylene nitrone (24). Mass spectrum of the nitrone metabolite dinorrecipavrin (20).

6. CONTROL EXPERIMENTS

6.1. (top) Total ion current for an extract of β-glucuronidase hydrolyzed conjugated biliary metabolites of recipavrin D_3. (bottom) Mass chromatogram m/z 167 for the same sample (control experiment for the sulfatase hydrolysis experiment)

6.2 (top) Mass chromatogram m/z 187 for an extract of β-glucuronidase hydrolyzed conjugated biliary metabolites of recipavrin D_3. (bottom) Mass chromatogram m/z 213 for the same sample (control experiment for the sulfatase hydrolysis experiment)

6.3. (top) Mass spectrum of the D_3 secondary formamide metabolite of recipavrin. (bottom) Mass spectrum of the D3 nitro O-methylcatechol.

6.4. (top) Total ion current for an extract of nonconjugated biliary metabolites of recipavrin (worked up after prolonged storage at room temperature). (bottom) A close-up of mass chromatogram m/z 167 for the same sample showing both the secondary formamide (12) and tertiary formamide (26).

6.5. (top) Mass spectrum of the secondary formamide (12). (bottom) Mass spectrum of the tertiary formamide observed in the delayed workup sample.

6.6. (top) Close-up of mass chromatogram m/z 167 for a control extract of β-glucuronidase hydrolyzed recipavrin metabolites. (bottom) Total ion current for the same sample.

6.6.1. (top) Close-up of mass chromatogram m/z 167 for a vitamin C treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a BHT treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.

6.6.2. (top) Close-up of mass chromatogram m/z 167 for a dimedone treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a formaldehyde treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.

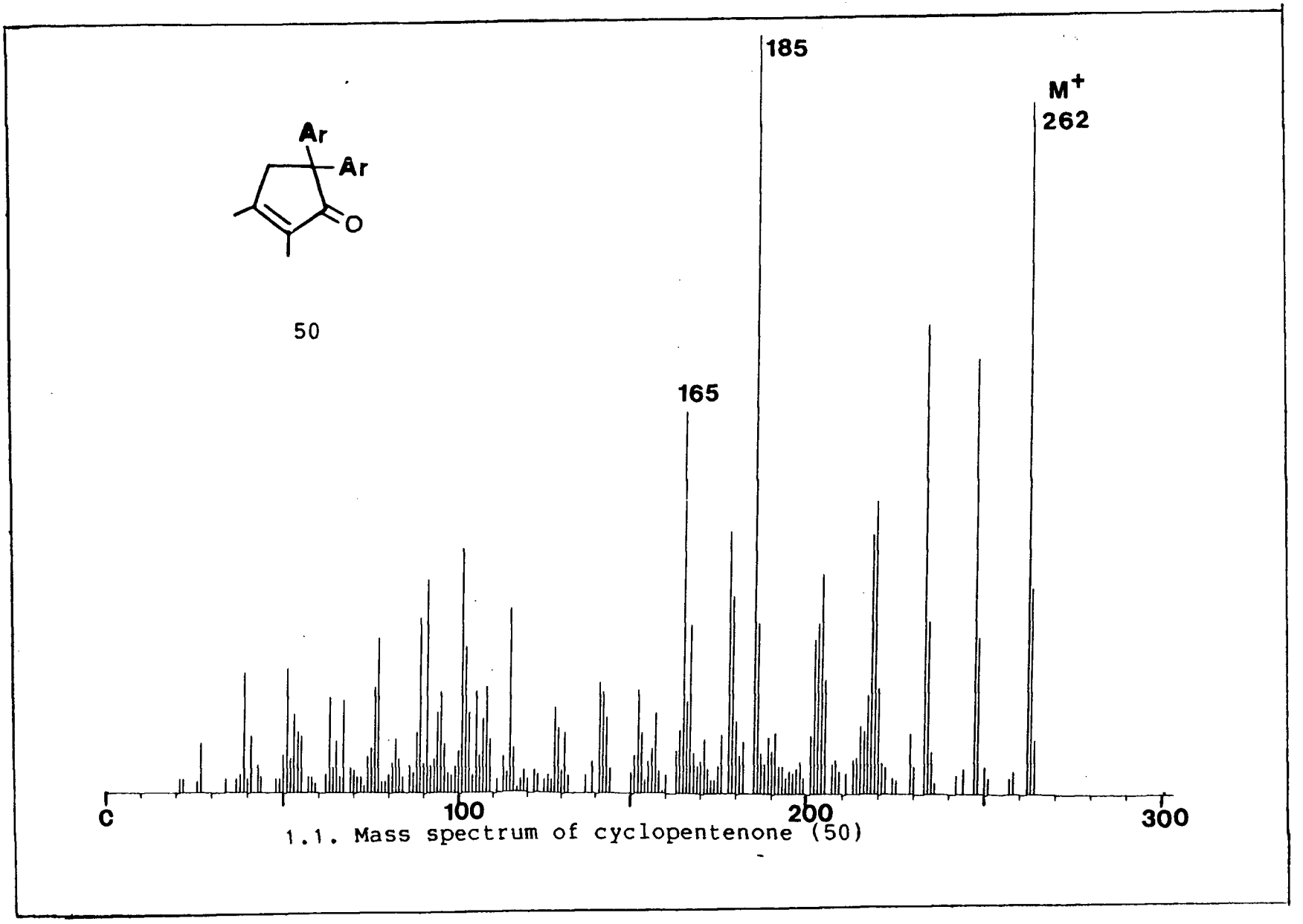
6.7. (top) Close-up of mass chromatogram m/z 167 for a control extract of β-glucuronidase hydrolyzed recipavrin metabolites. (bottom) Mass chromatogram m/z 167 for the same sample.

6.7.1. (top) Close-up of mass chromatogram m/z 167 for a formate treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a formaldehyde treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.

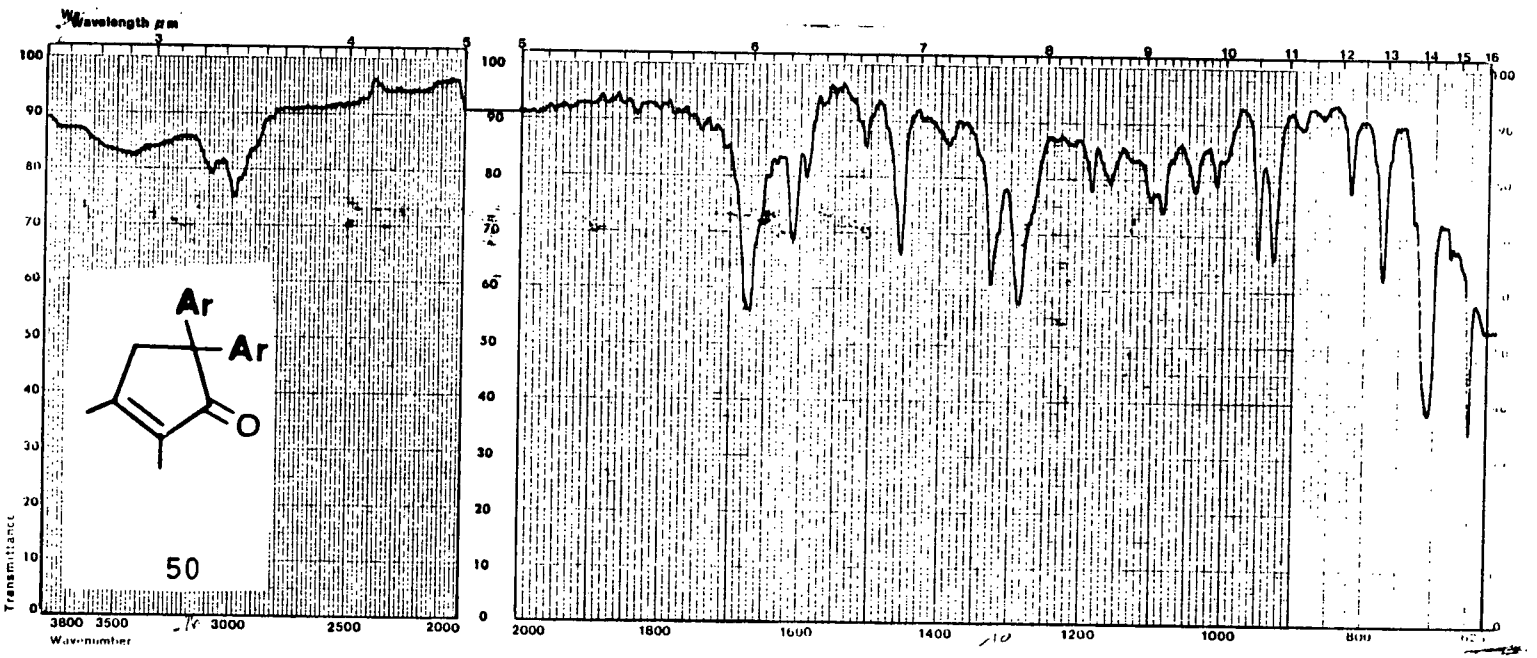
6.7.2. (top) Close-up of mass chromatogram m/z 167 for a hydrogen peroxide treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a formaldehyde and hydrogen peroxide treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.

6.8. (top) Mass chromatogram m/z 167 for a control extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Mass chromatogram m/z 167 for a formaldehyde and hydrogen peroxide treated extract of β-glucuronidase hydrolyzed recipavrin metabolites, showing the secondary formamide (solid) and tertiary formamide (hatched).

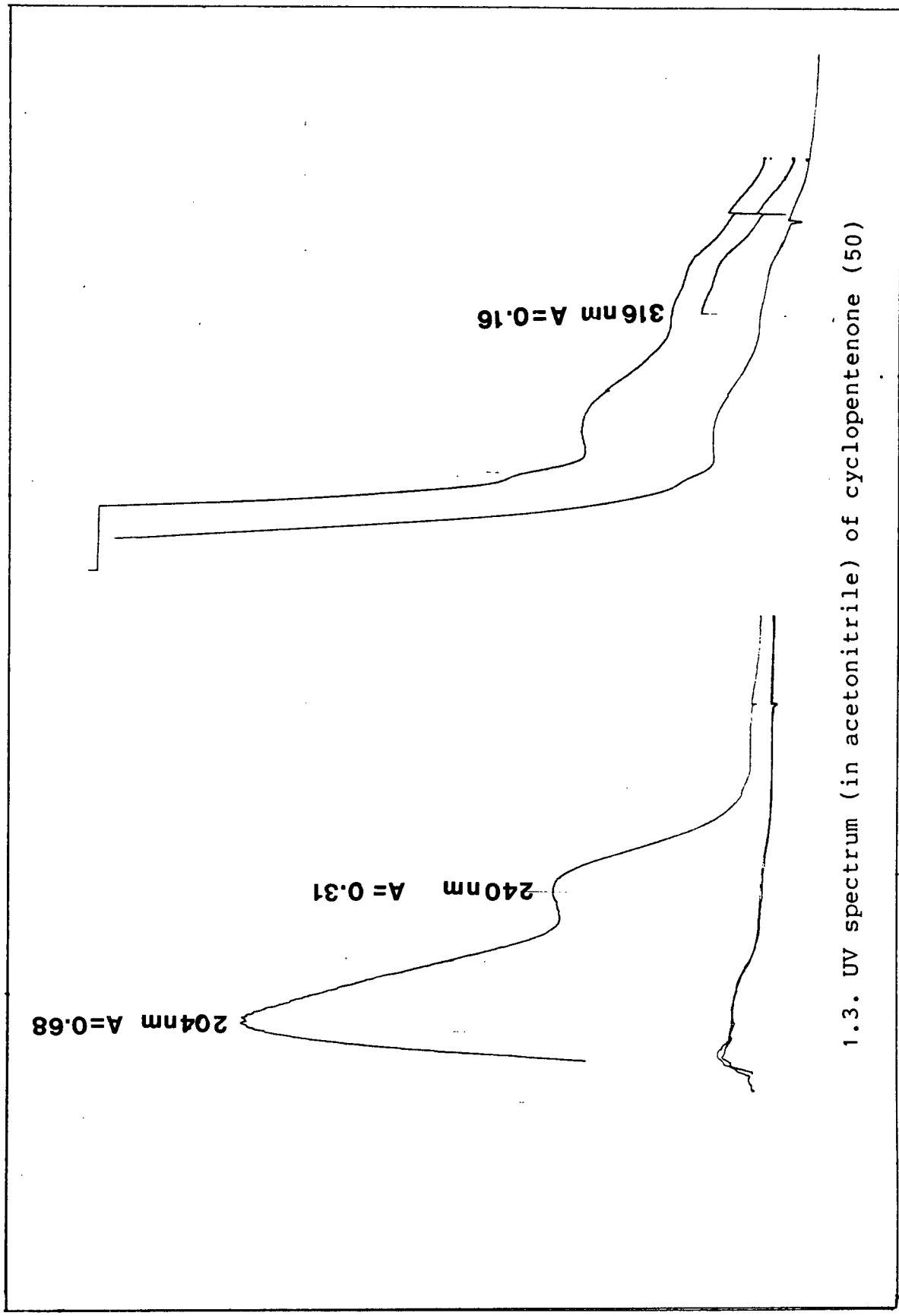
6.9. (top) Mass chromatogram m/z 167 for a control extract of nonconjugated recipavrin metabolites.(bottom) Mass chromatogram m/z 167 for a formaldehyde and hydrogen peroxide treated extract of nonconjugated recipavrin metabolites showing the secondary formamide (hatched).



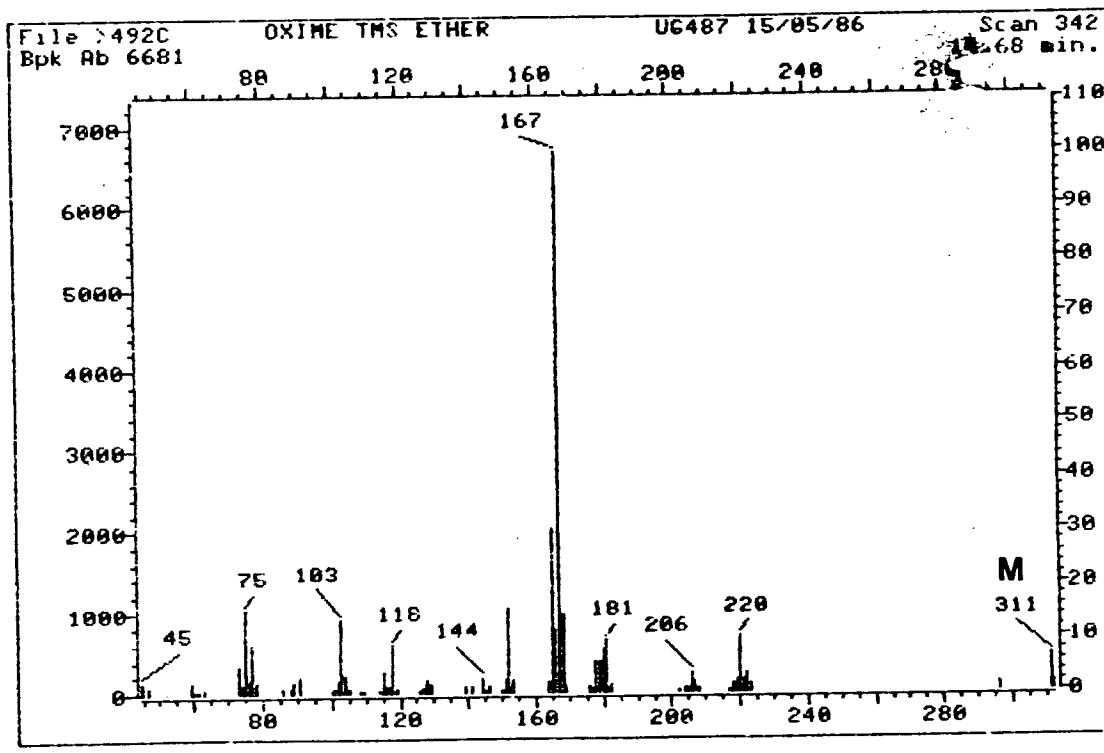
1.1. Mass spectrum of cyclopentenone (50)



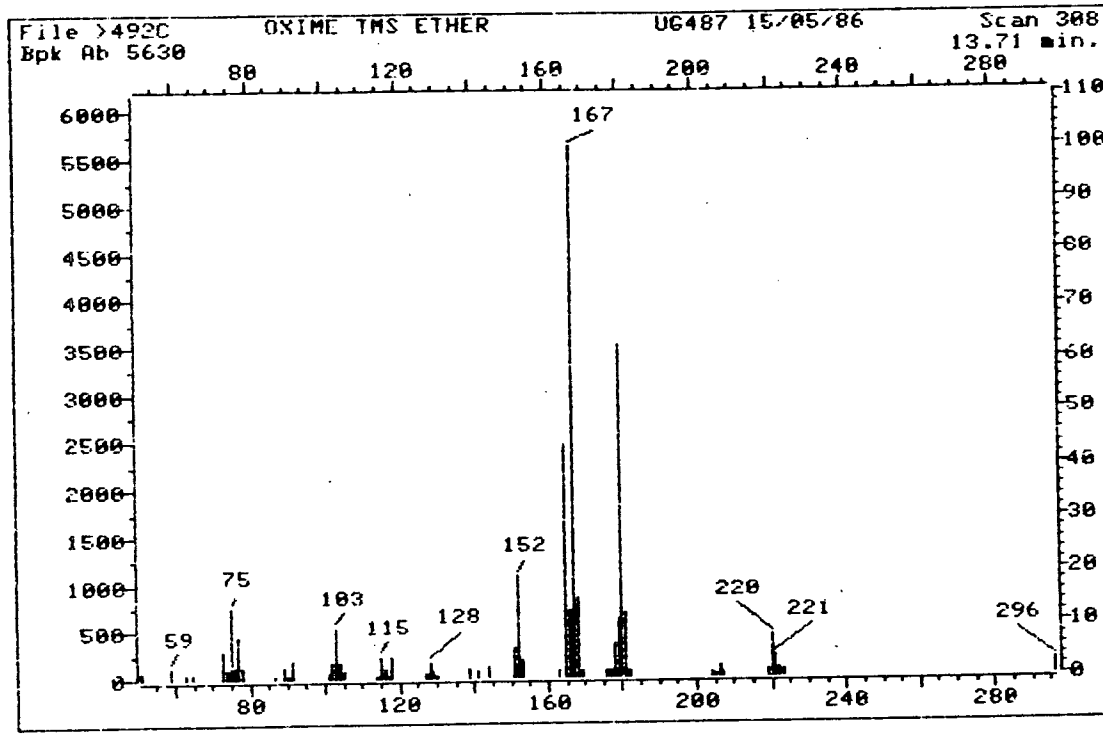
1.2.IR spectrum (film) of cyclopentenone (50)



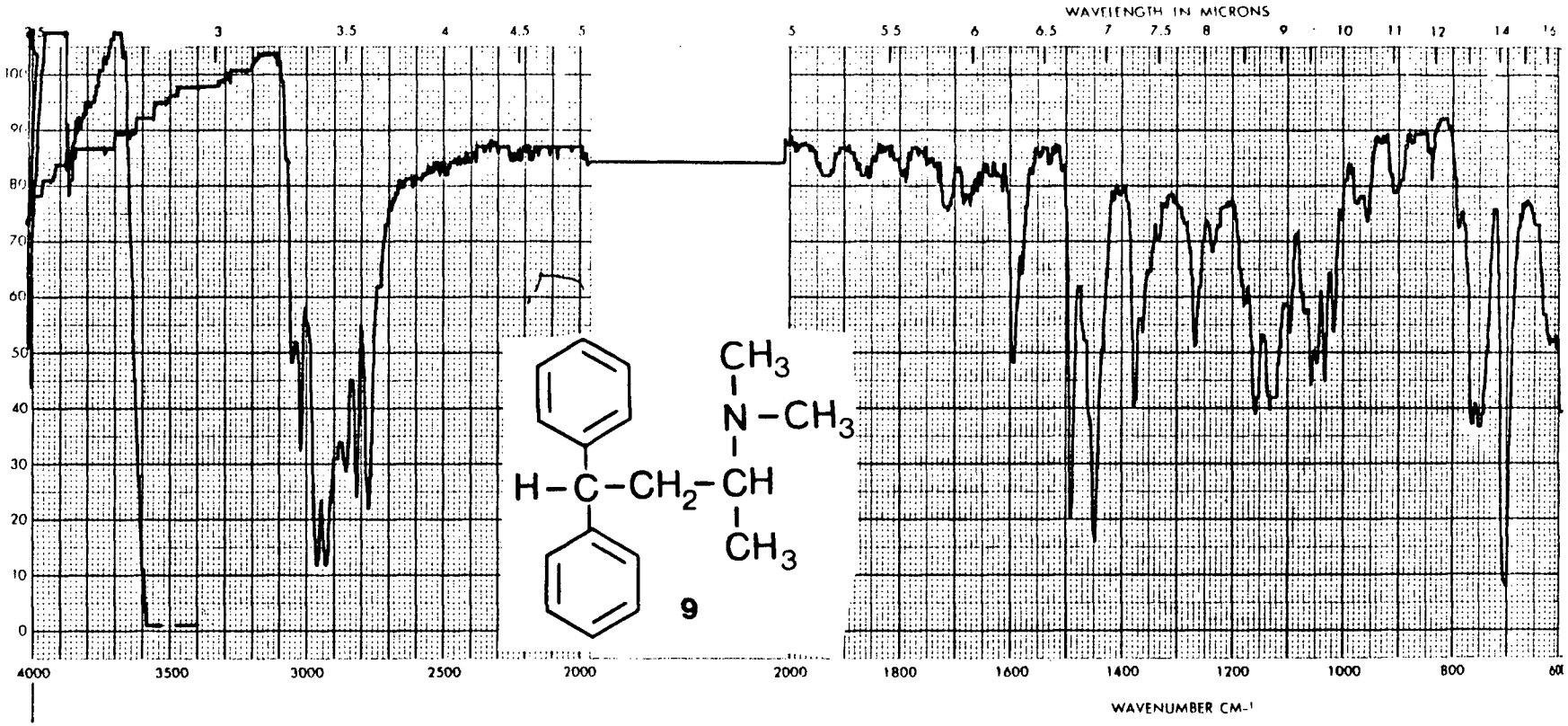
1.3. UV spectrum (in acetonitrile) of cyclopentenone (50)



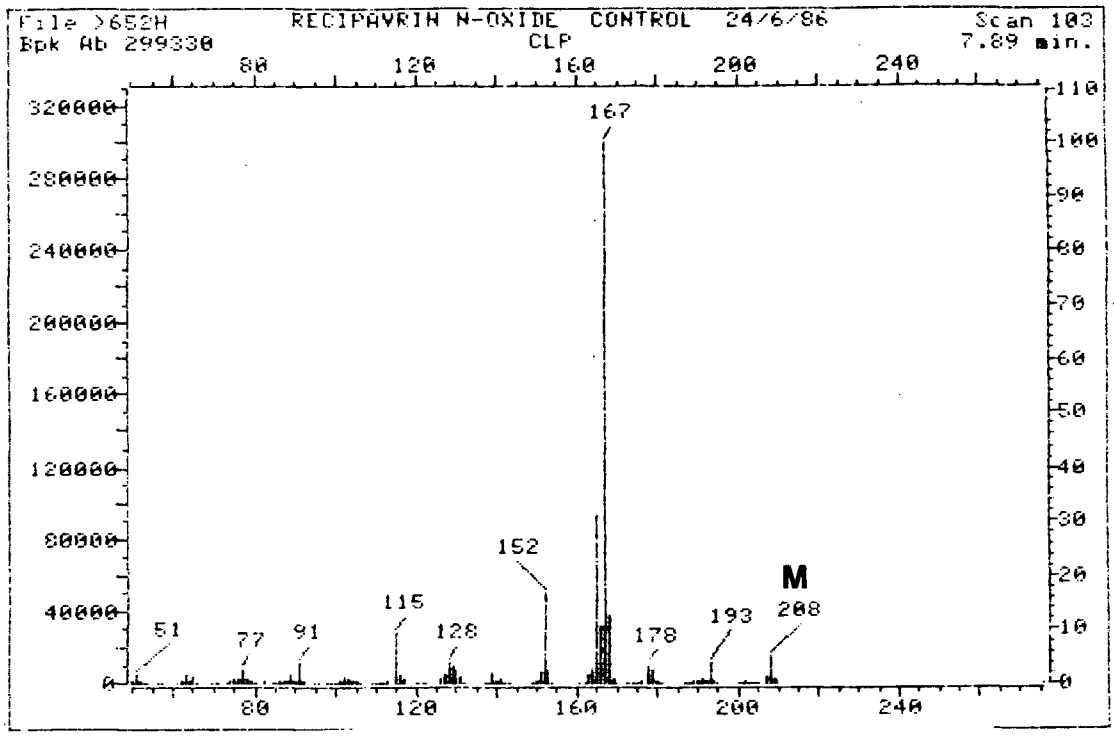
1.4. Mass spectrum of anti TMS oxime (52b)



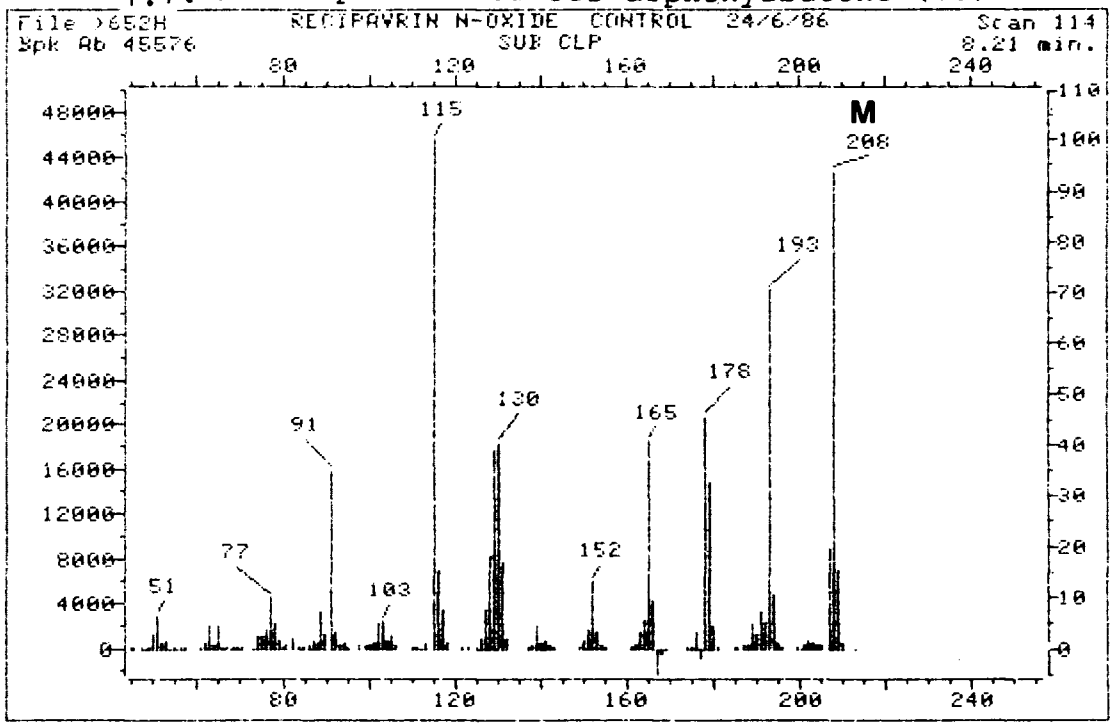
1.5. Mass spectrum of syn TMS oxime (52)



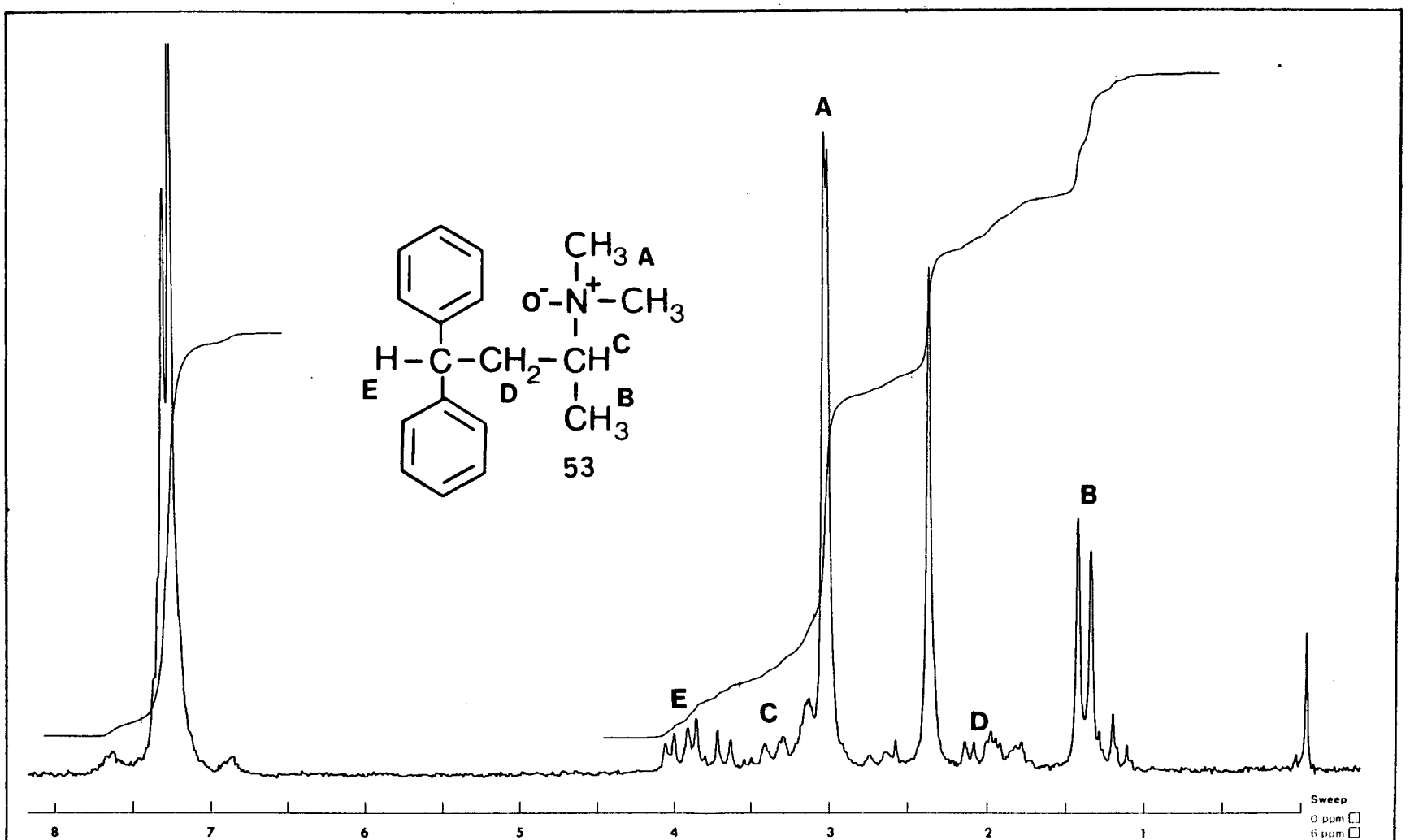
1.6. IR spectrum (film) of recipavrin free base



1.7. Mass spectrum of cis diphenylbutene (54)

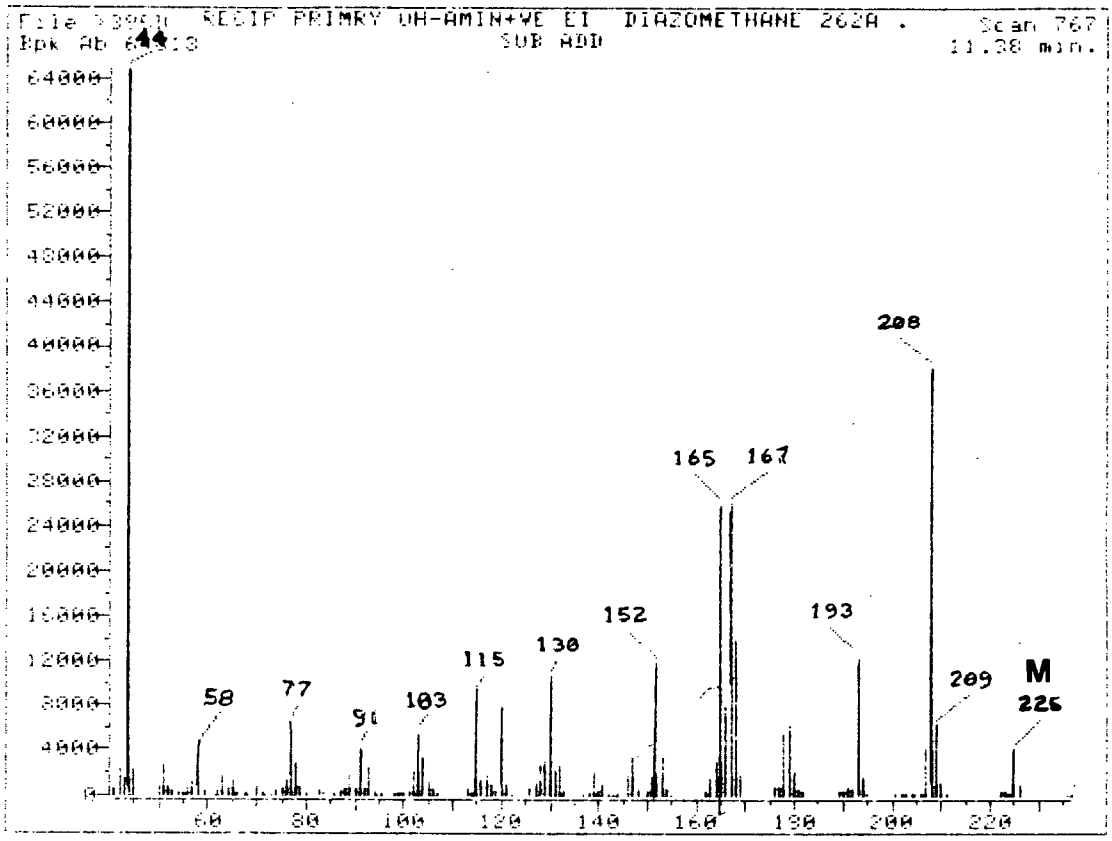


1.8. Mass spectrum of trans diphenylbutene (55)

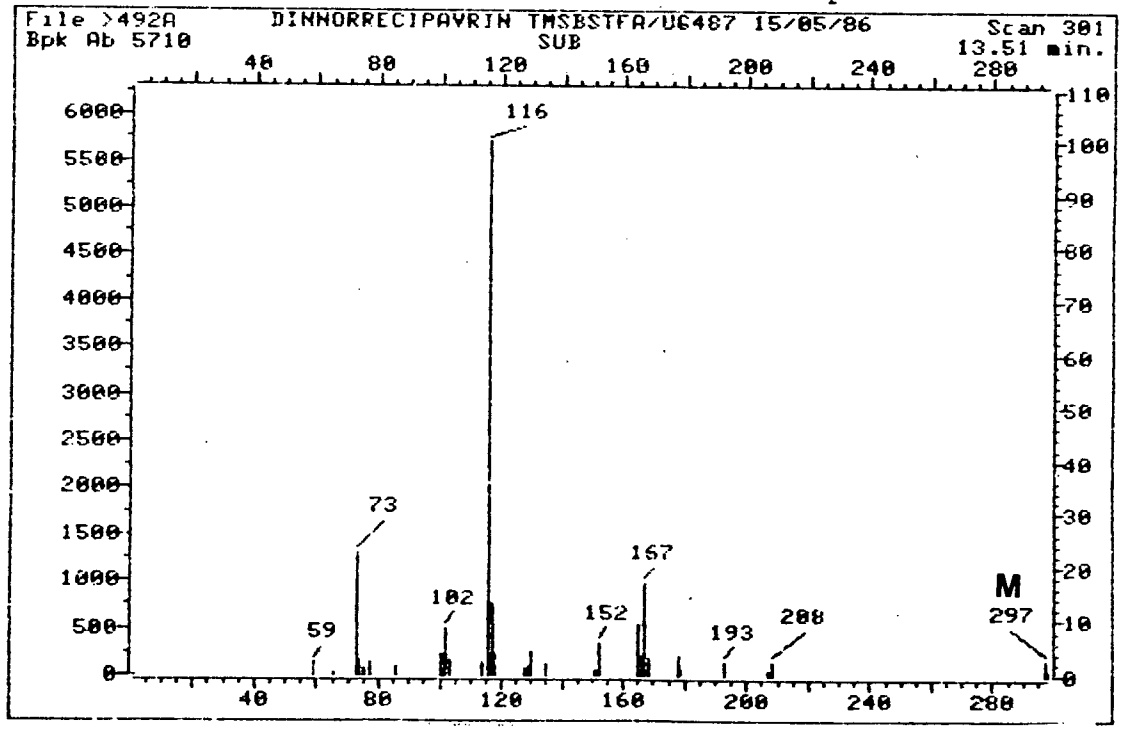


53

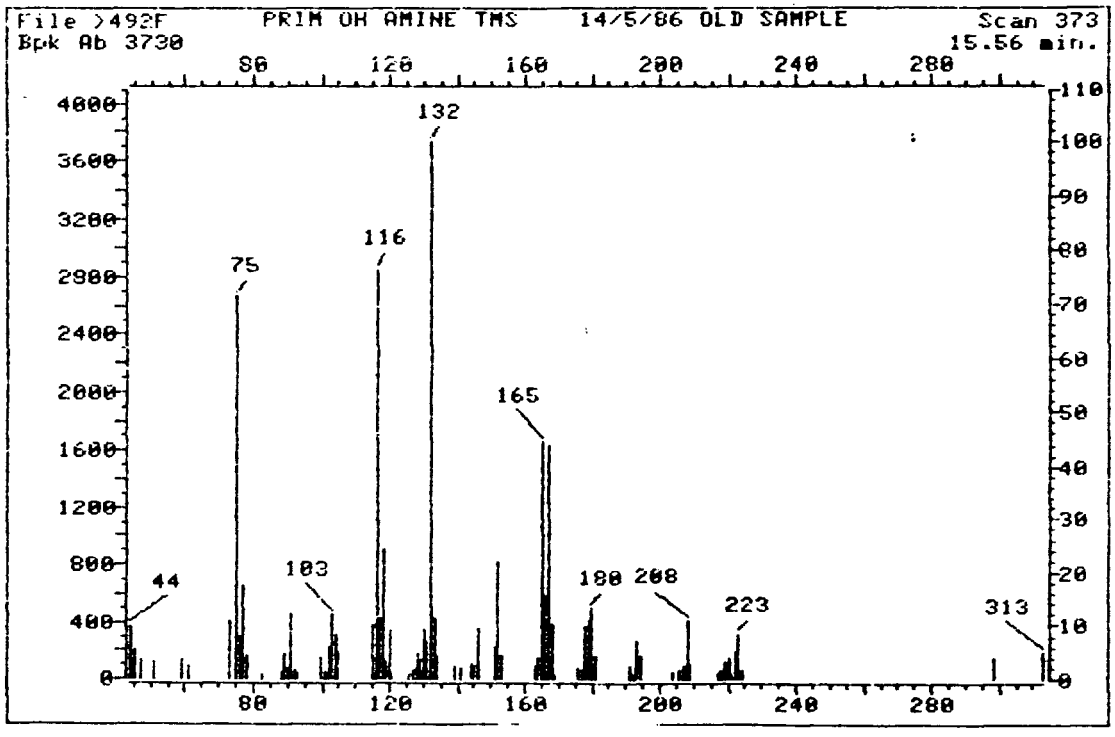
1.9. 80 MHz NMR spectrum of recipavrin N-oxide (53) (contaminated with some recipavrin)



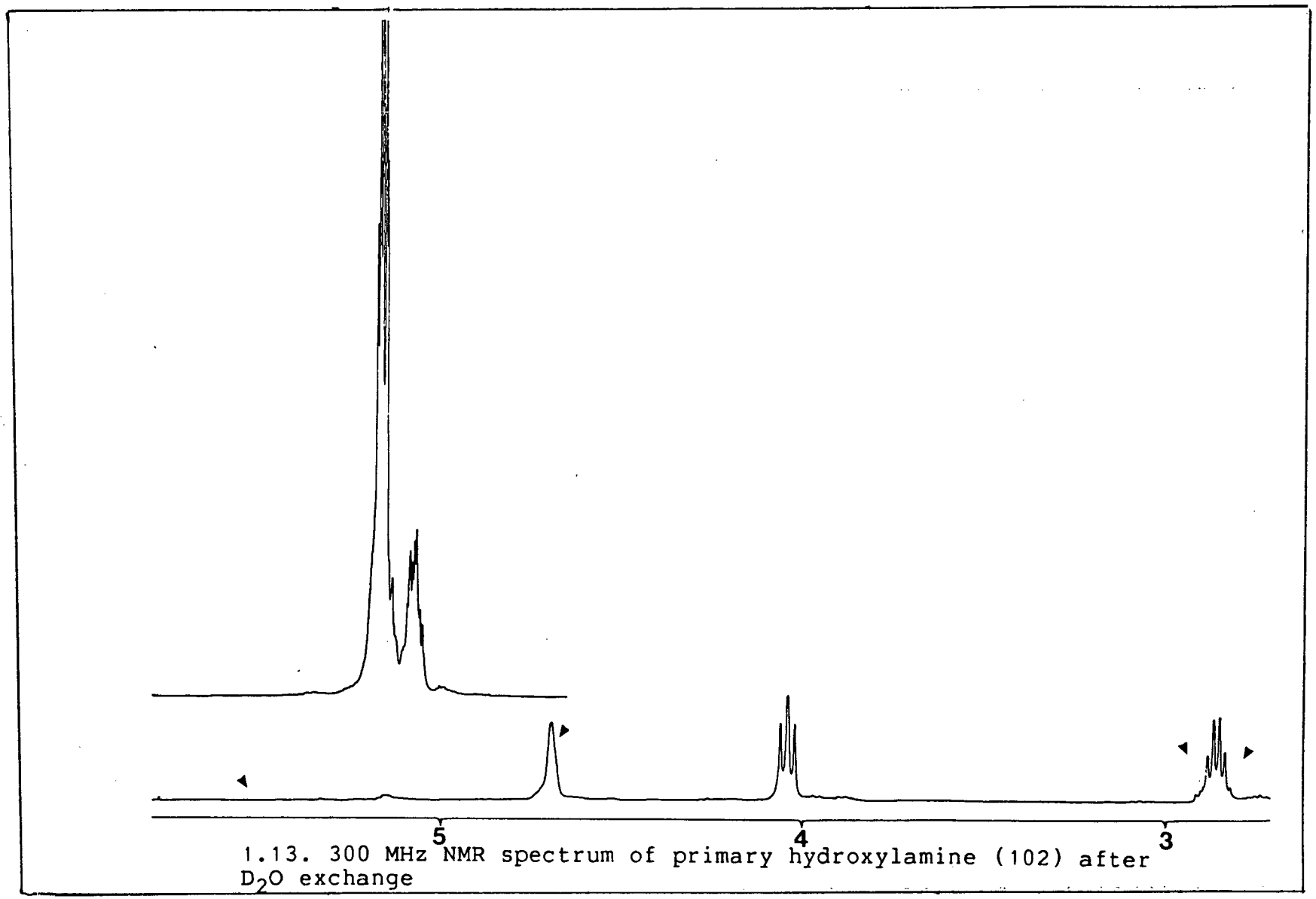
1.10. Mass spectrum of dinorrecipavrin (20)



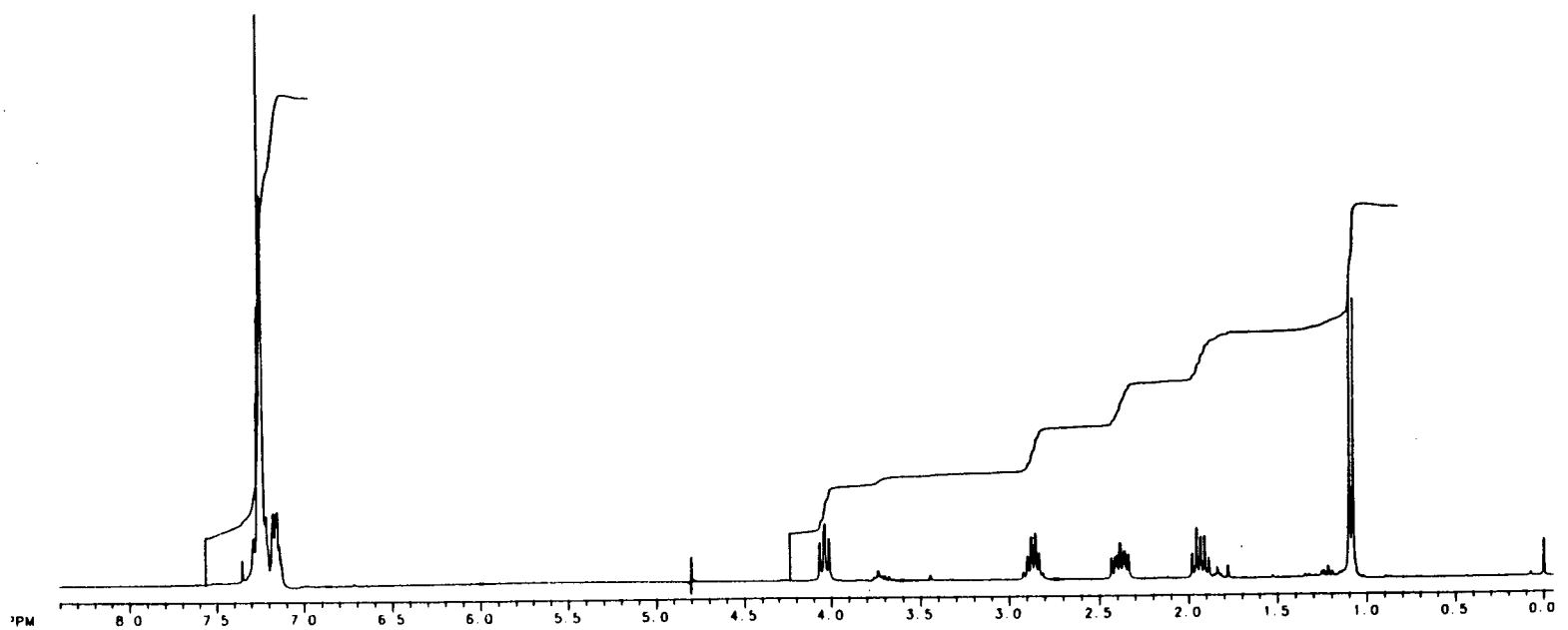
1.11. Mass spectrum of dinorrecipavrin TMS (104)



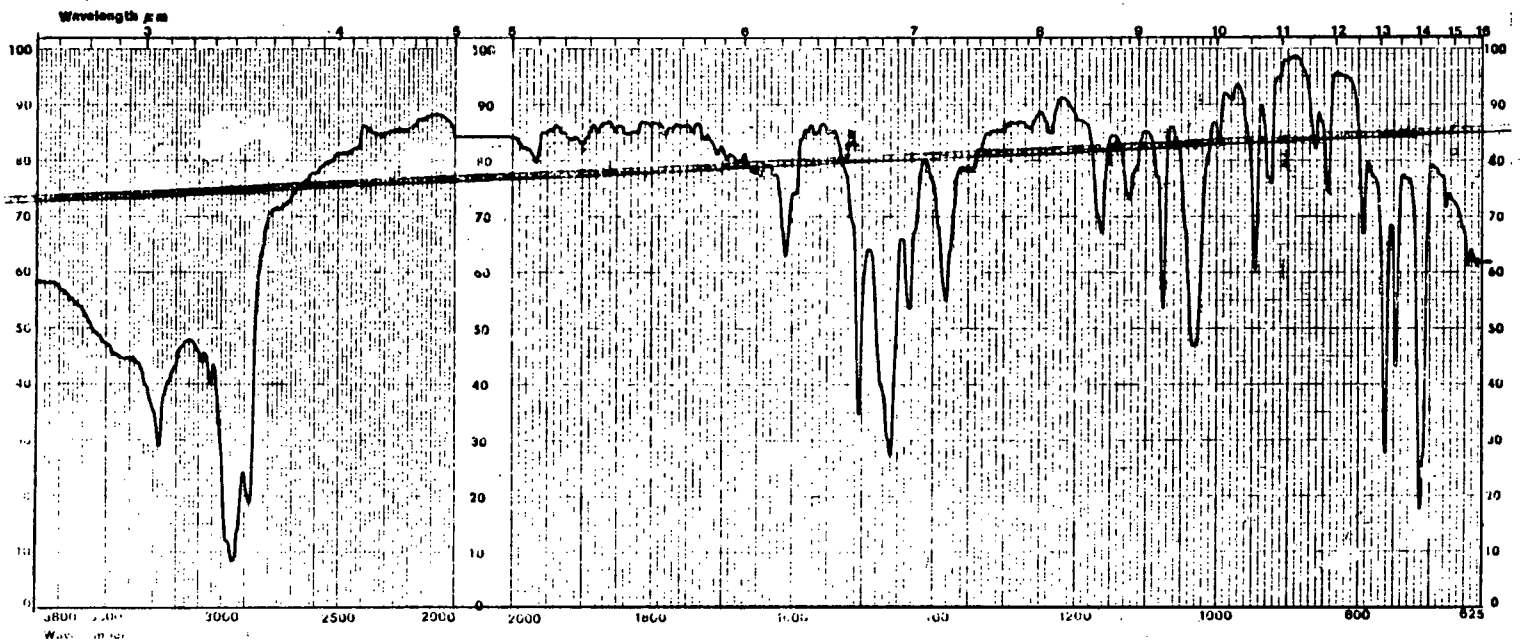
1.12. Mass spectrum of the TMS primary hydroxylamine (102)



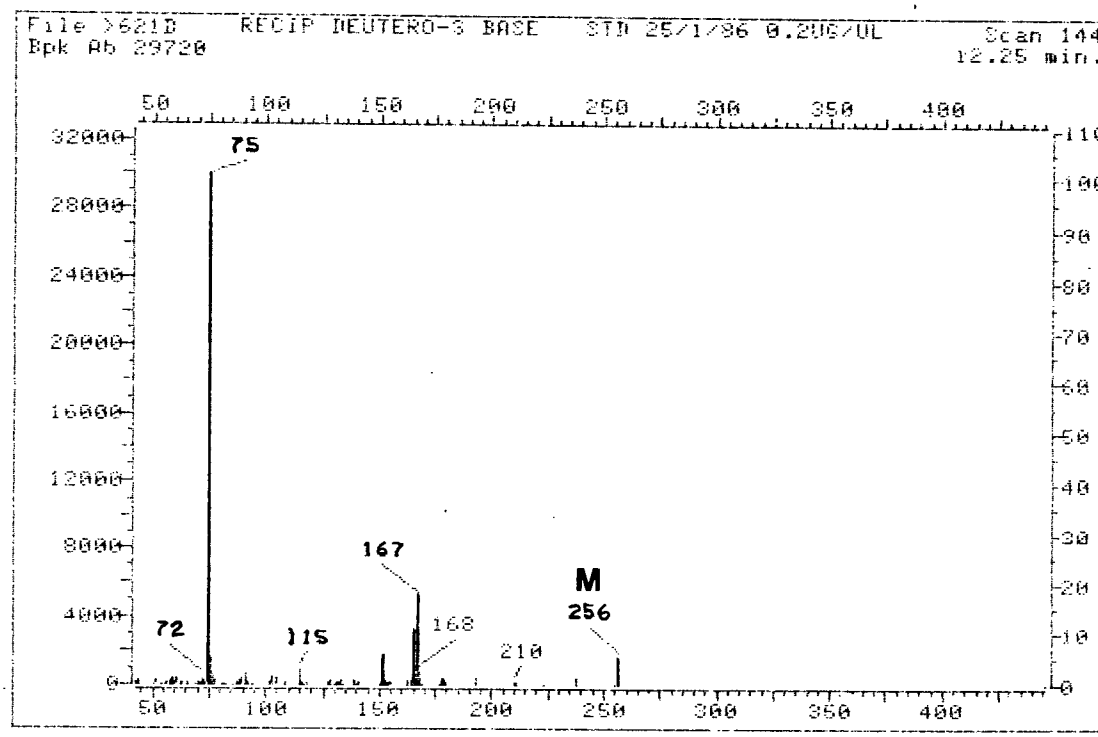
1.13. 300 MHz NMR spectrum of primary hydroxylamine (102) after D_2O exchange



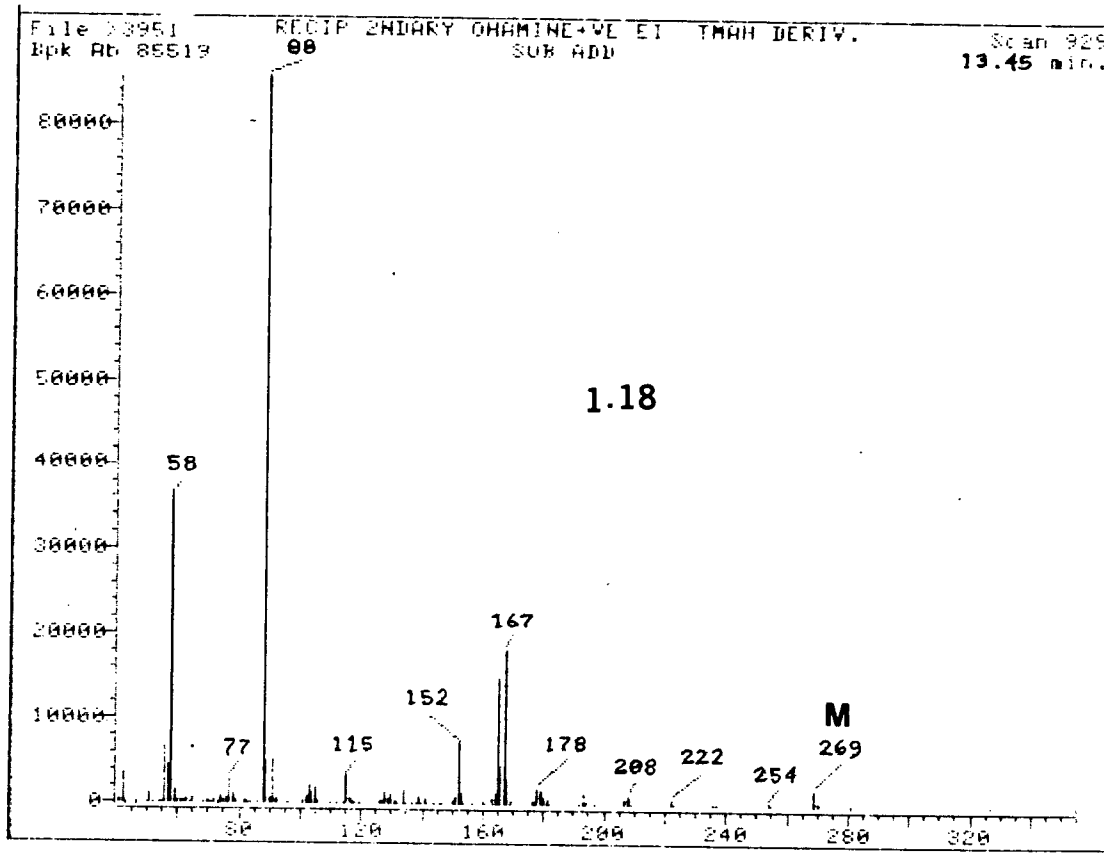
1.14. 300 MHz NMR spectrum of autoxidation product (56)

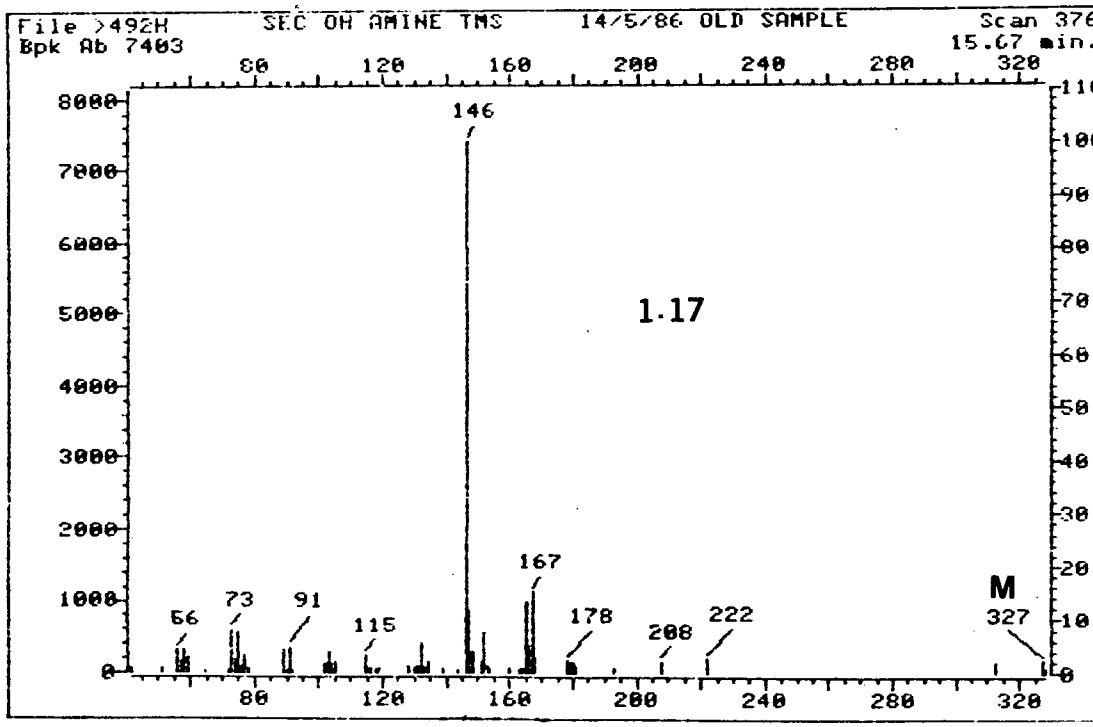


1.15. IR spectrum (mull) of autoxidation product (56)



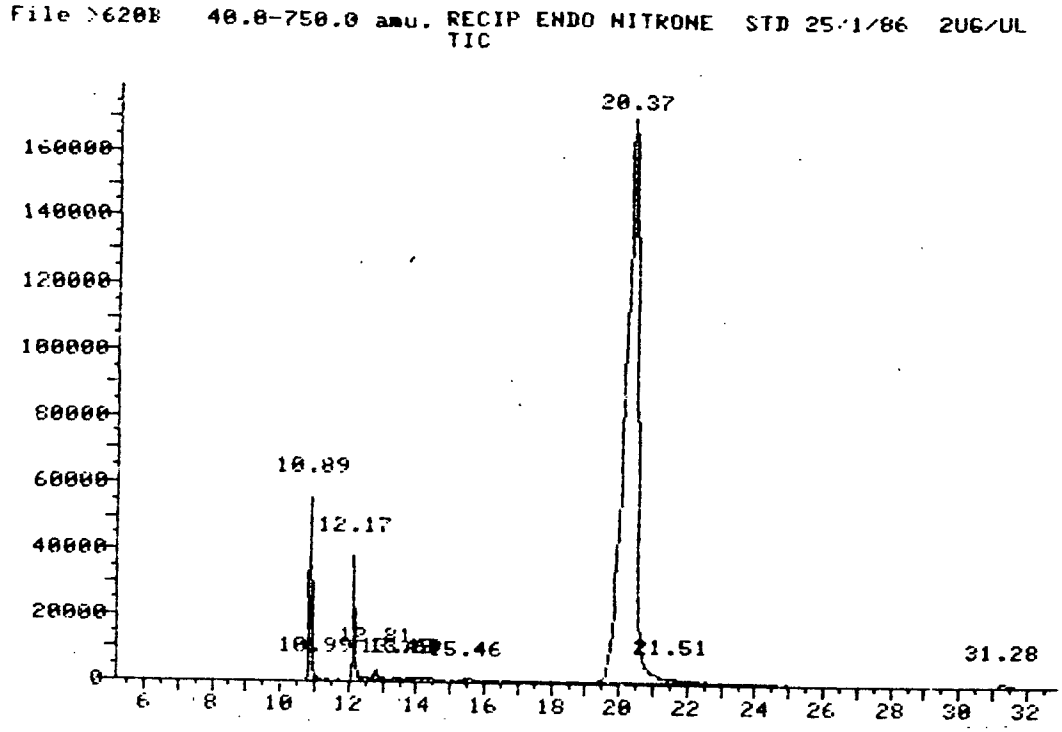
1.16. Mass spectrum of recipavrin-D_3

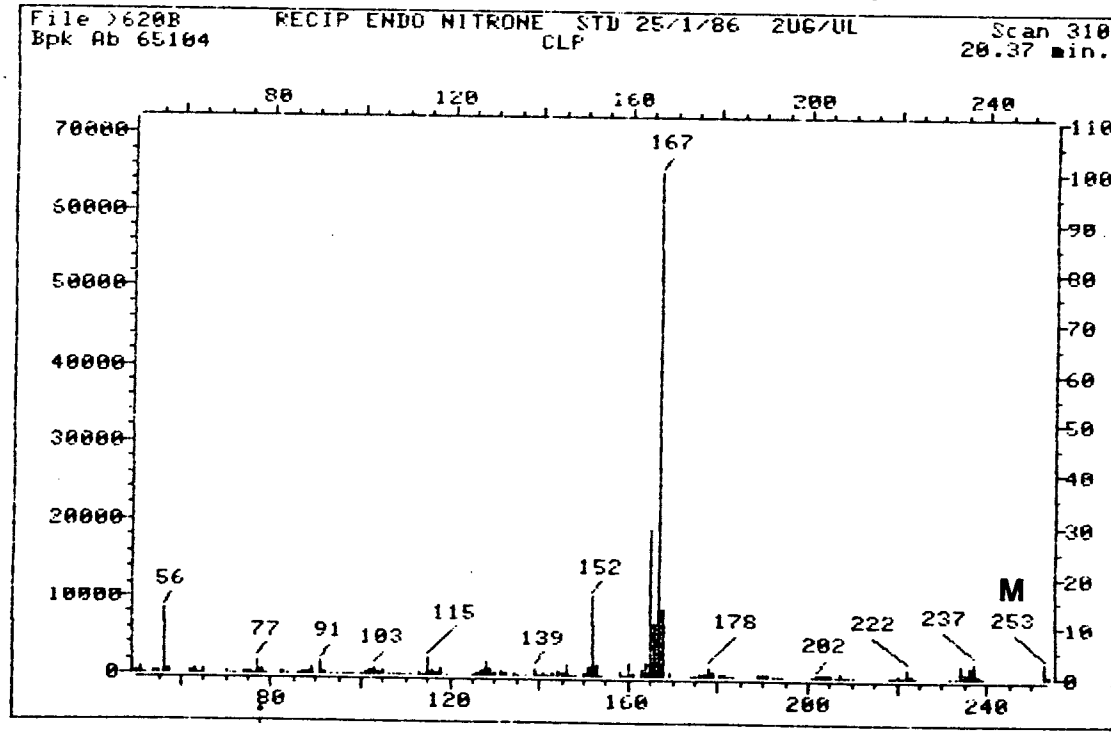




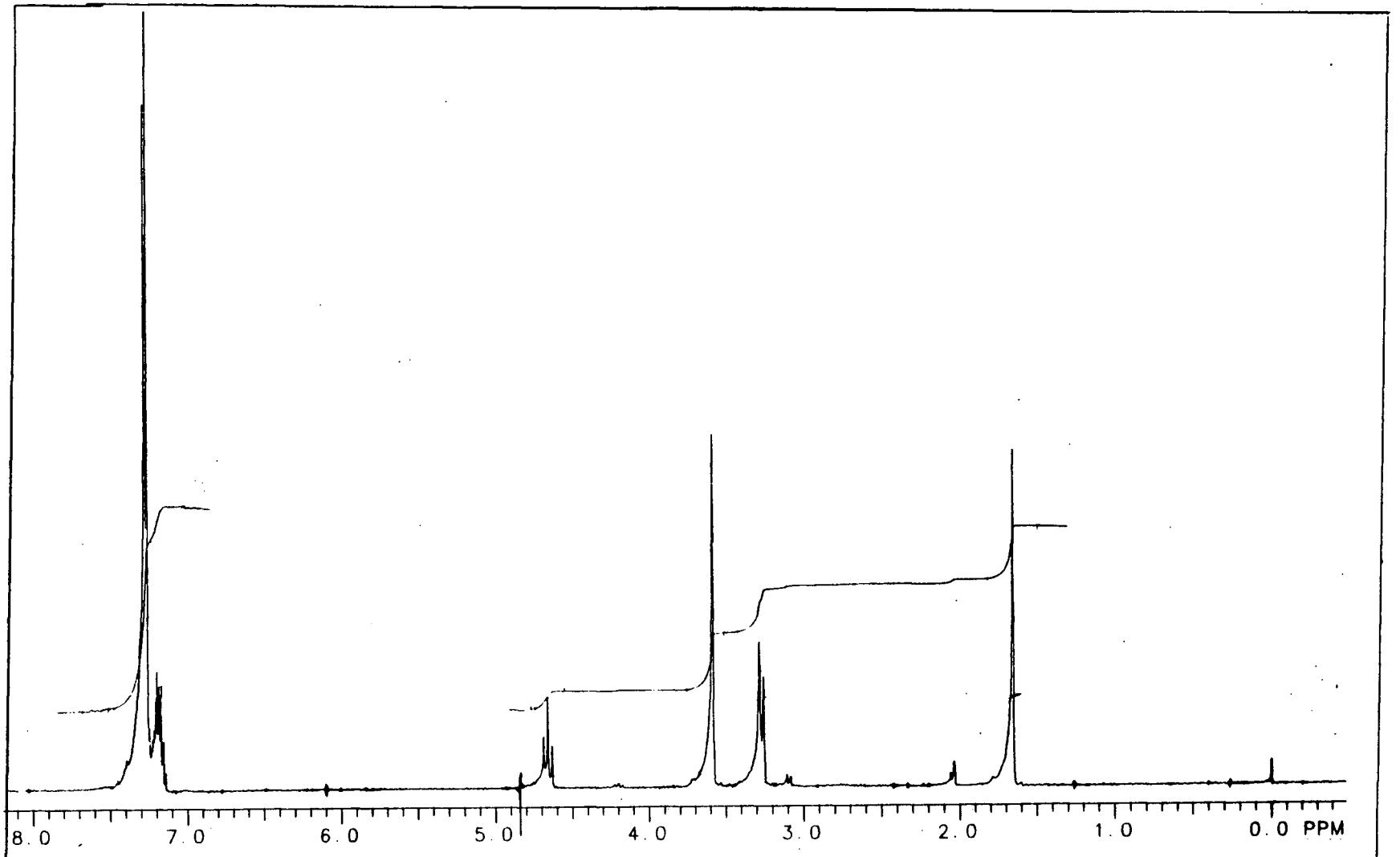
1.17. Mass spectrum of secondary hydroxylamine TMS derivative (18)

1.18. Mass spectrum of secondary hydroxylamine O-methyl ether (58)

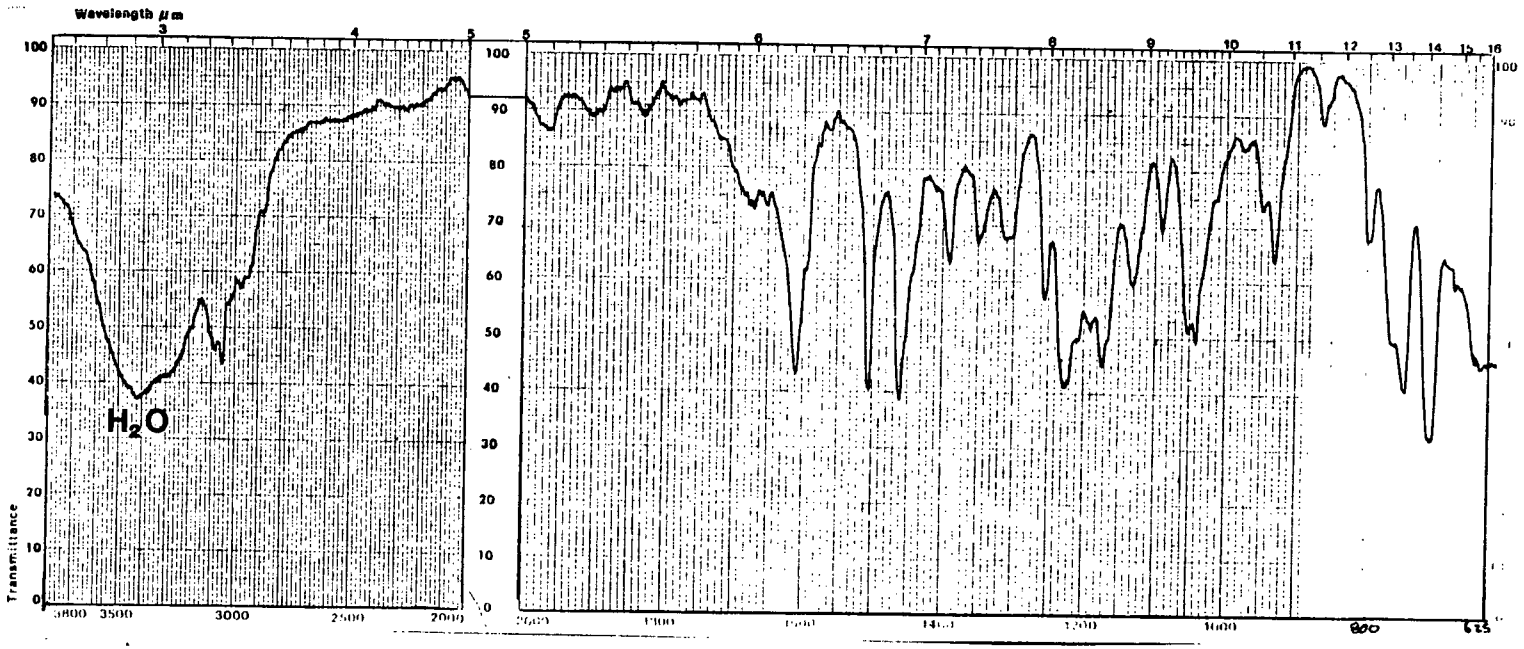




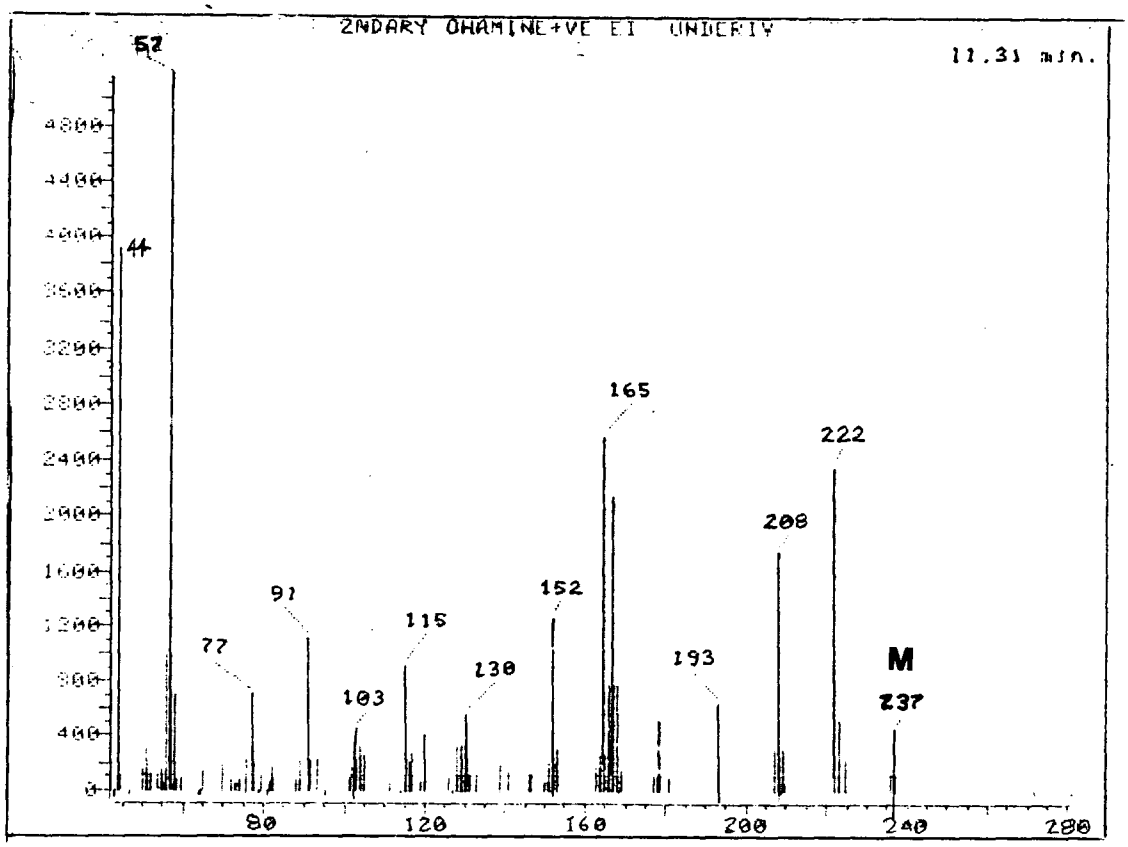
1.19. Total ion current and mass spectrum of N-methyl nitrone (44)



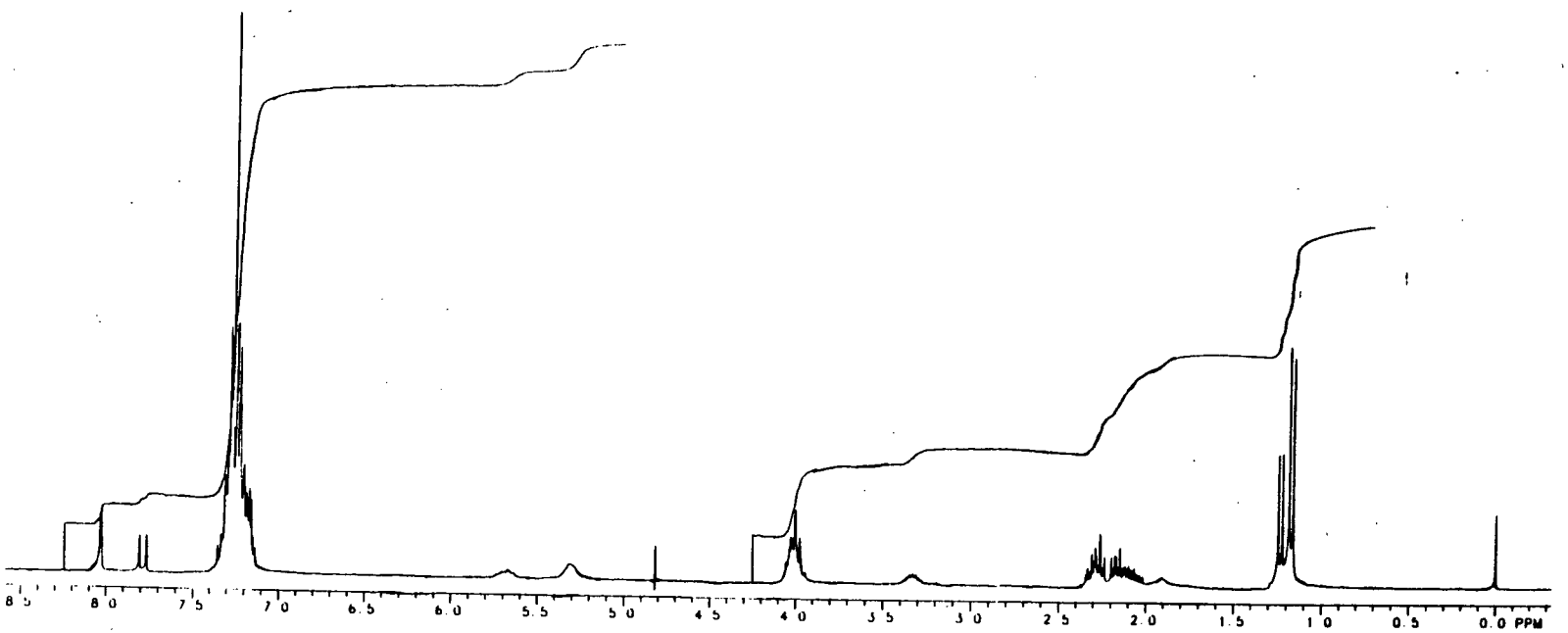
1.20. 300 MHz NMR spectrum of N-methyl nitrone (44) showing only trans isomer



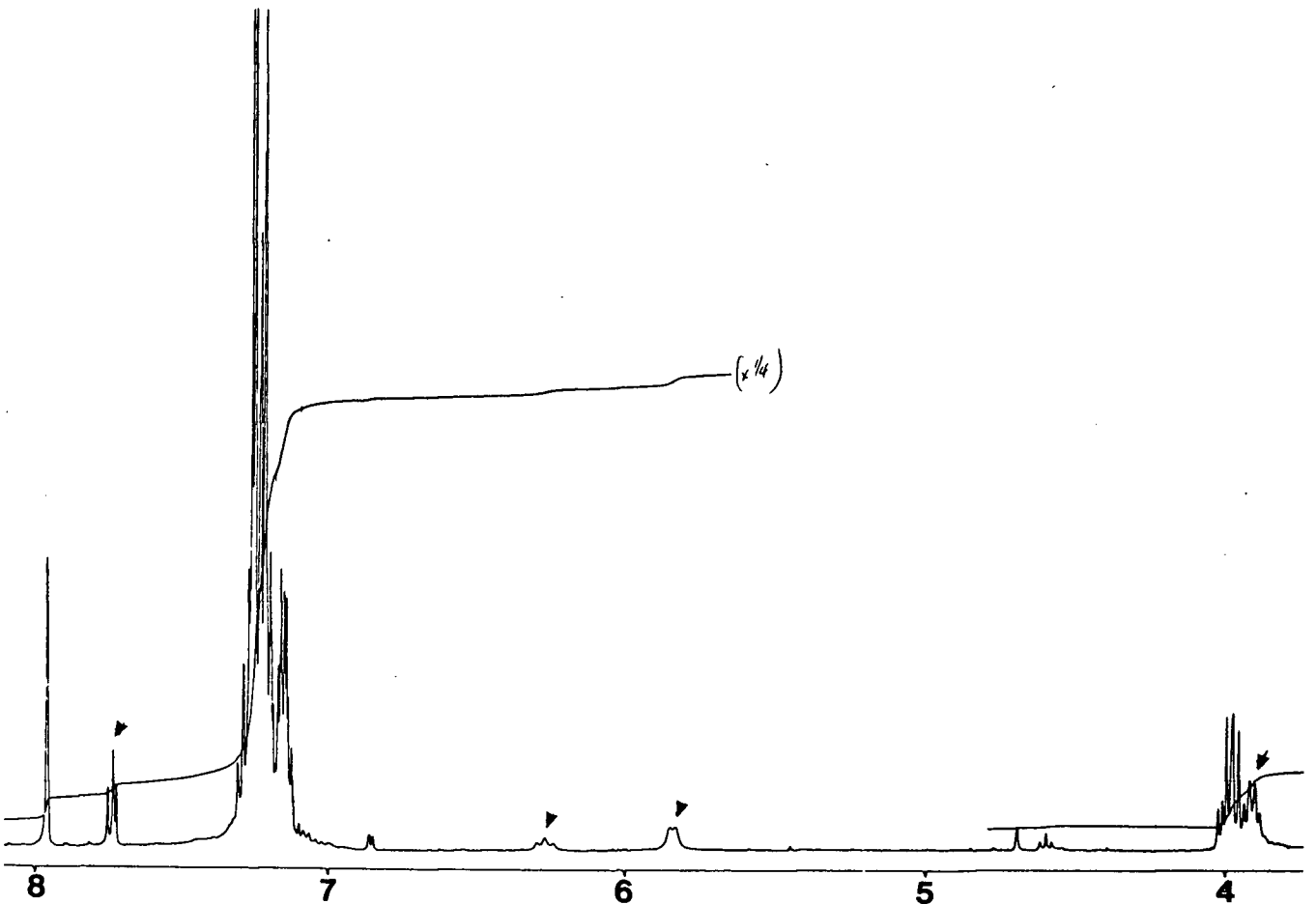
1.21. IR spectrum (mull) of N-methyl nitrone (44)



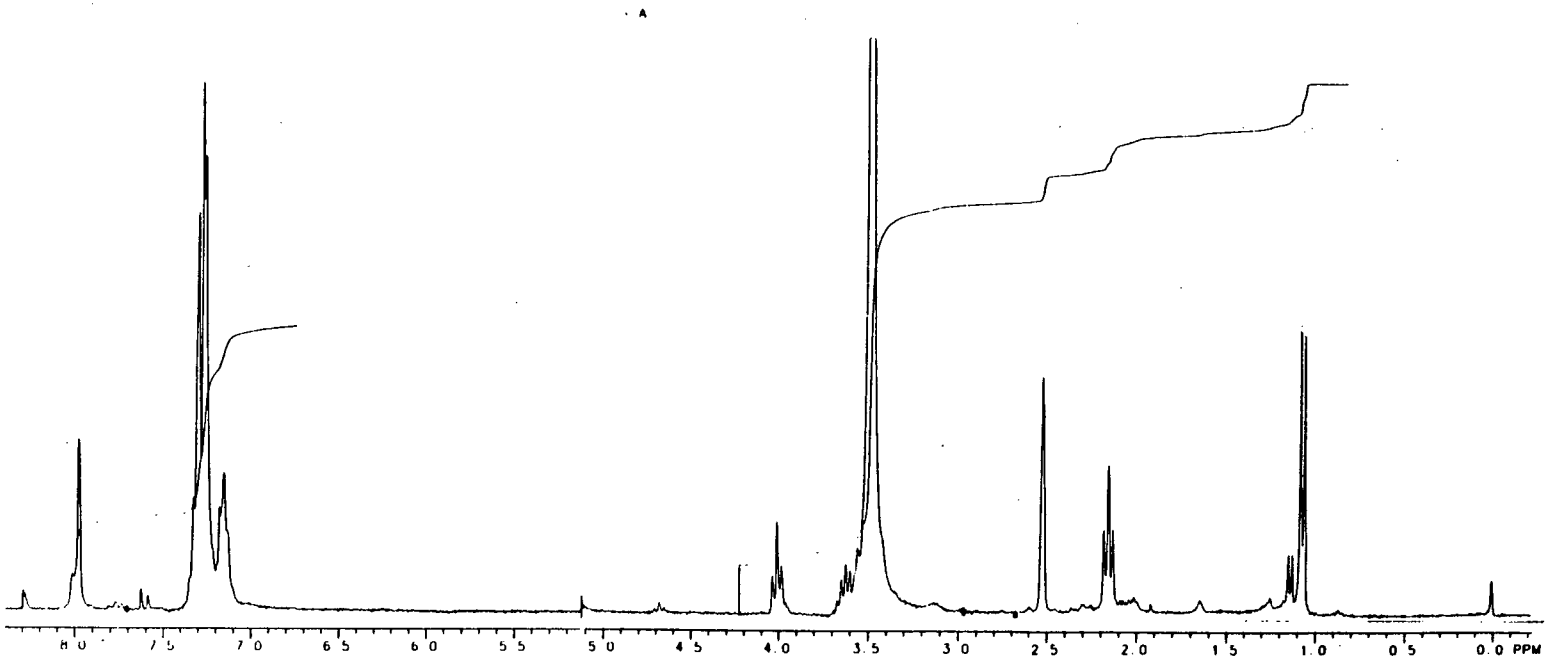
1.22. Mass spectrum of methylene imine (23)



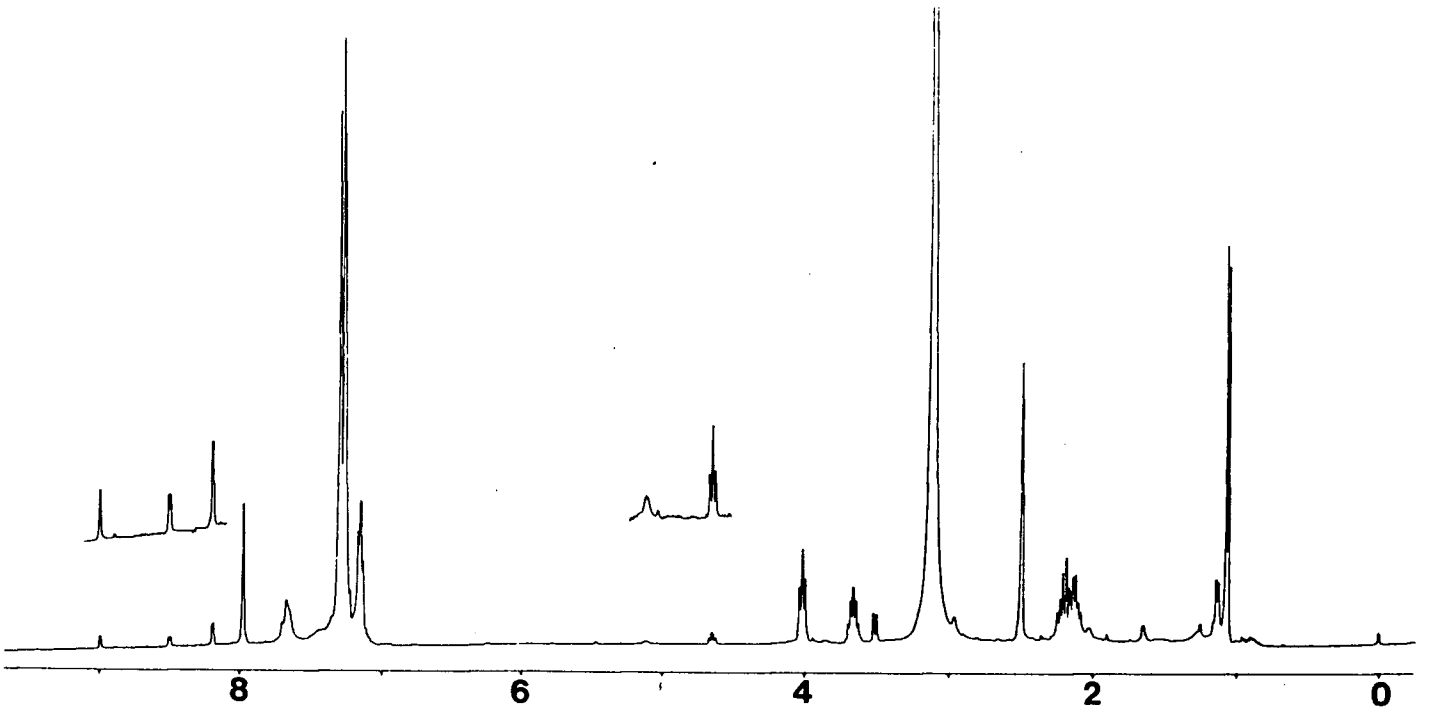
1.23. 300 MHz NMR spectrum of secondary formamide (12) (from ethyl formate reaction) ($CDCl_3$ at 25°)



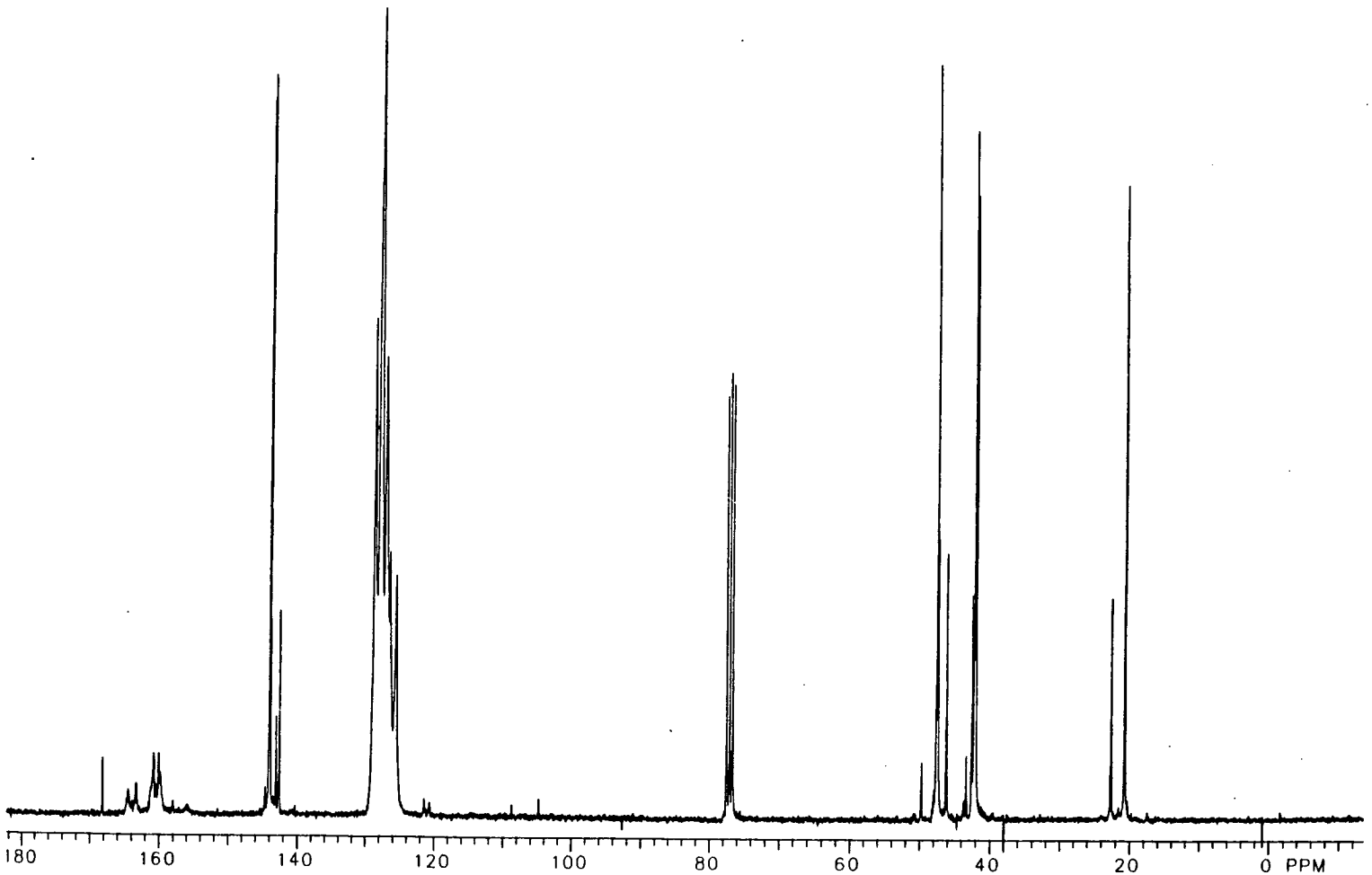
1.24. 300 MHz NMR spectrum of secondary formamide (12) (Leuckart reaction) Brief D_2O exchange. Arrows denote changes in spectrum



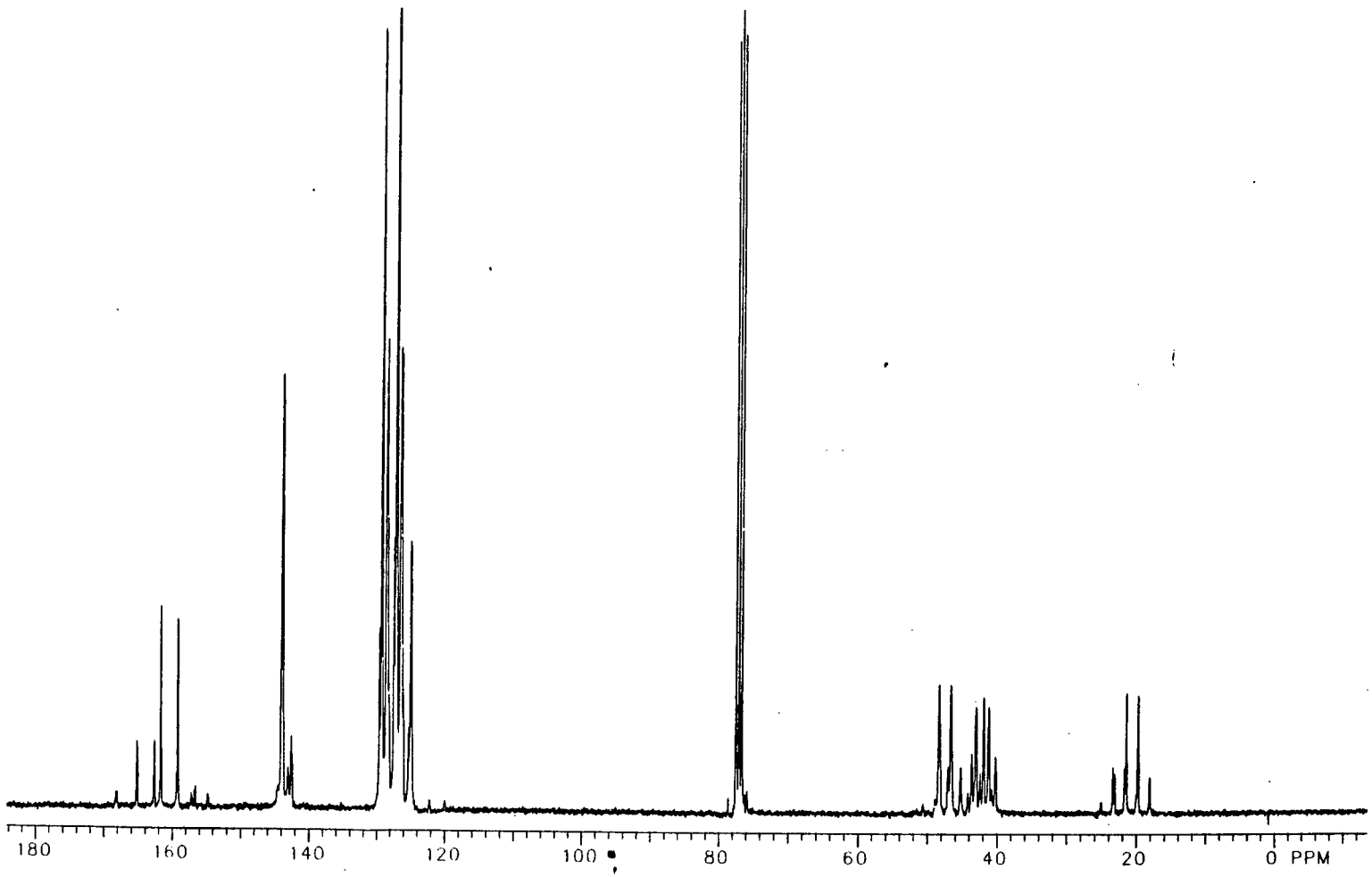
1.25. 300 MHz NMR spectrum of secondary formamide (12) (Leuckart reaction) (DMSO-D_6 at 25°)



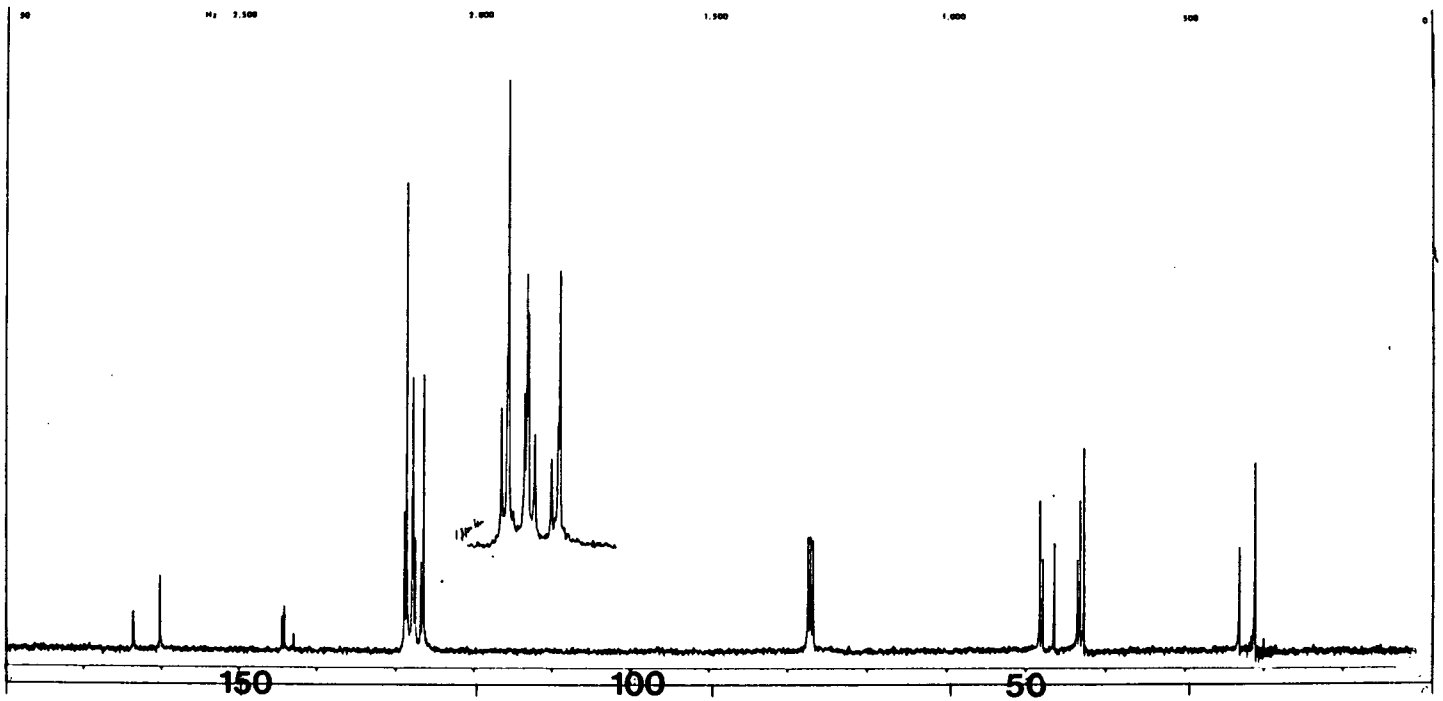
1.26. 300 MHz NMR spectrum of secondary formamide (12) (Leuckart reaction) (DMSO-D_6 at 90°)



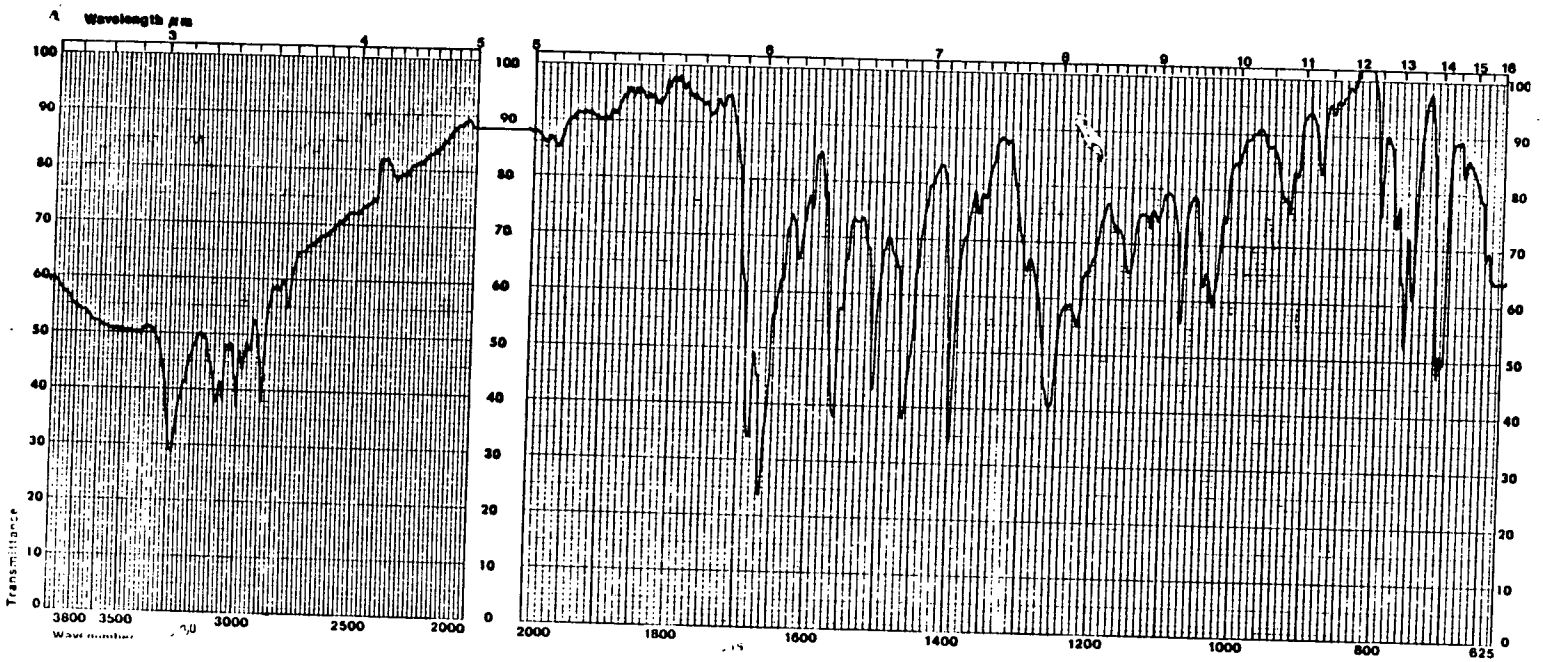
1.27. 75 MHz ^{13}C NMR spectrum of secondary formamide (12) (Leuckart reaction) (BB decoupled)



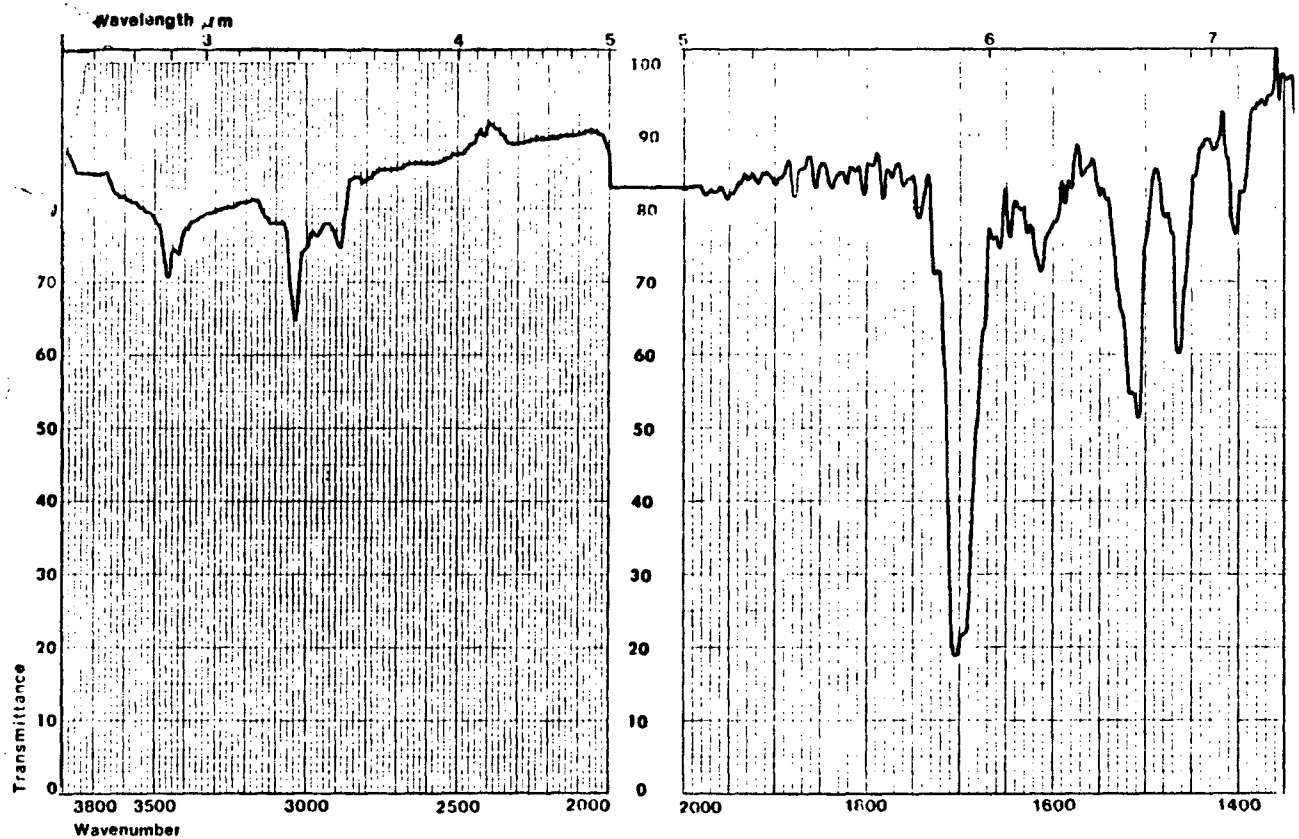
1.28. 75 MHz ^{13}C NMR spectrum of secondary formamide (12) (Leuckart reaction) (SFORD)



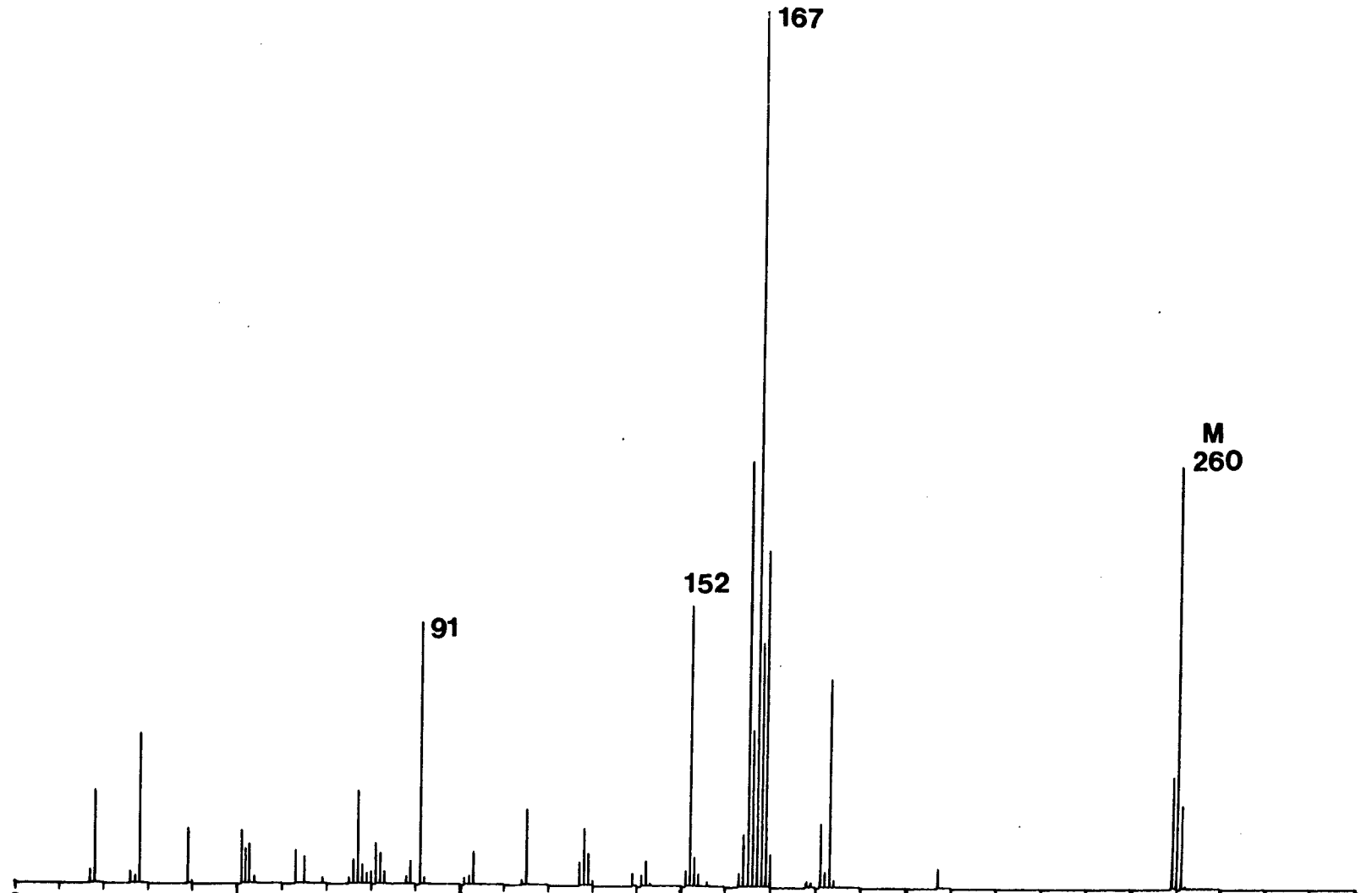
1.29. 100 MHz ^{13}C NMR spectrum of secondary formamide (12) (ethyl formate reaction) (BB decoupled)



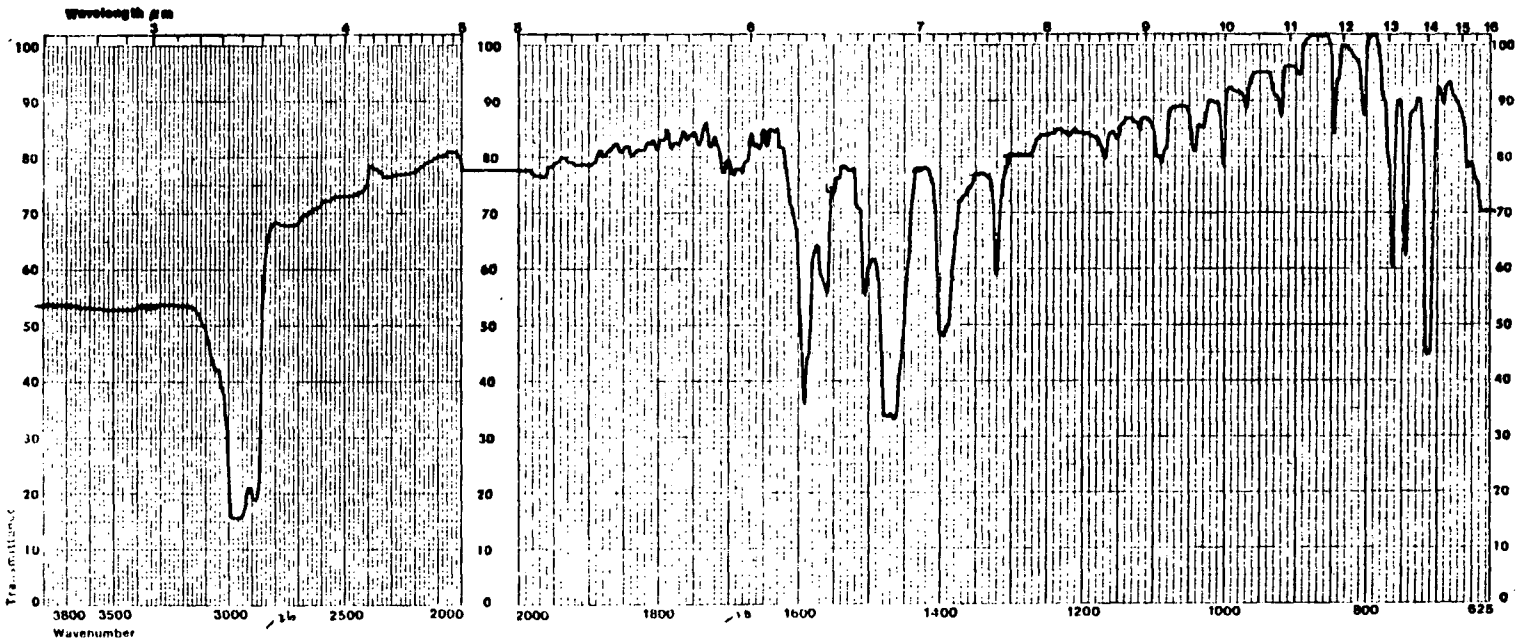
1.30. IR spectrum (film) of secondary formamide (12) (ethyl formate reaction)



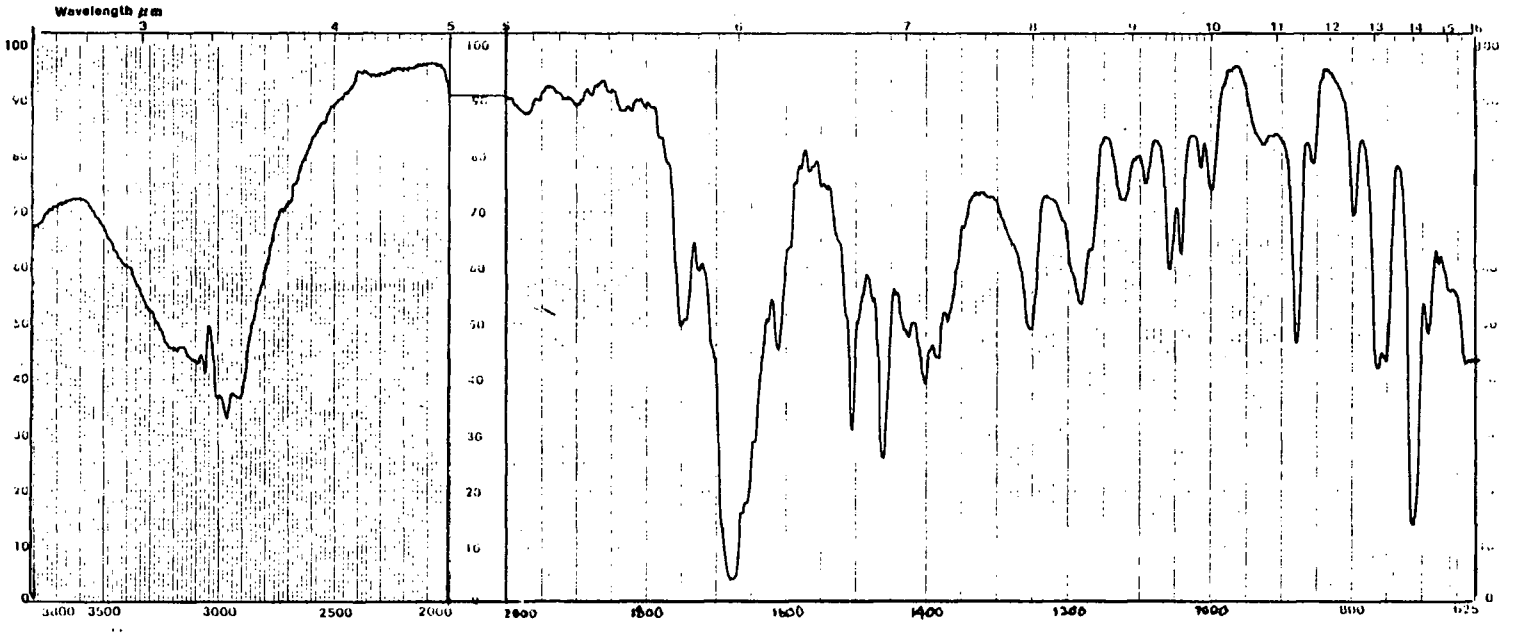
1.31. IR spectrum (0.1 mm cell in $CHCl_3$) of secondary formamide (12) (Leuckart reaction)



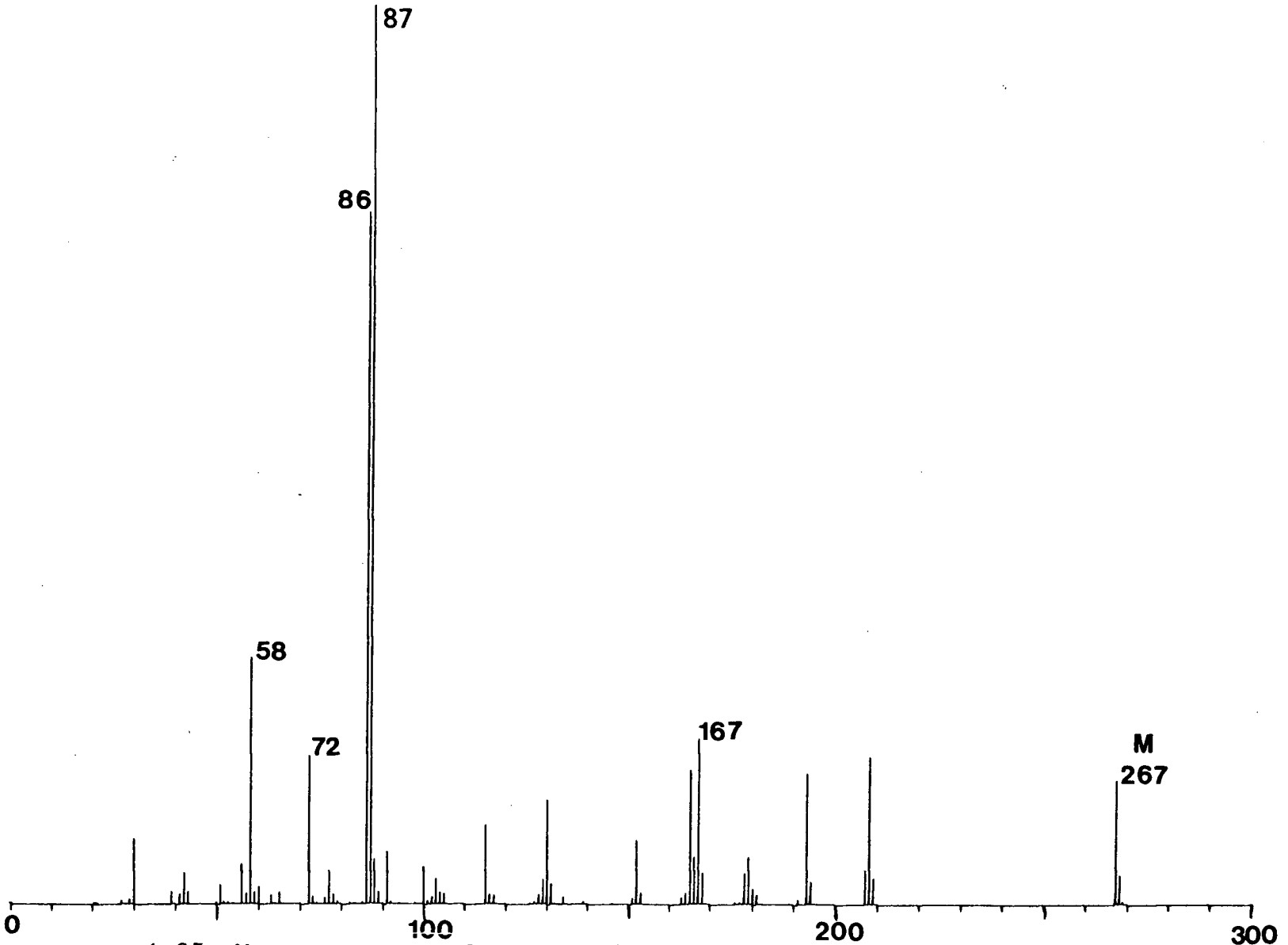
1.32. Mass spectrum of pyrimidine (59)



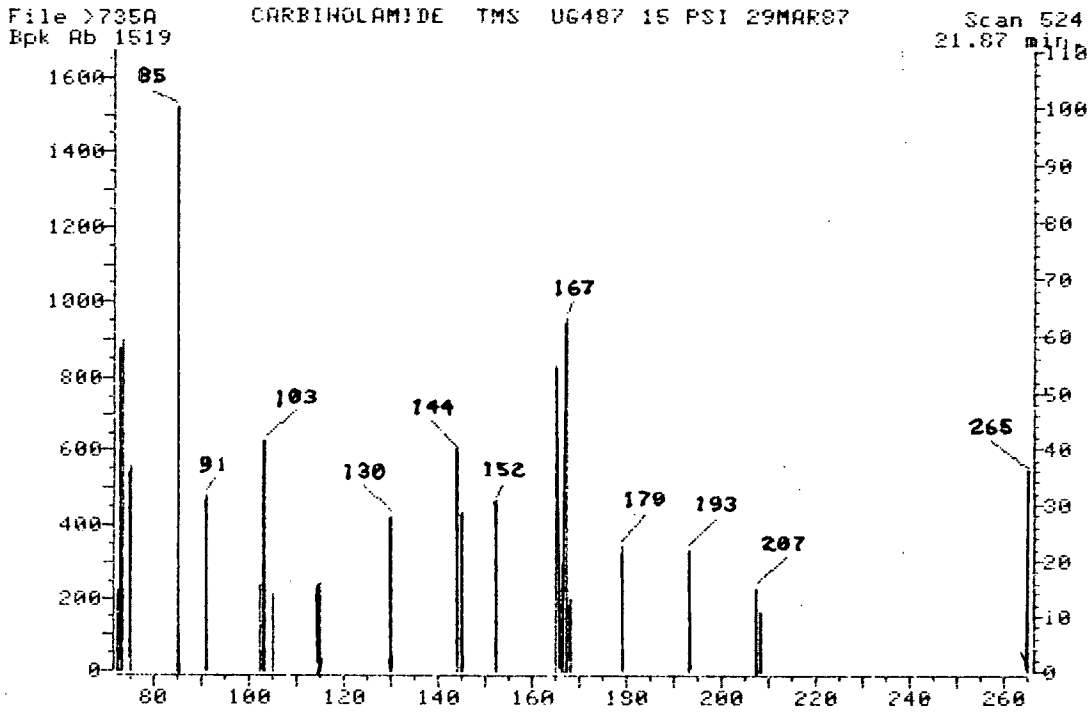
1.33. IR spectrum (film) of pyrimidine (59)



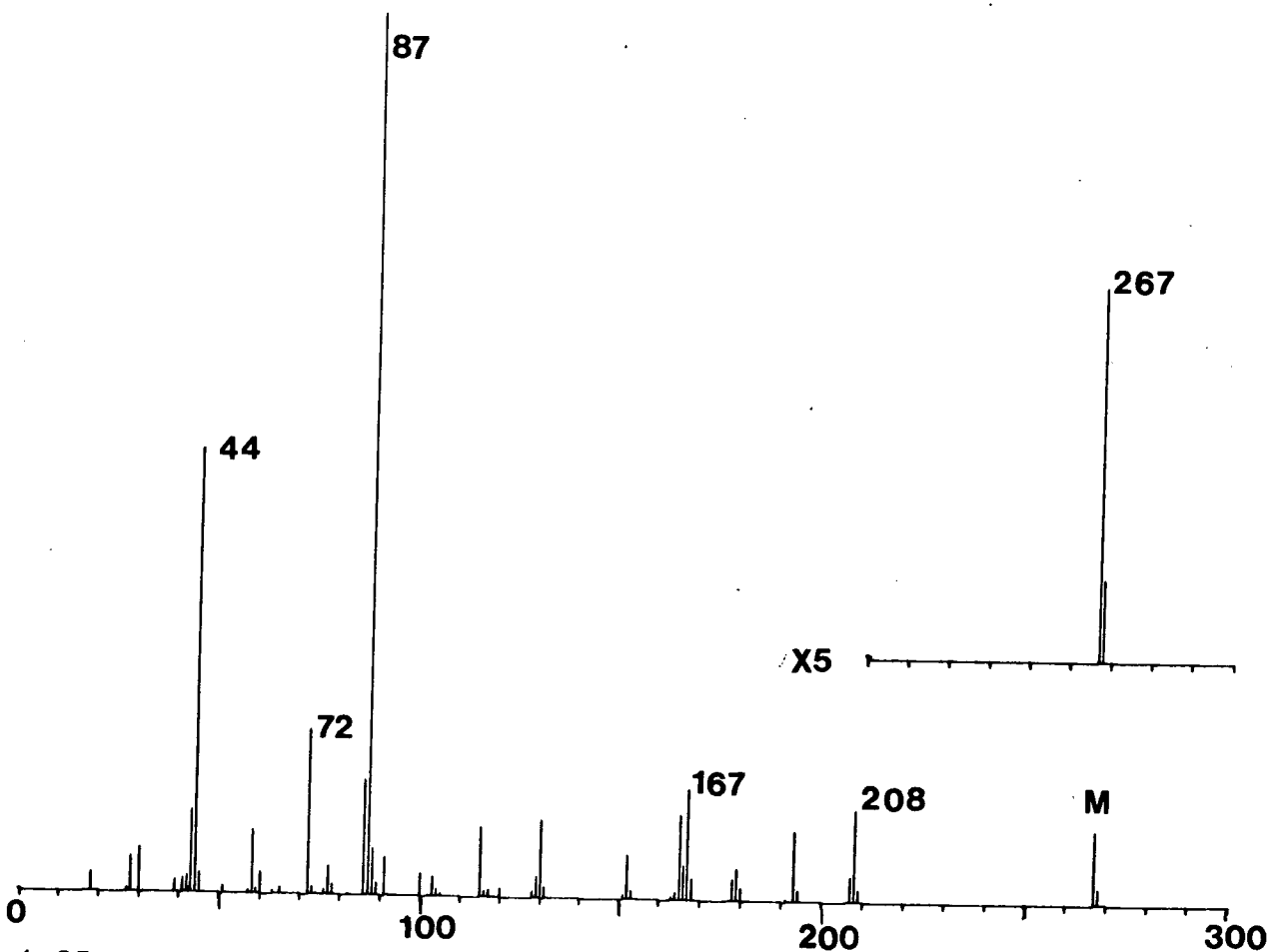
1.34. IR spectrum (film) of suspected formohydroxamic acid (61)



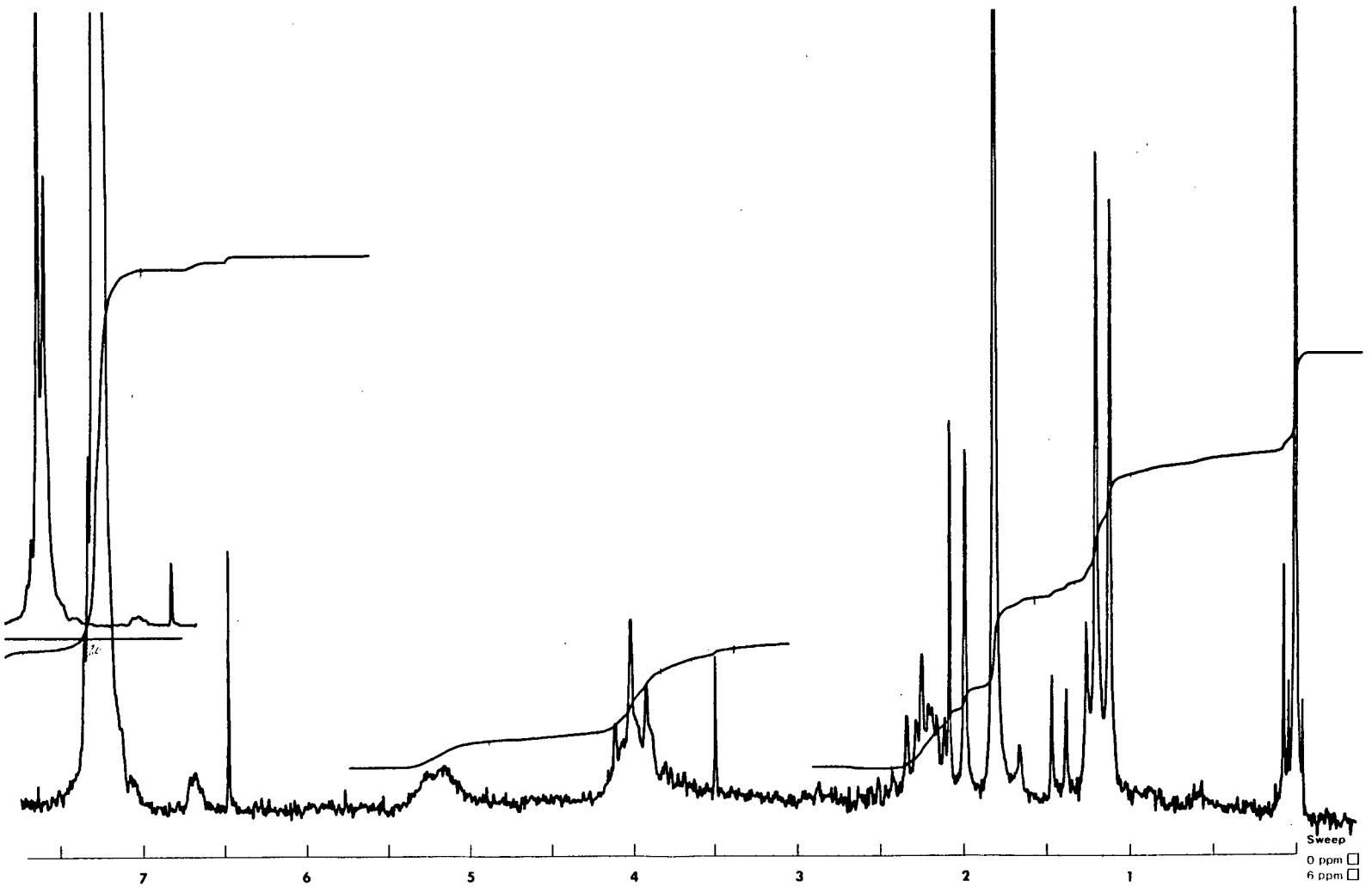
1.35. Mass spectrum of the tertiary formamide (26)



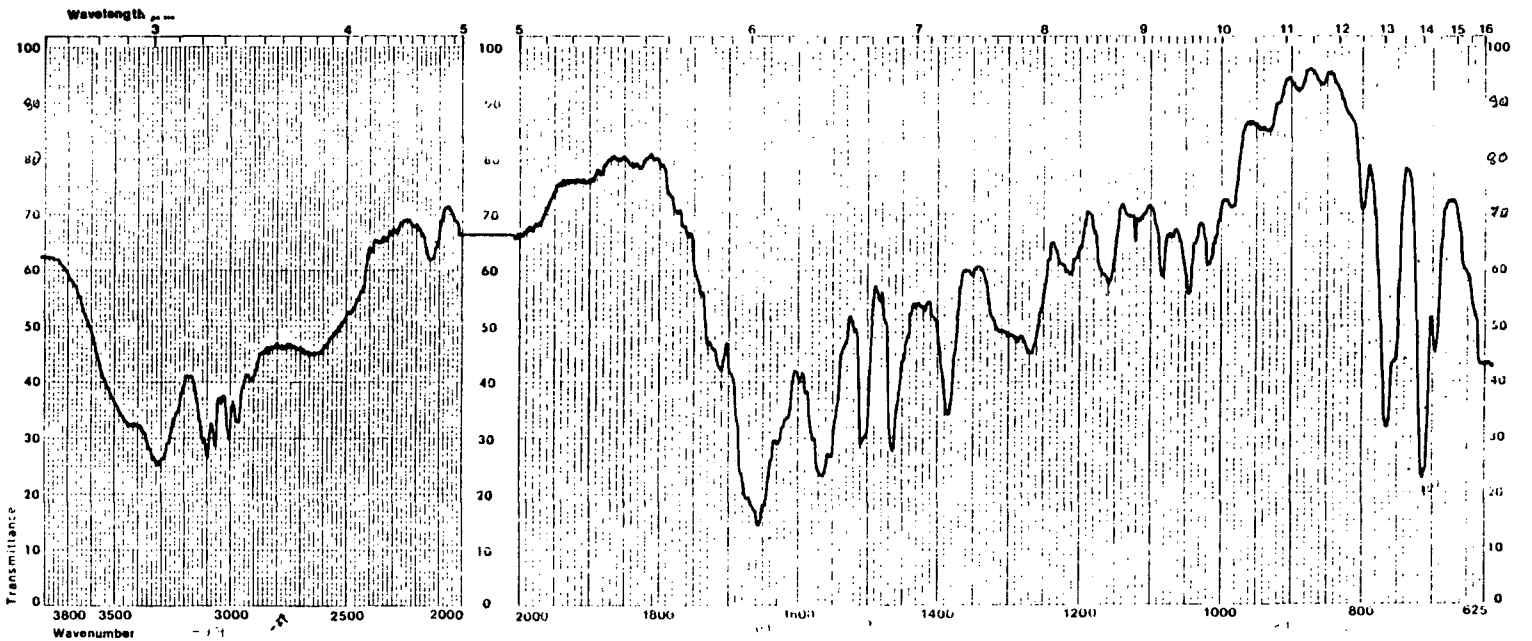
1.36. Mass spectrum of the synthetic TMS carbinolamide (62)



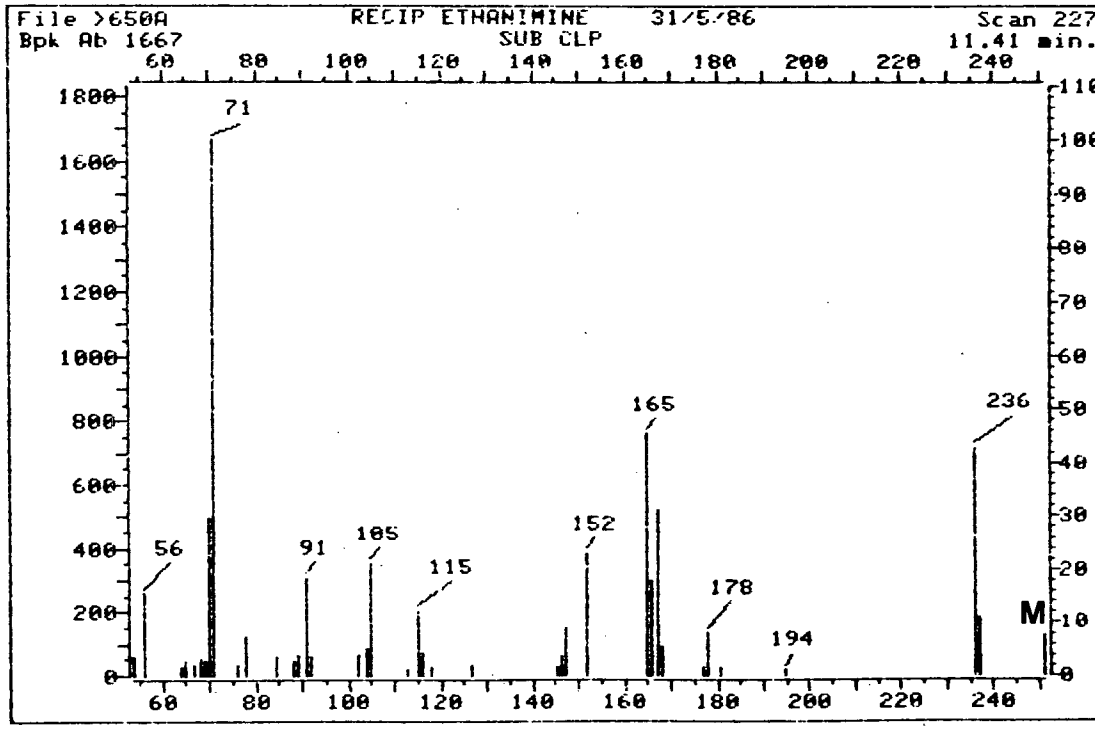
1.37. Mass spectrum of the acetamide (63)



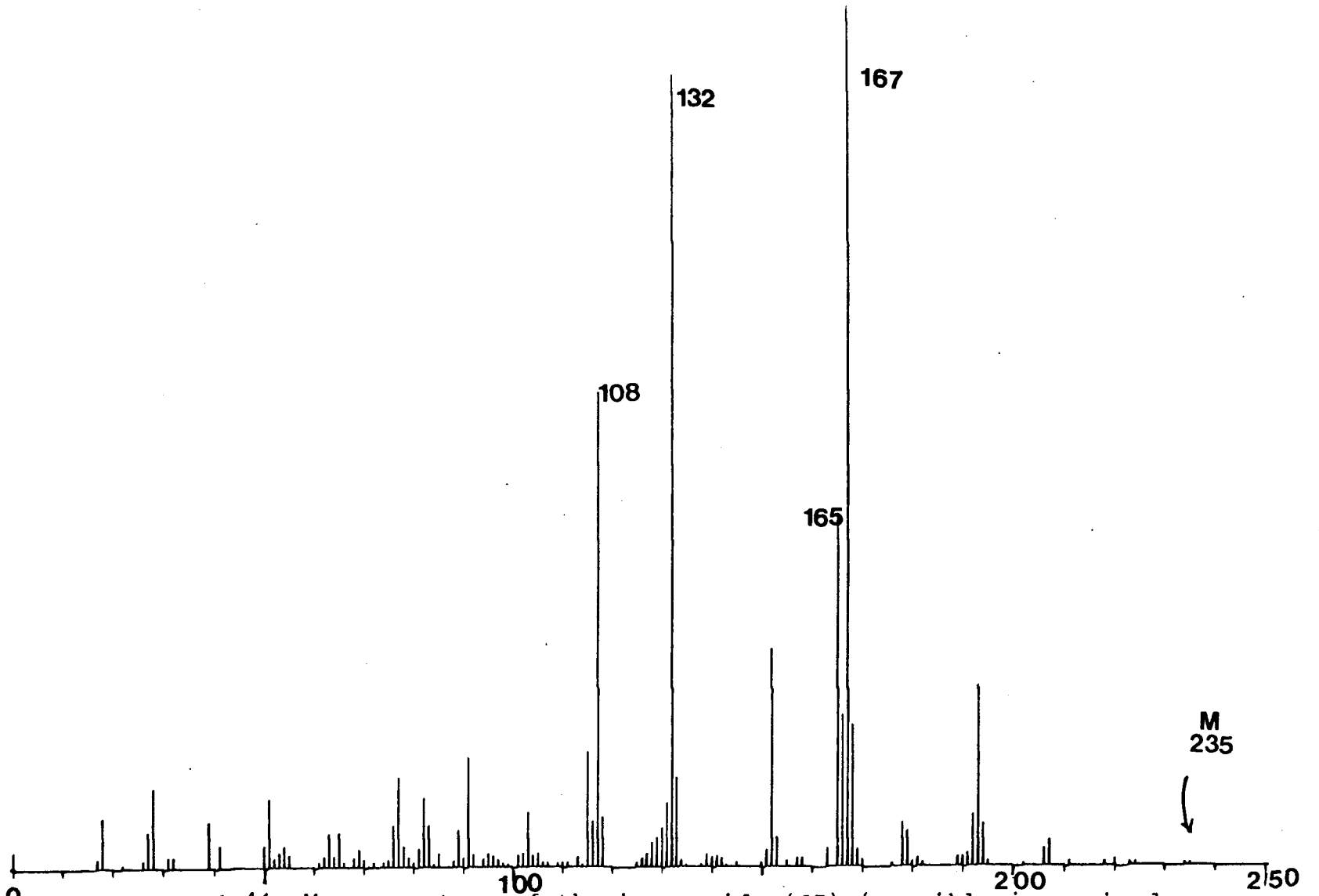
1.38. 80 MHz NMR spectrum of the acetamide (63)



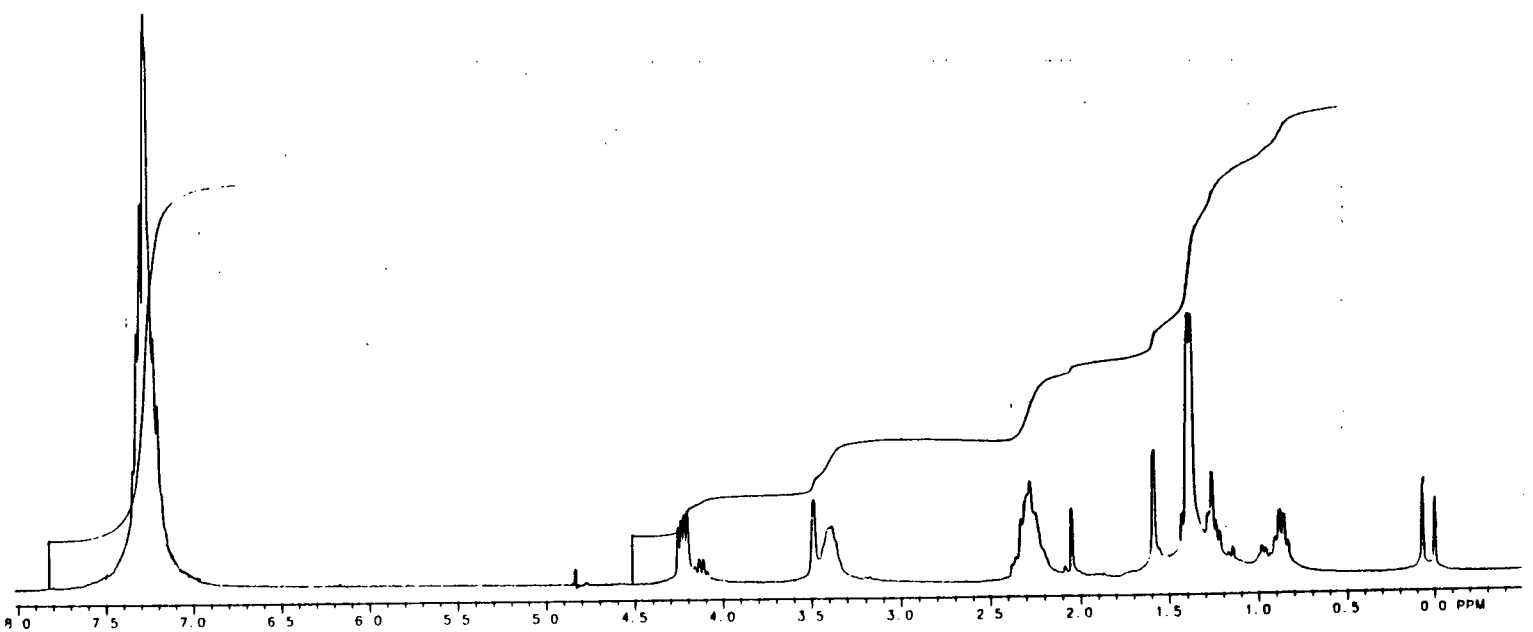
1.39. IR spectrum (film) of the acetamide (63)



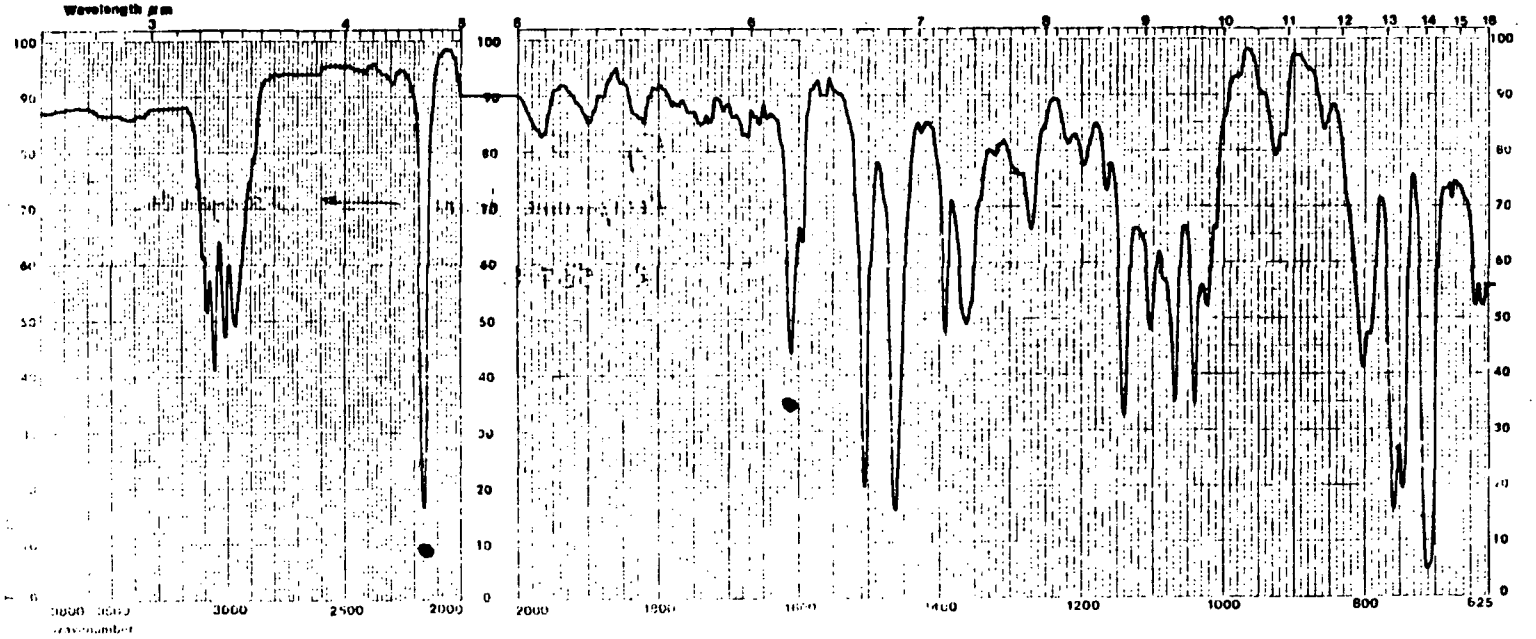
1.40. Mass spectrum of the synthetic ethanimine (64)



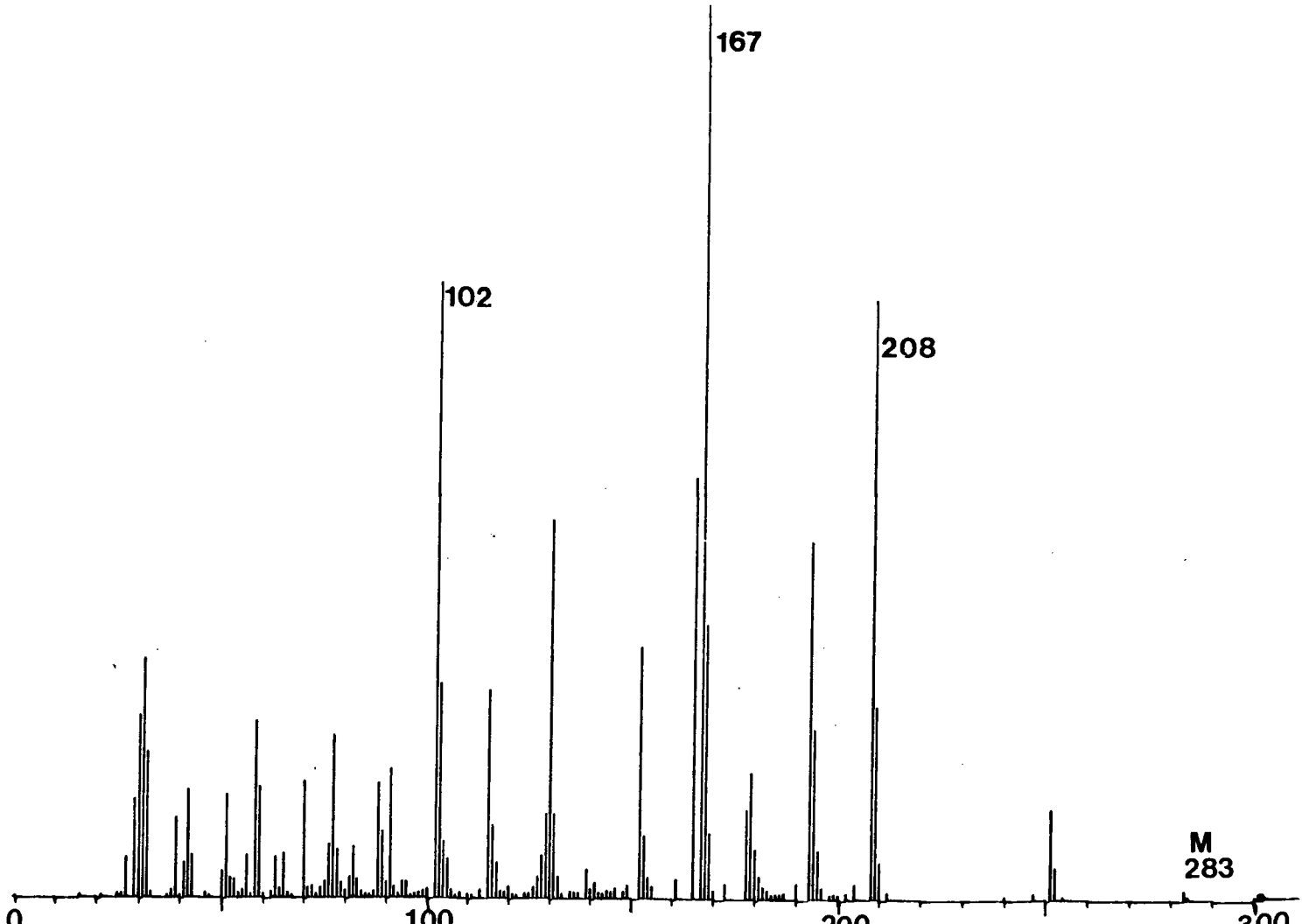
1.41. Mass spectrum of the isocyanide (65) (possibly isomerized to the nitrile)



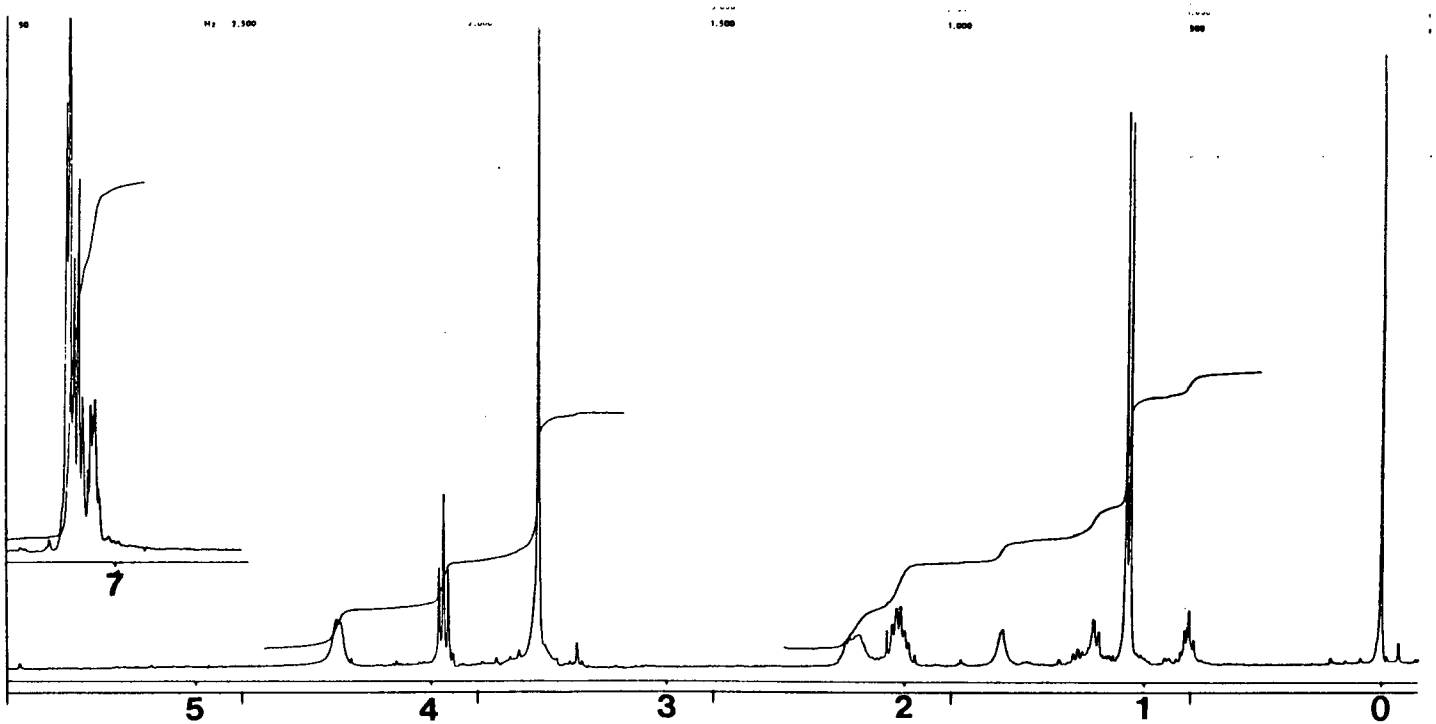
1.42. 300 MHz NMR spectrum of the isocyanide (65)



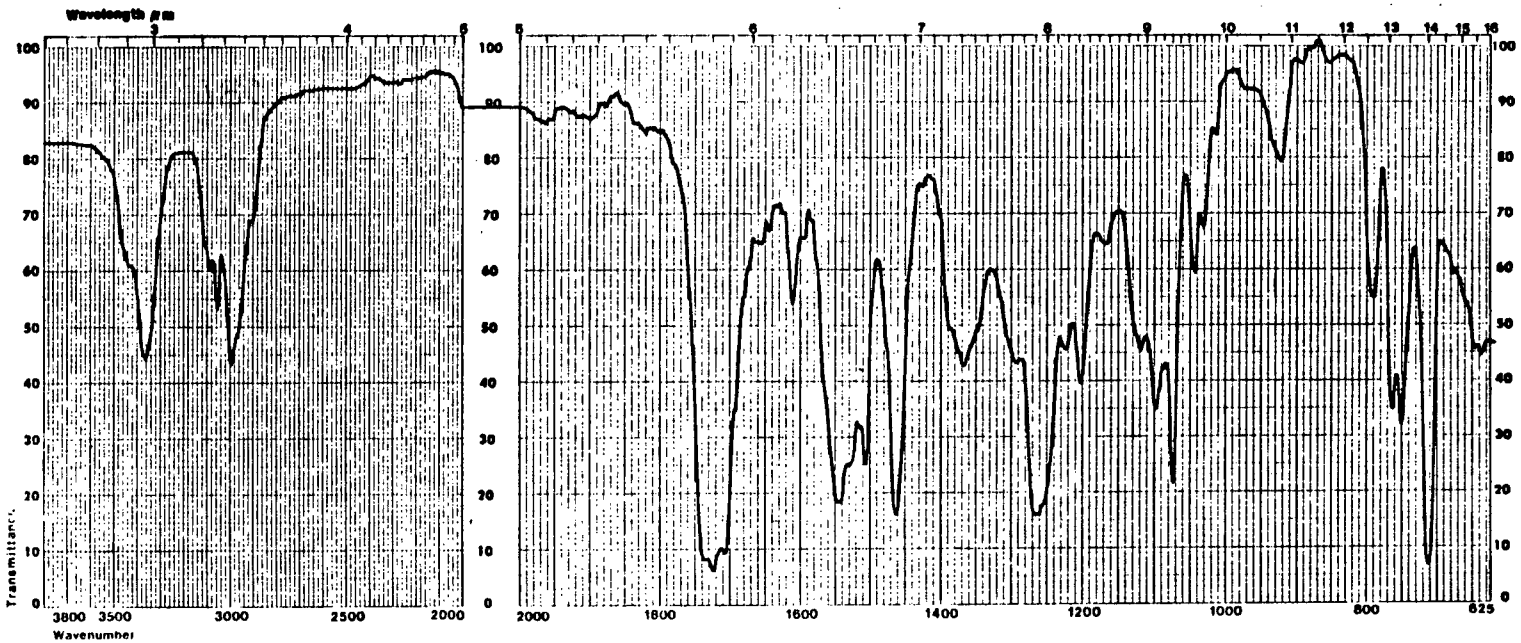
1.43. IR spectrum (film) of the isocyanide (65)



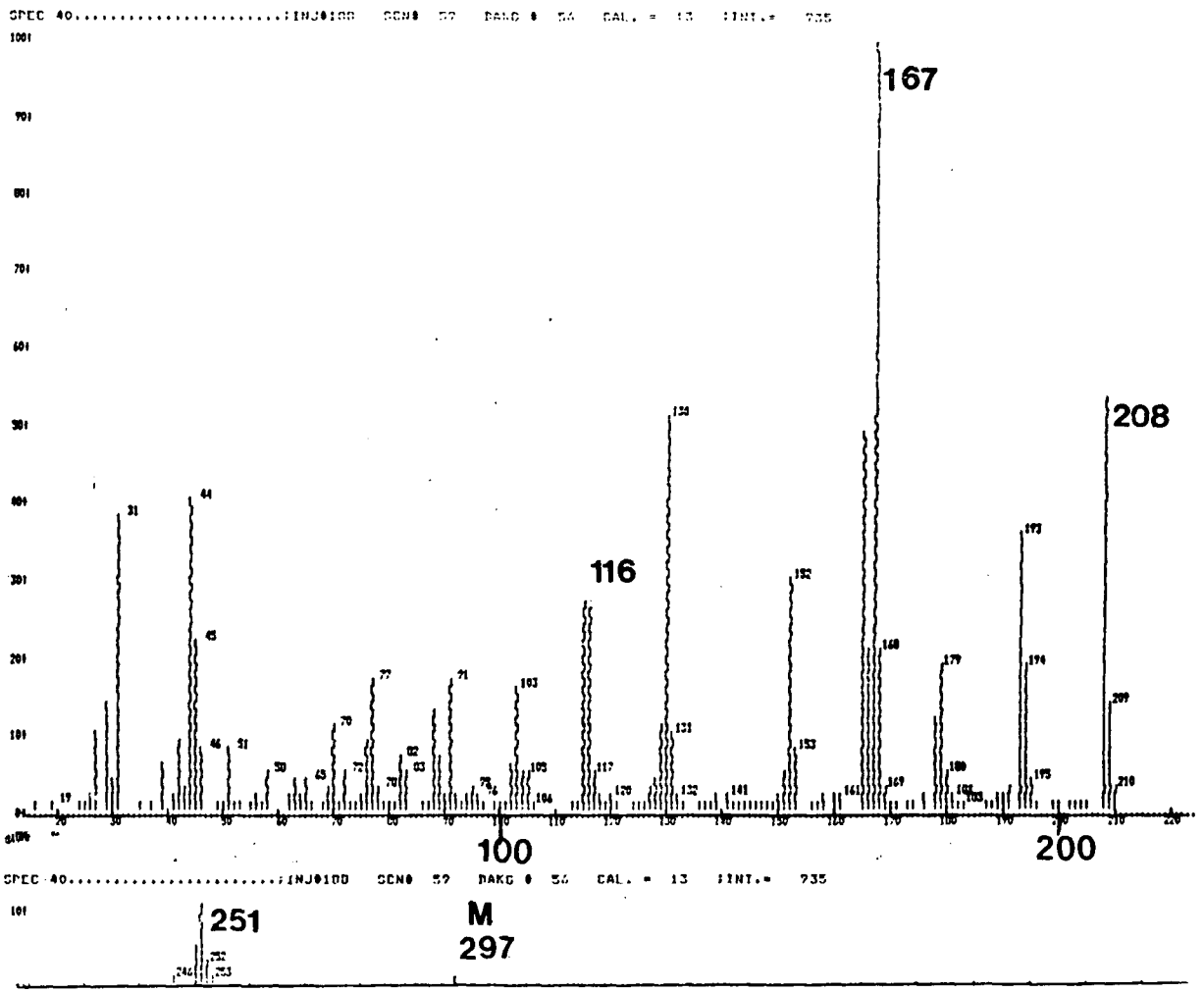
1.44. Mass spectrum of the carbamate (66)



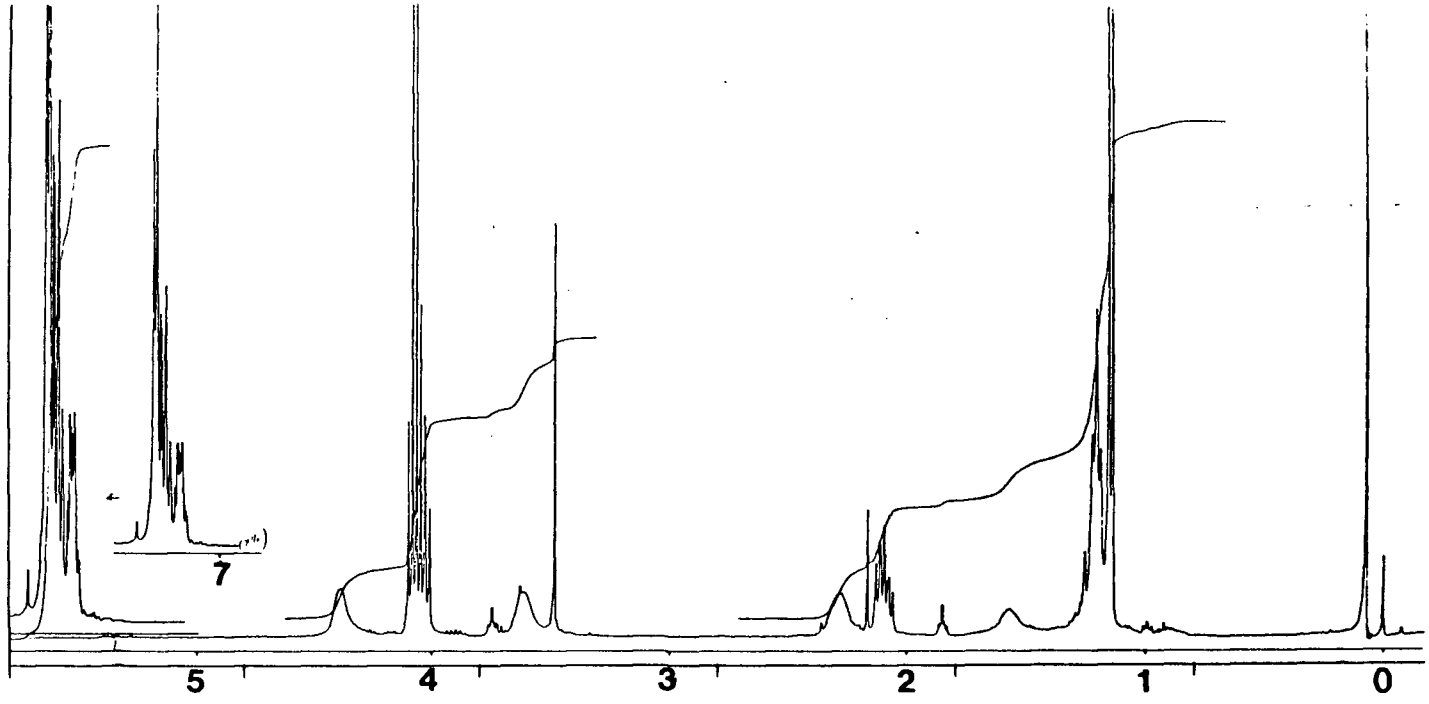
1.45. 300 MHz NMR spectrum of the carbamate (66)



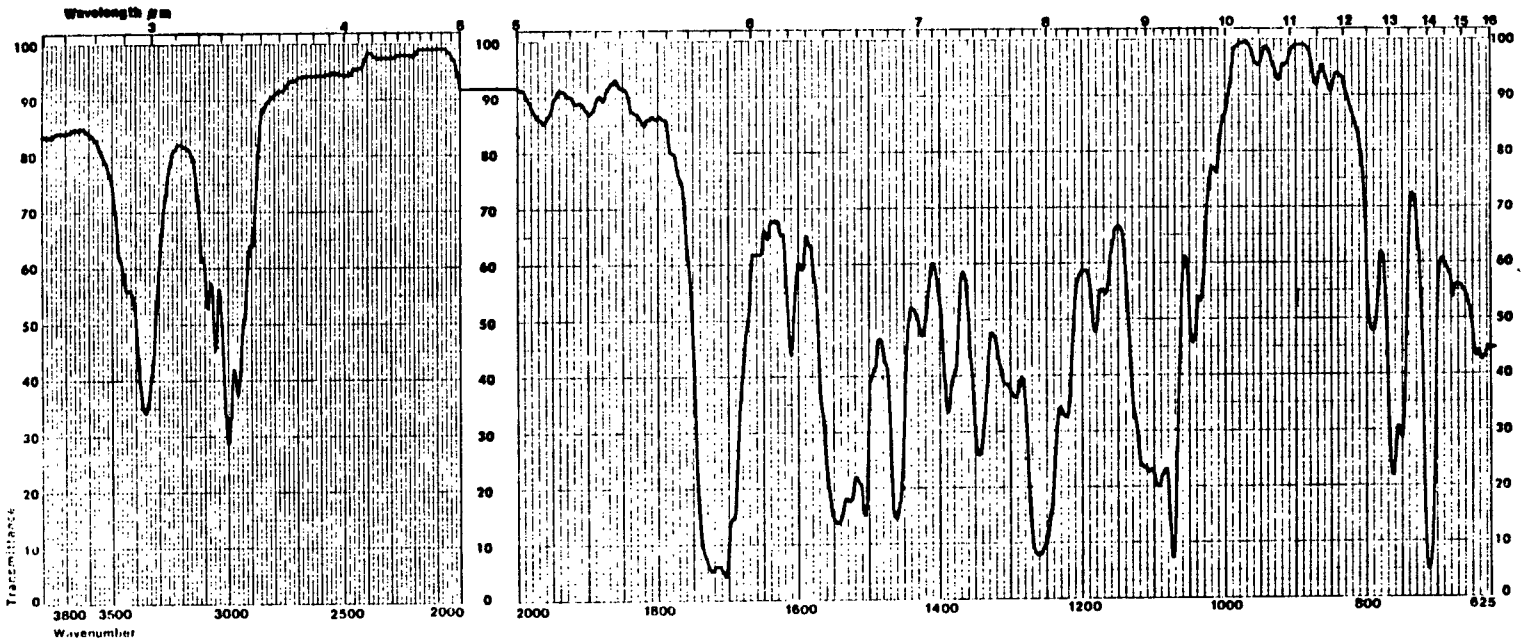
1.46. IR spectrum (film) of the carbamate (66)



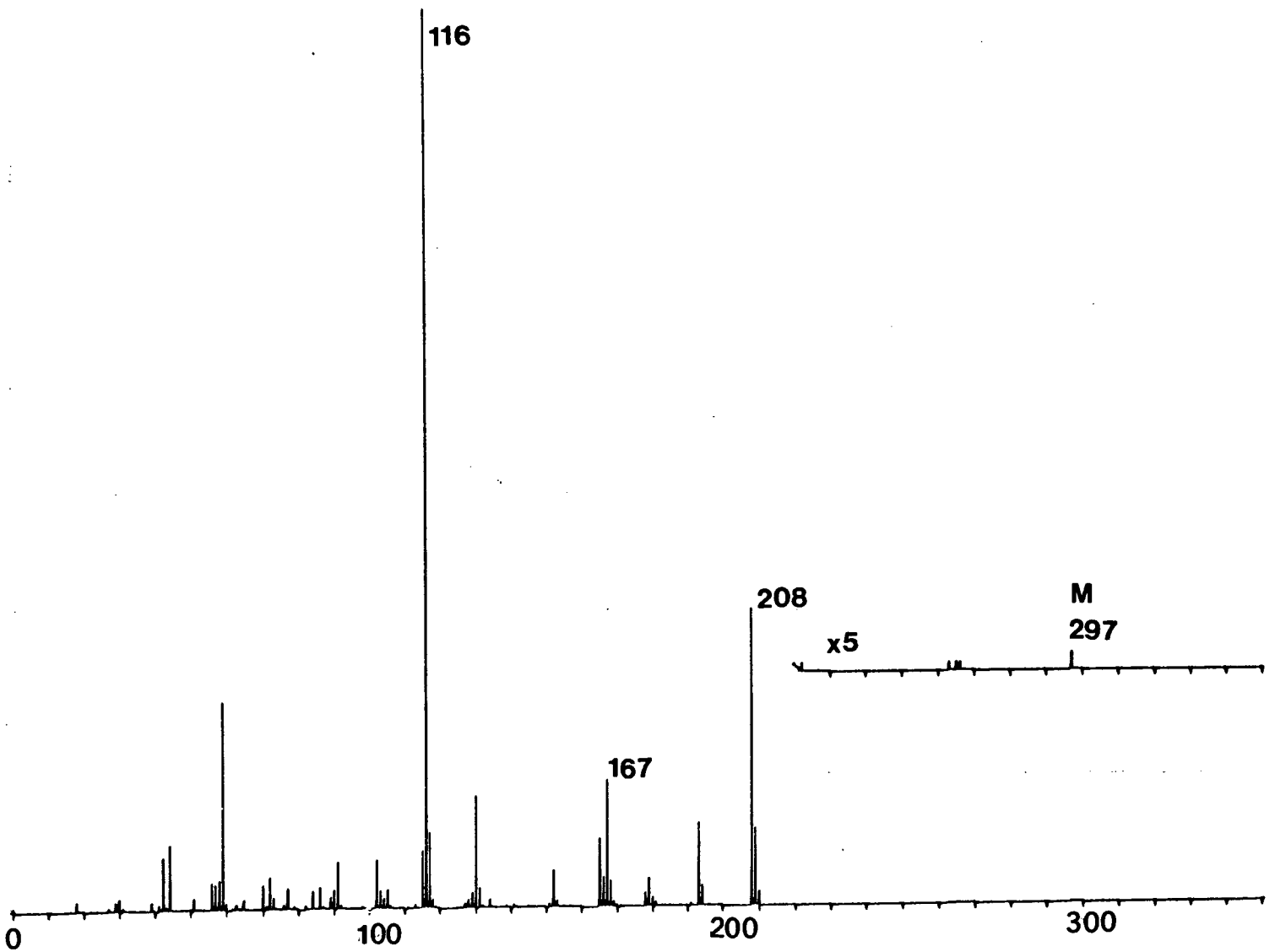
1.47. Mass spectrum of the carbamate (67)



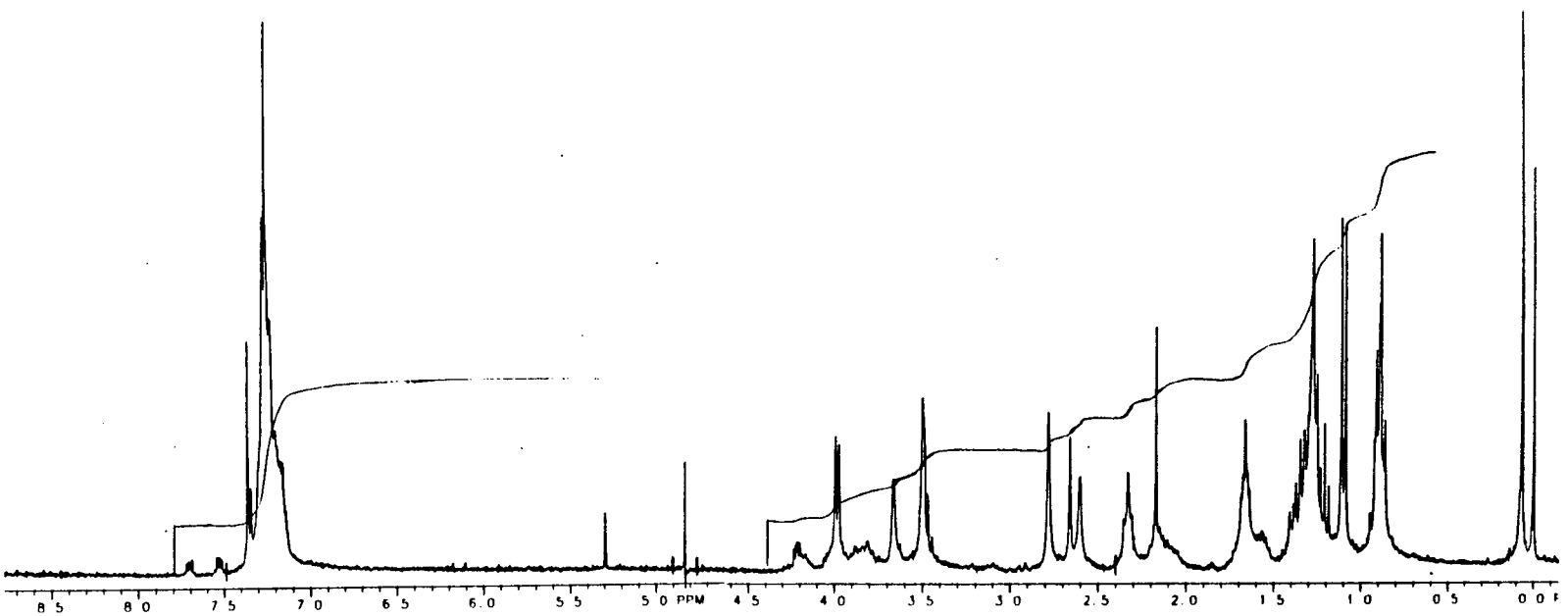
1.48. 300 MHz NMR spectrum of the carbamate (67)



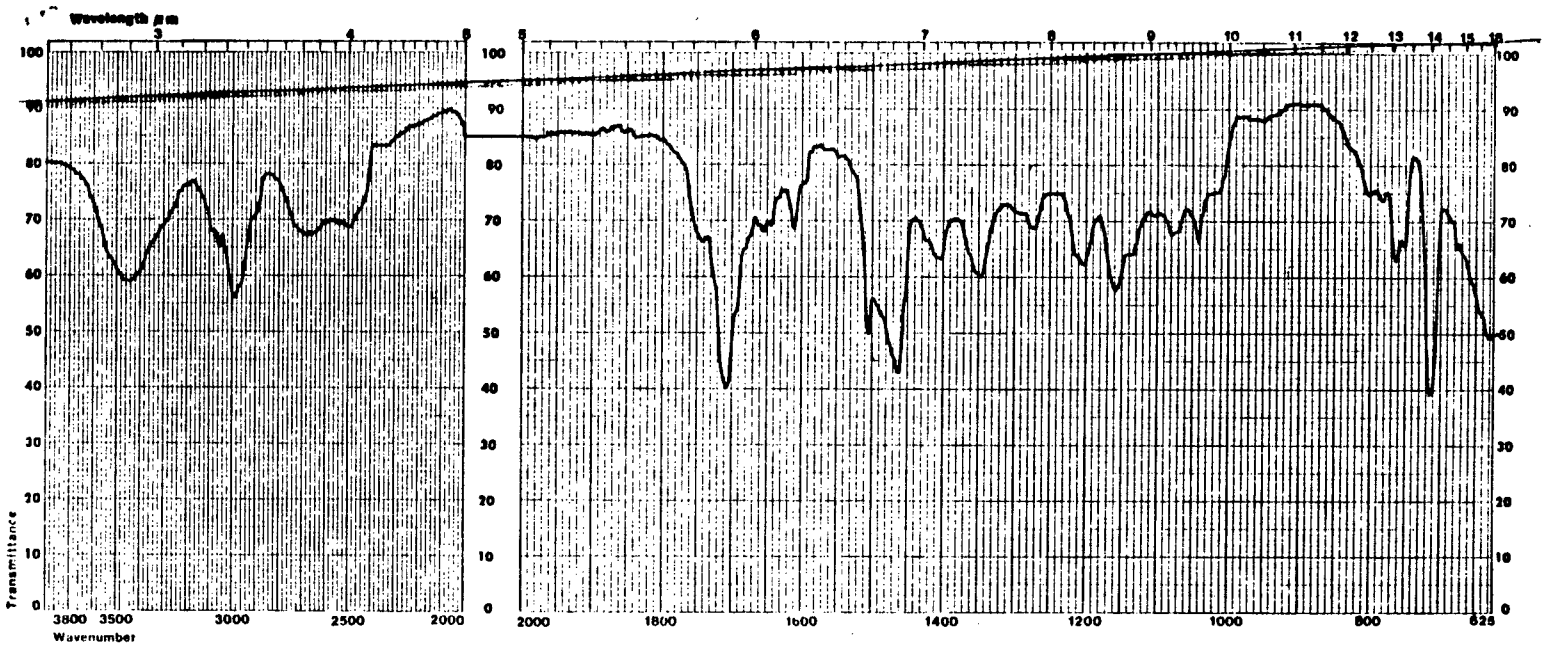
1.49. IR spectrum (film) of the carbamate (67)



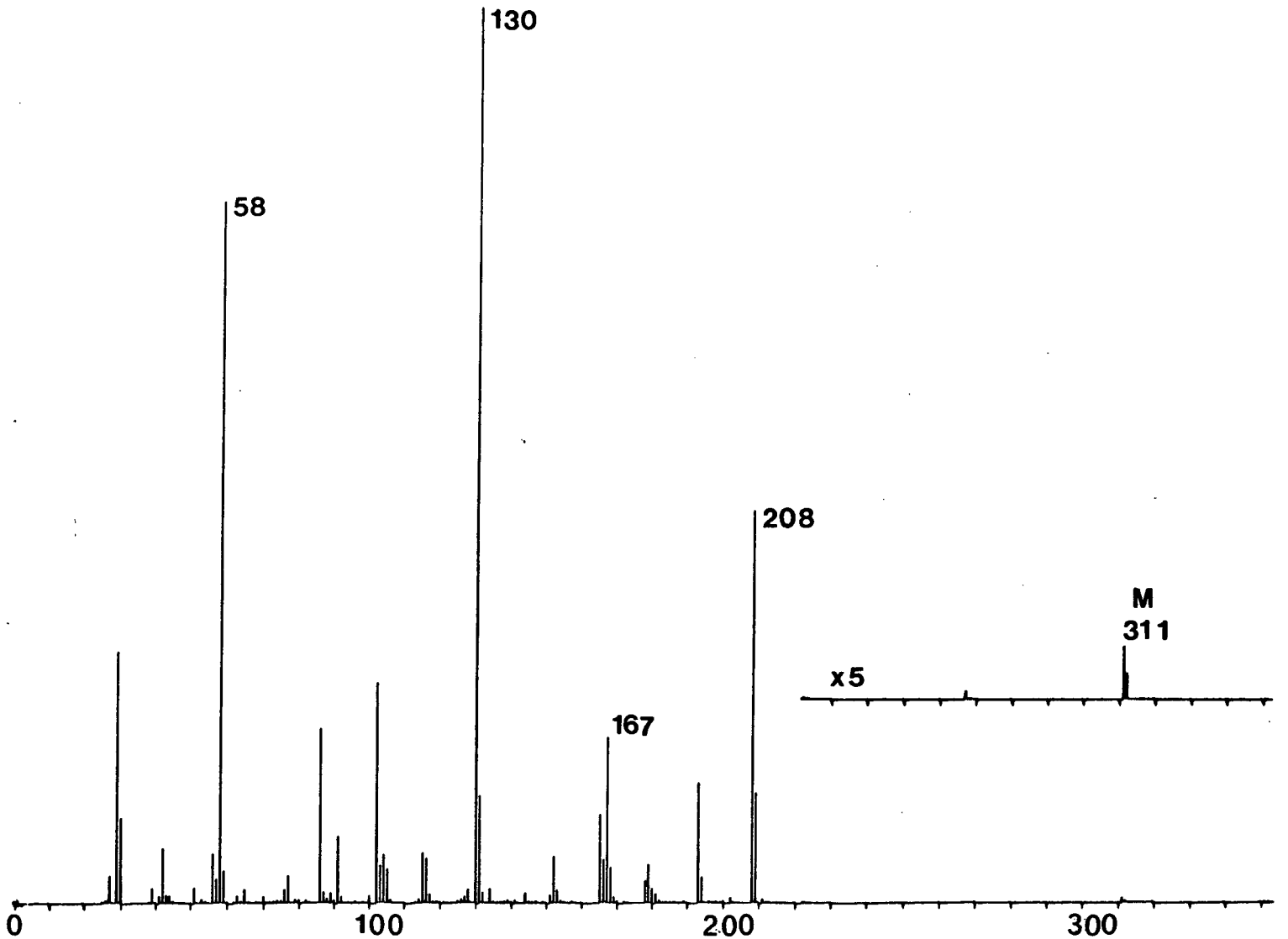
1.50. Mass spectrum of the carbamate (68)



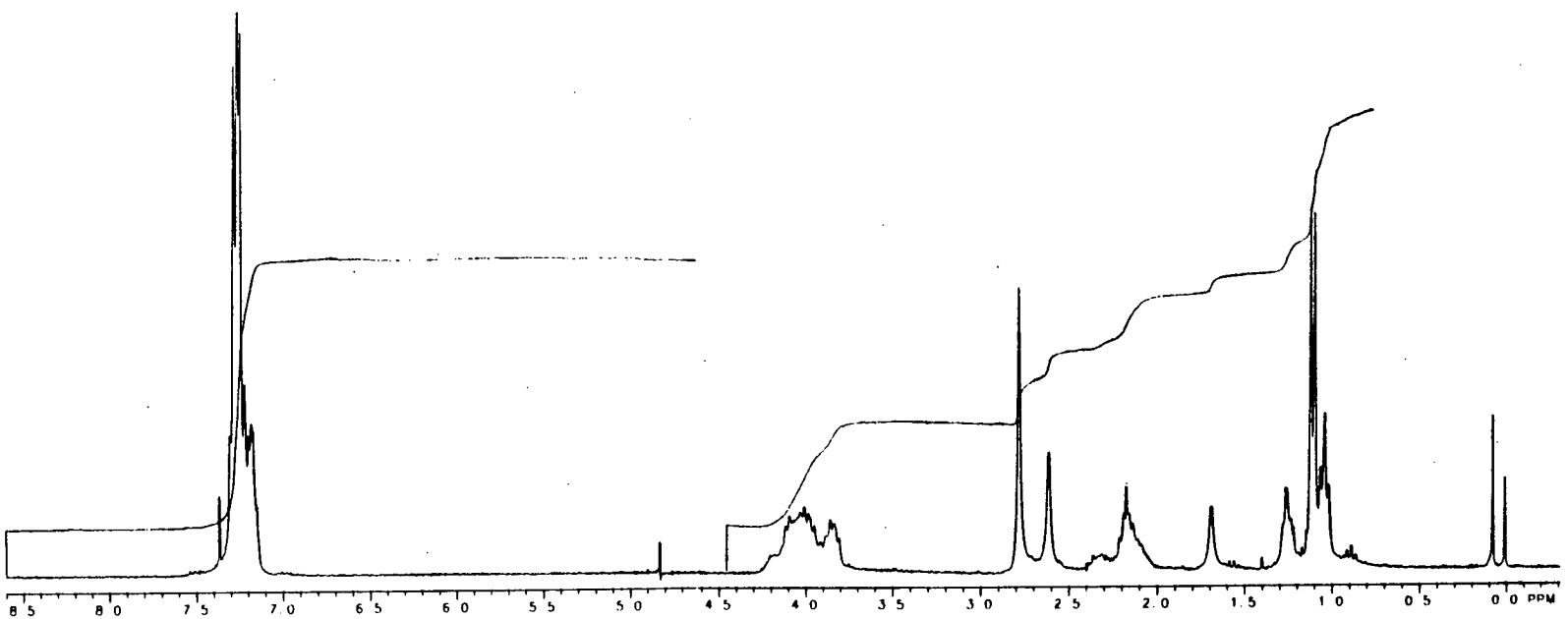
1.51. 300 MHz NMR spectrum of the carbamate (68)



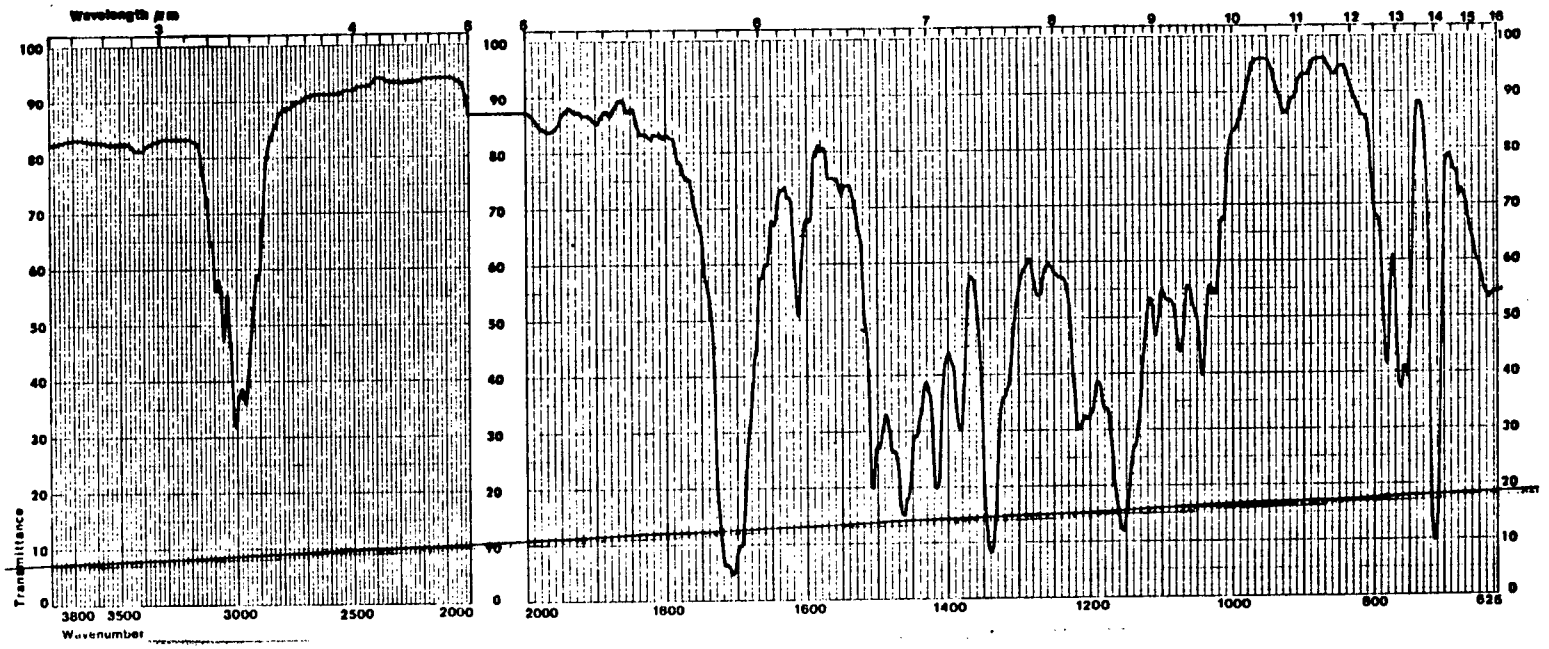
1.52. IR spectrum (film) of the carbamate (68)



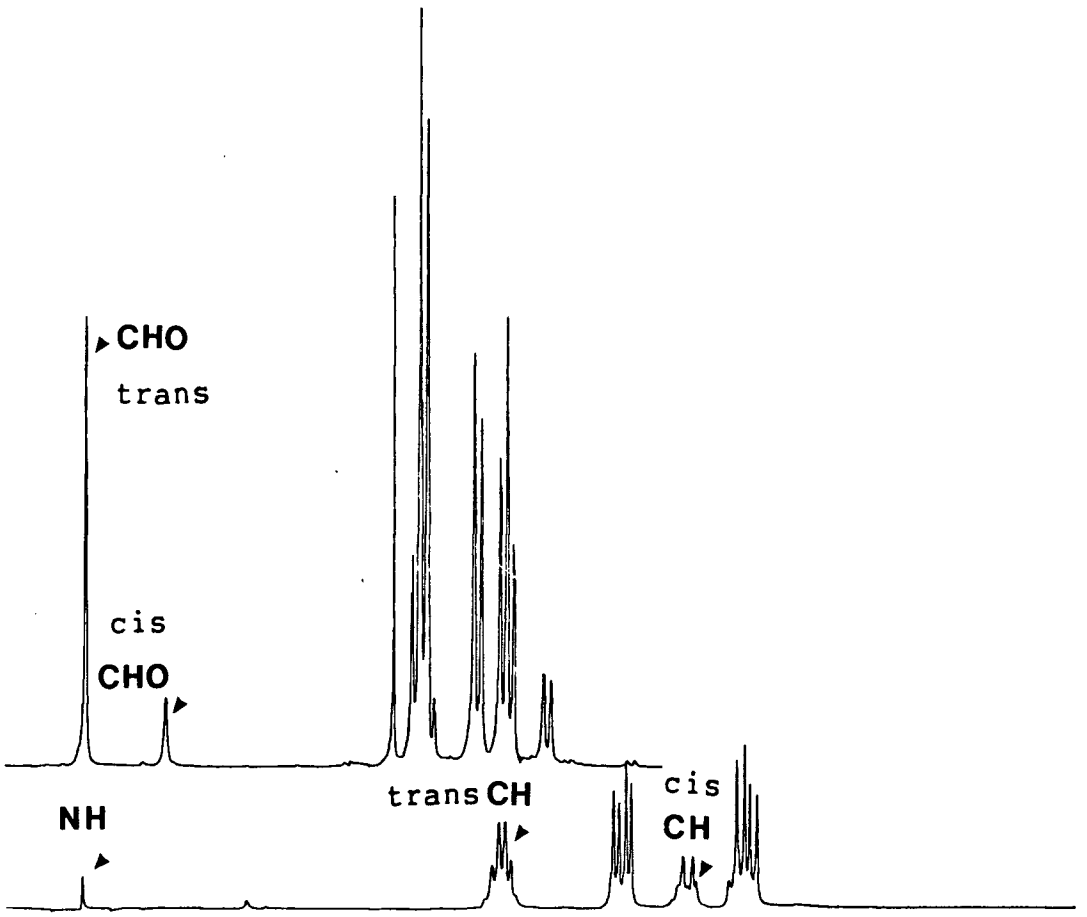
1.53. Mass spectrum of the carbamate (69)



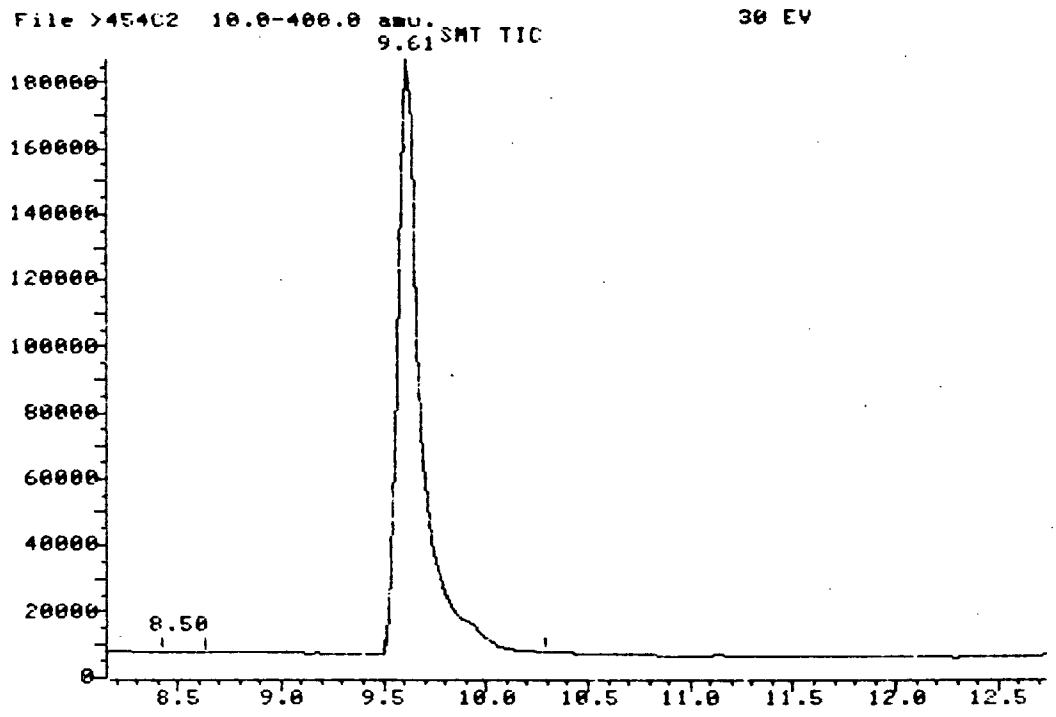
1.54. 300 MHz NMR spectrum of the carbamate (69)

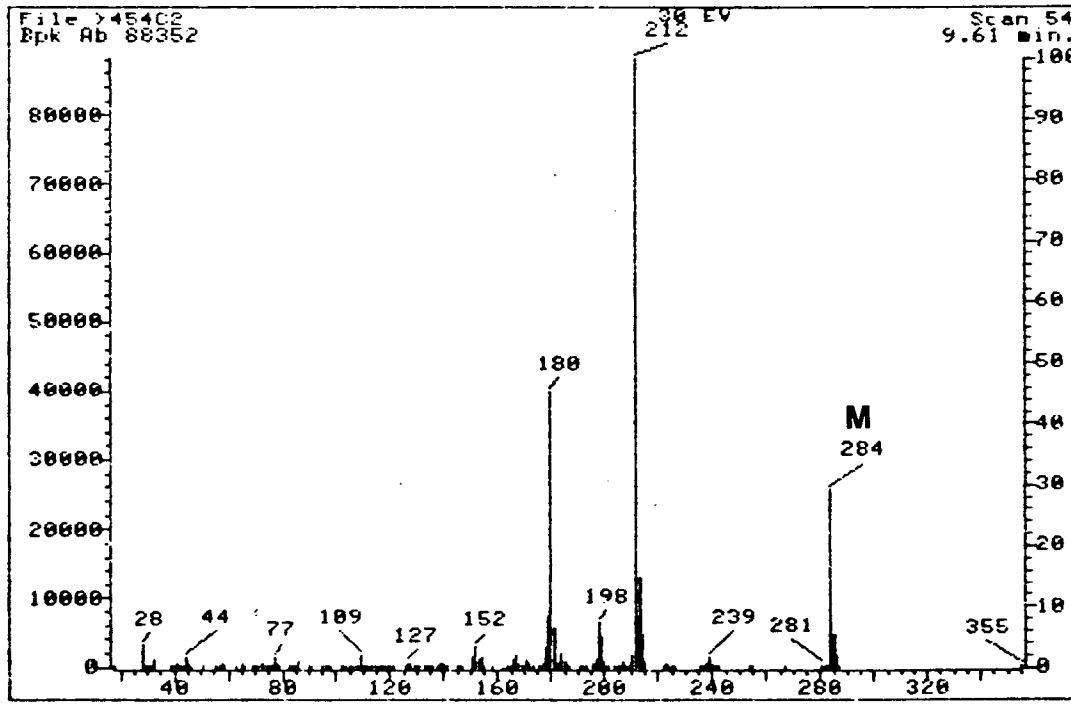


1.55. IR spectrum (film) of the carbamate (69)

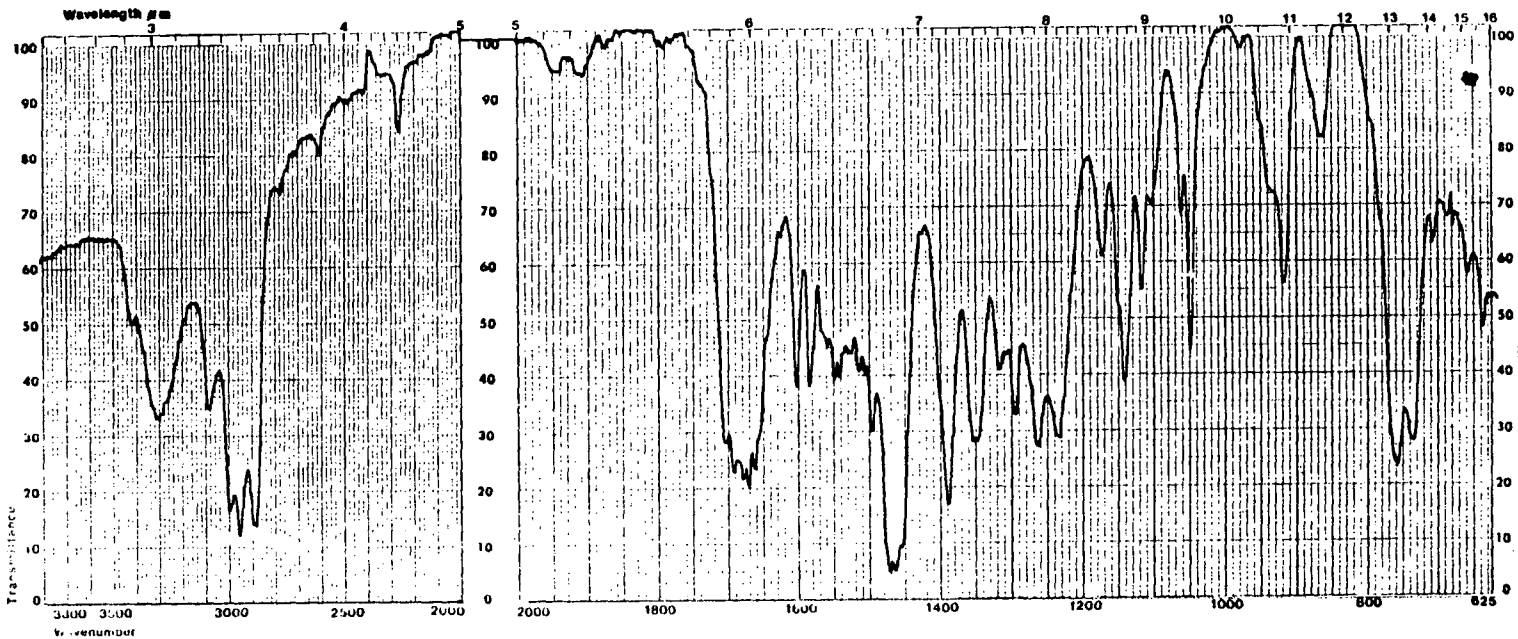


1.56. 400 MHz NMR spectrum of promethazine secondary formamide (72). NH decoupled.

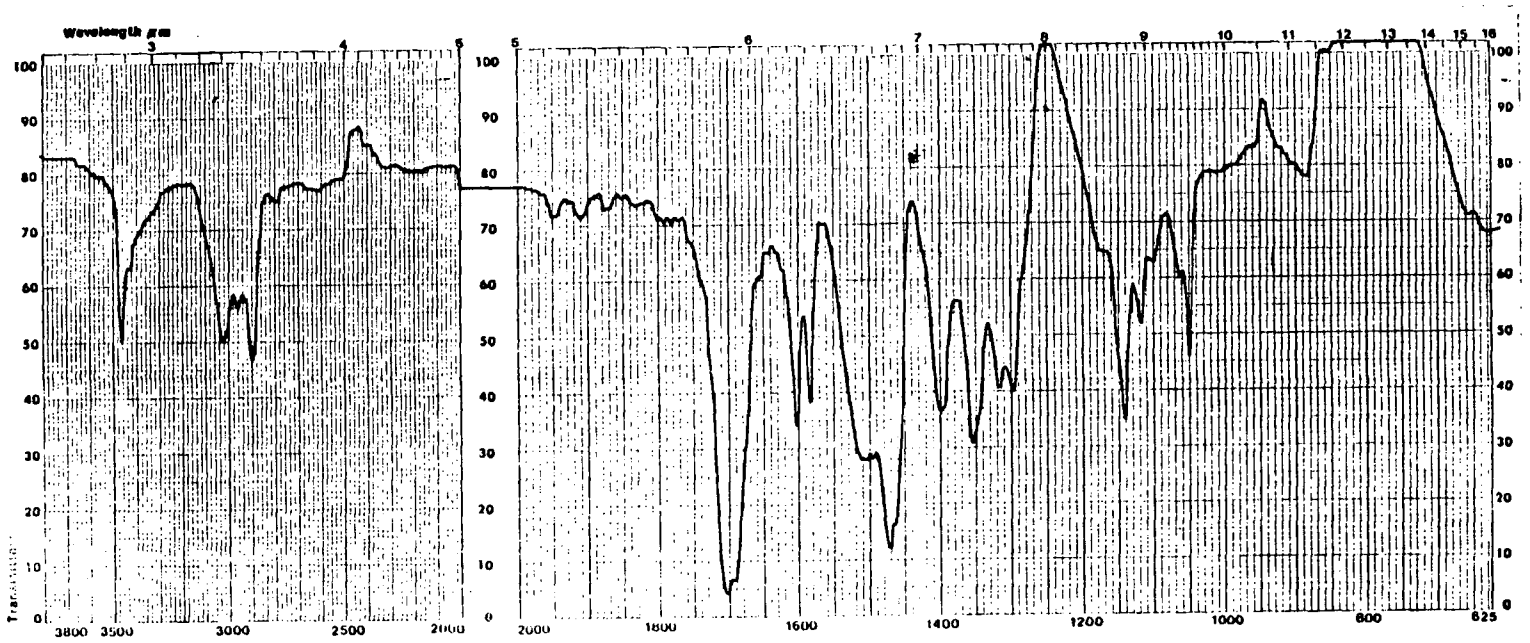




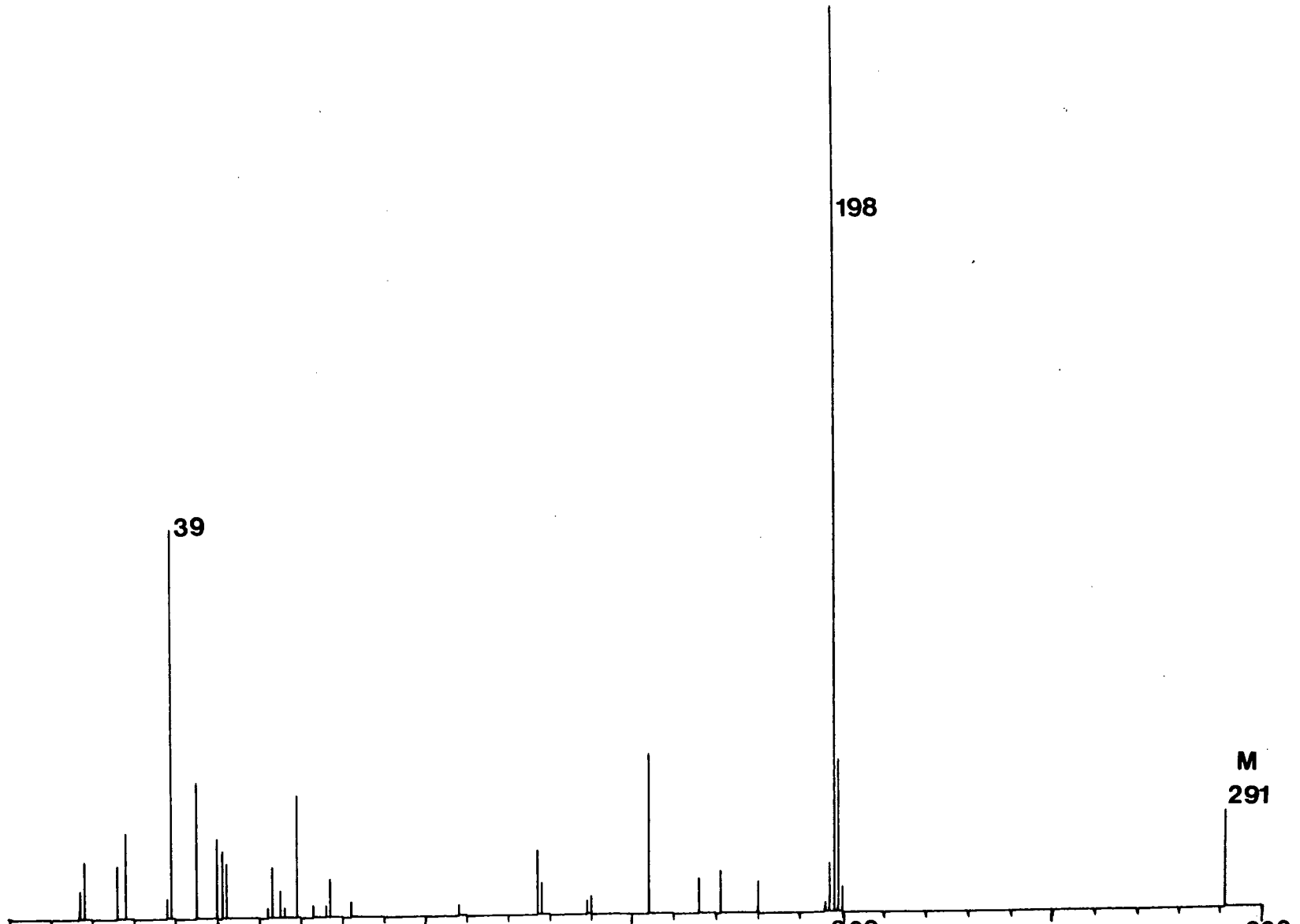
1.57. Total ion current and mass spectrum of promethazine secondary formamide (72) (30 eV)



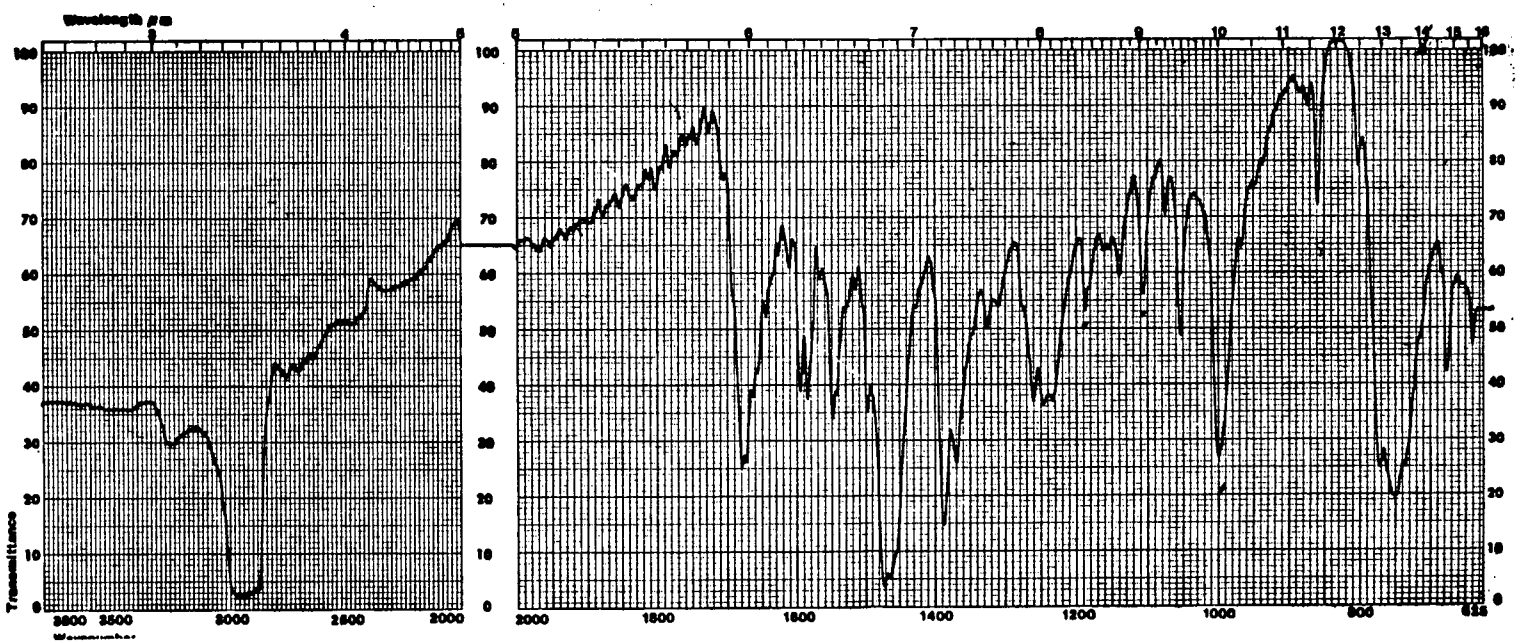
1.58. IR spectrum (mull) of promethazine secondary formamide (72)



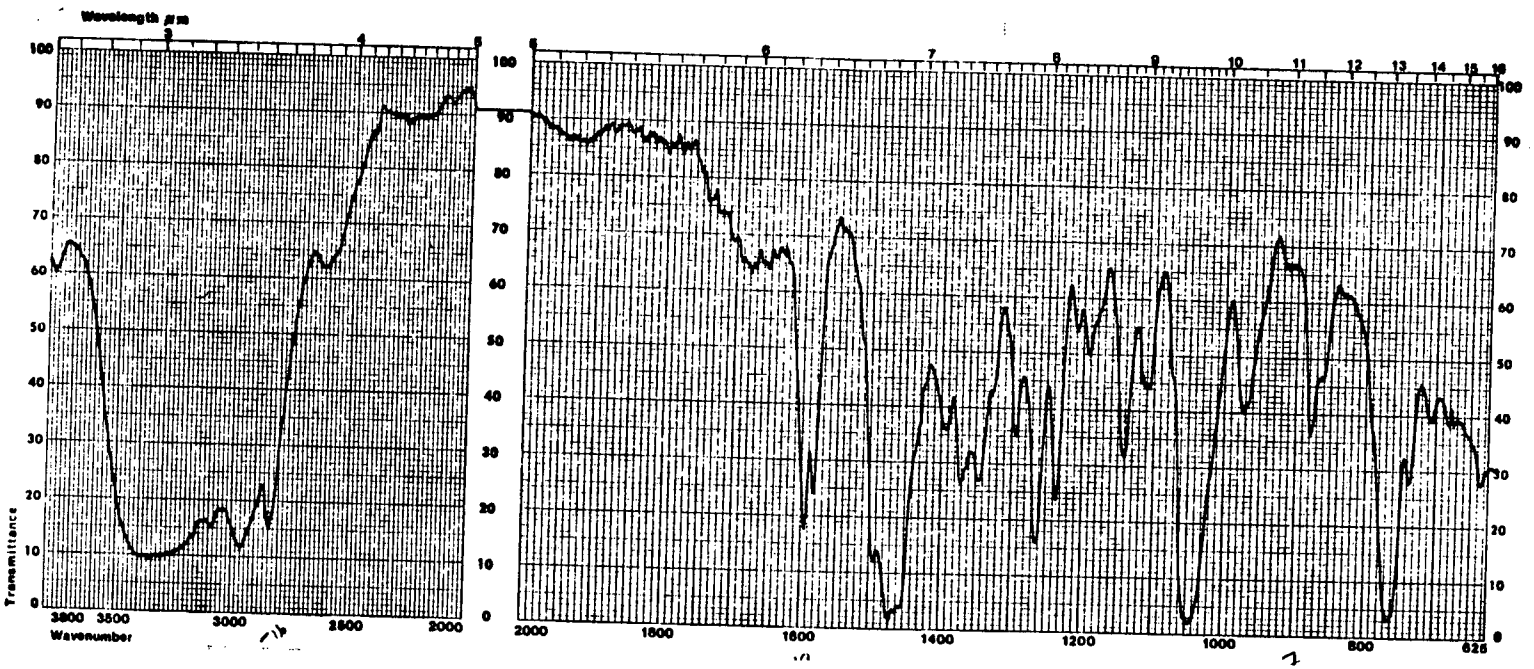
1.59. IR spectrum ($CHCl_3$ solution) of promethazine secondary formamide (72)



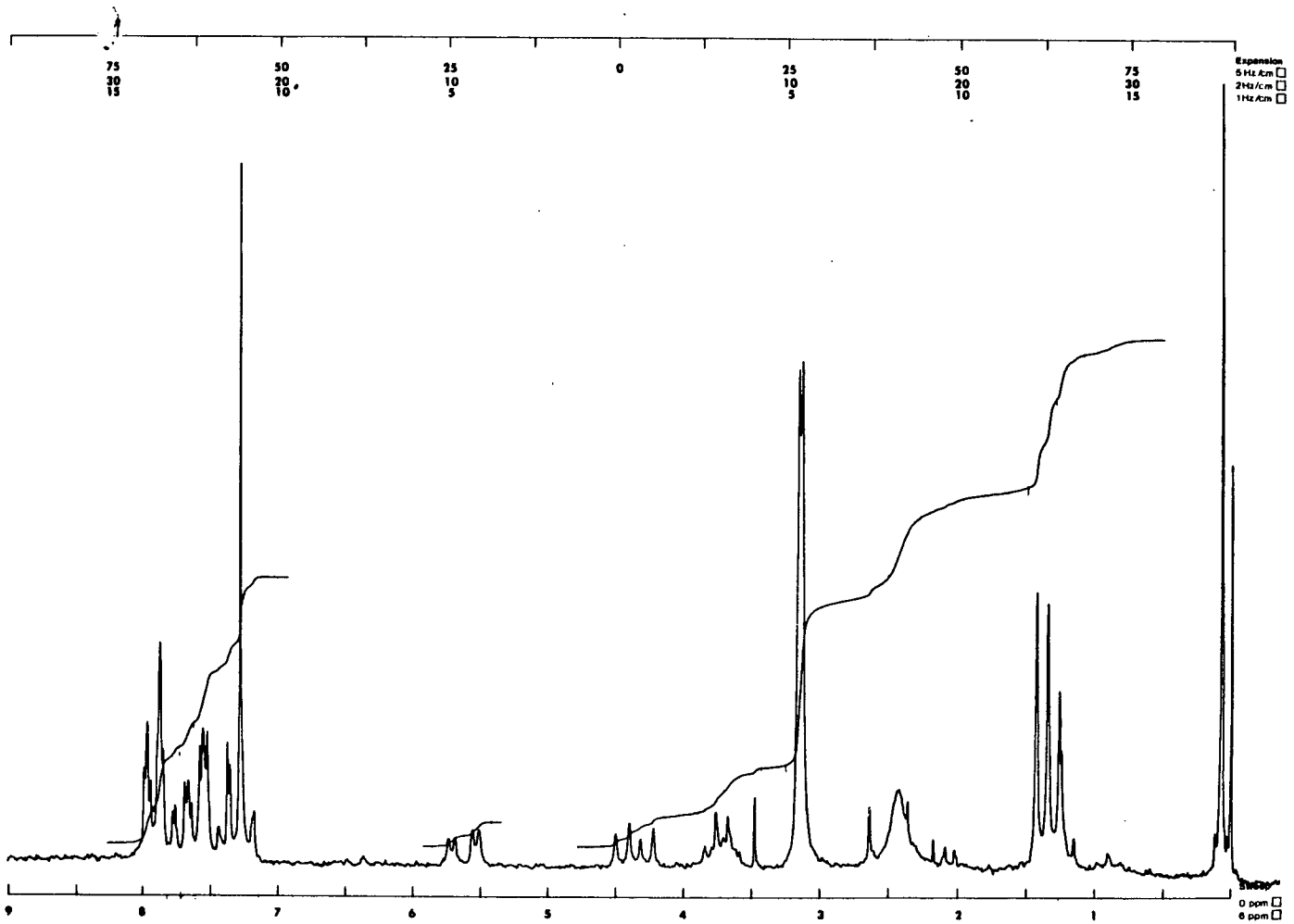
1.60. Mass spectrum of pyrimidine (73)



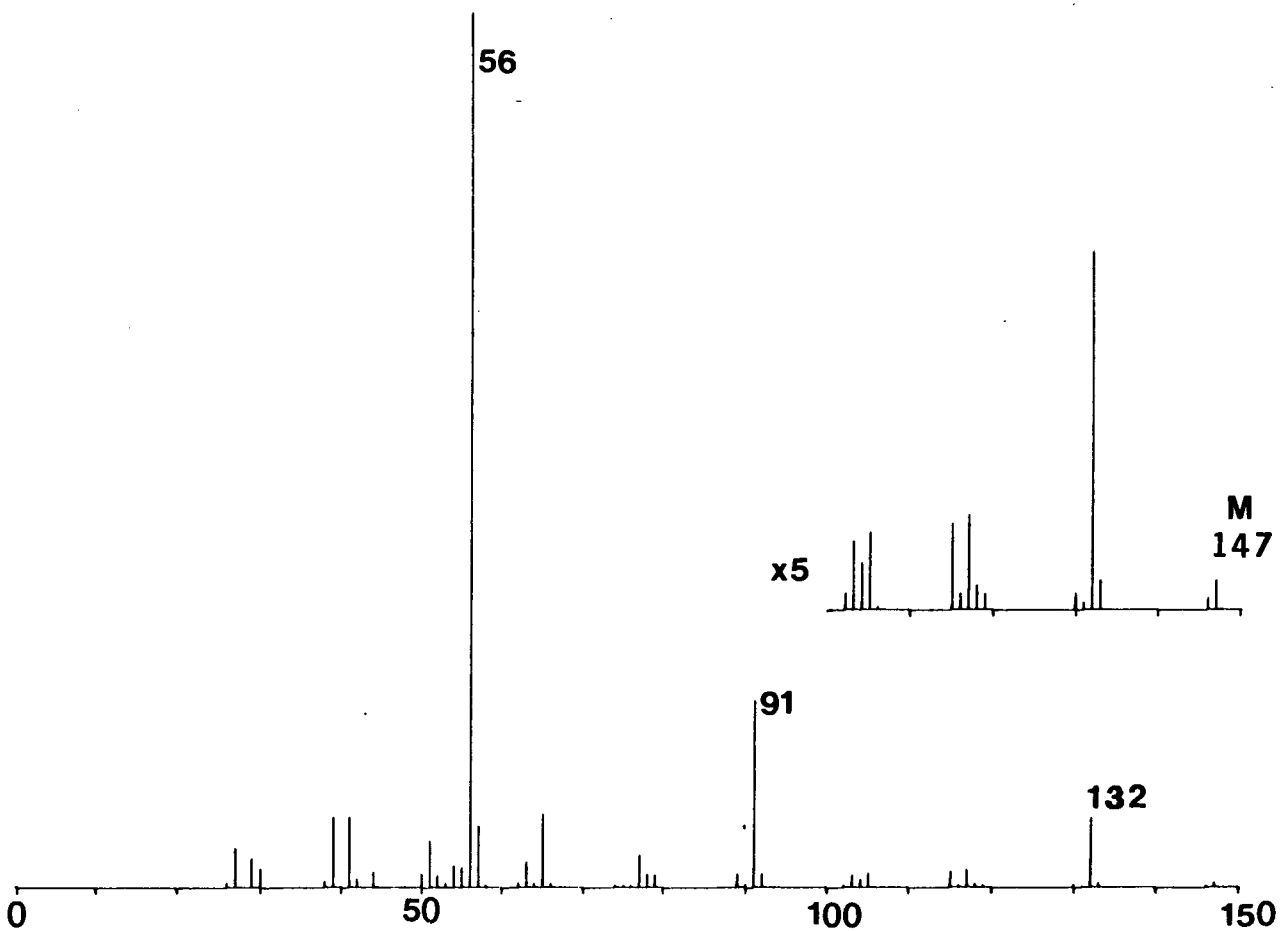
1.61. IR spectrum (mull) of promethazine secondary formamide N_{10}-oxide (75)



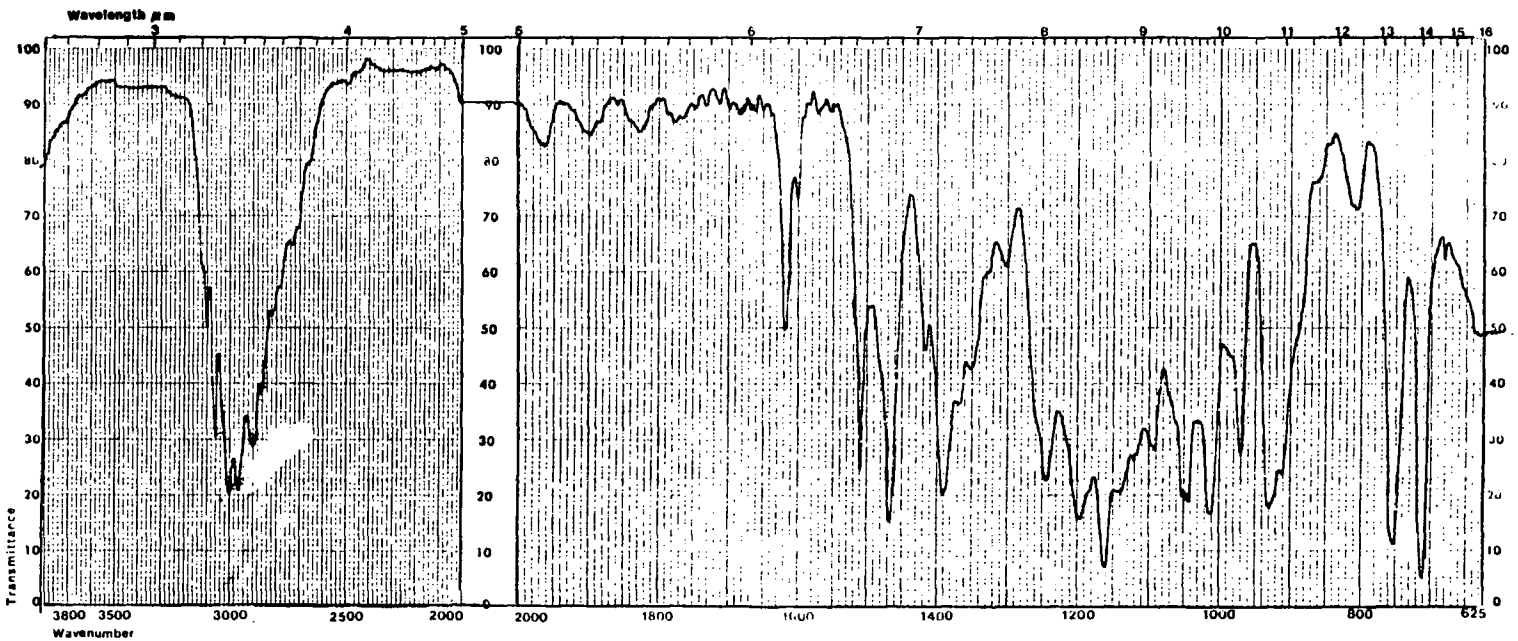
1.62. IR spectrum (film) of promethazine N-oxide (76)



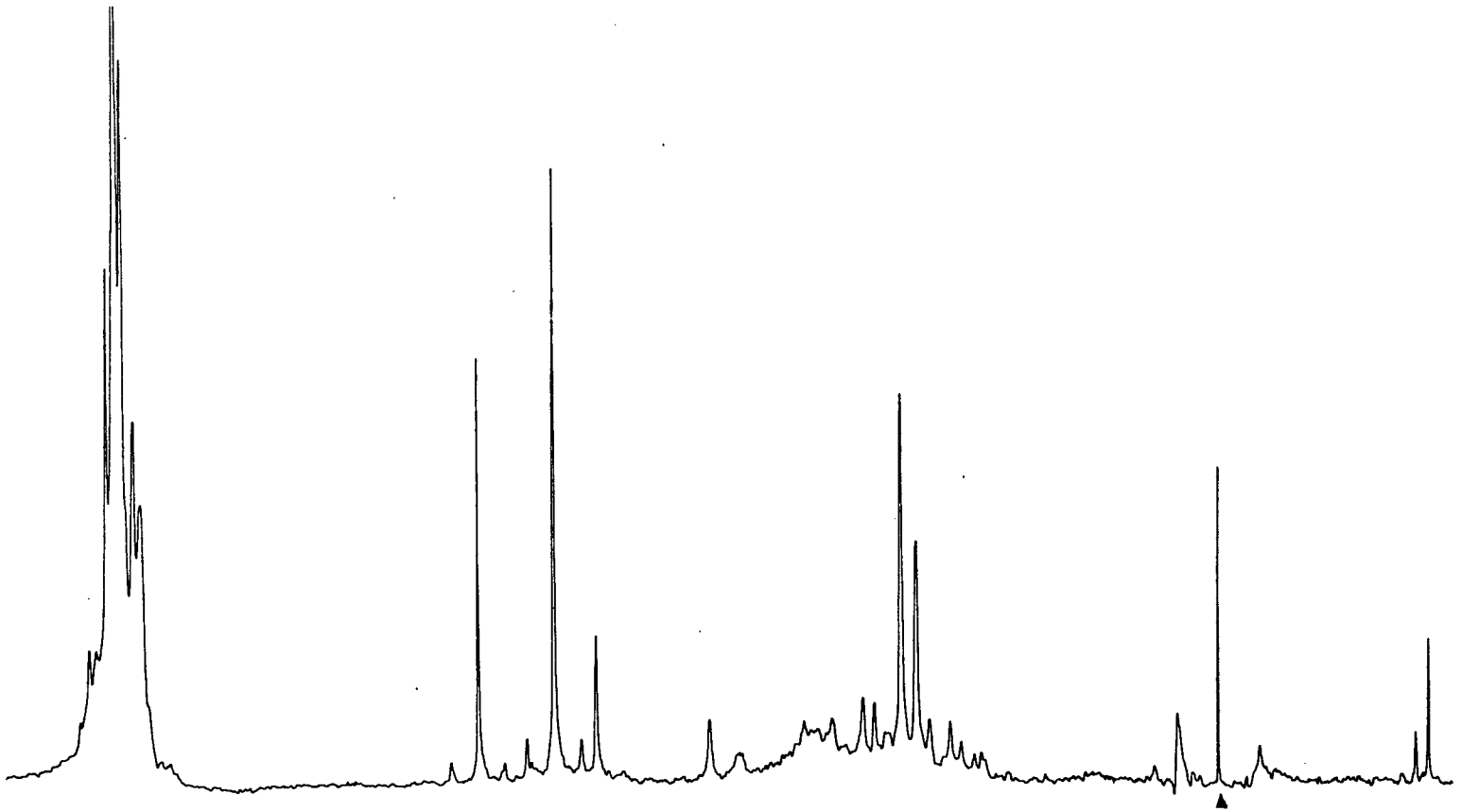
1.63. 80 MHz NMR spectrum of promethazine N-oxide (76)



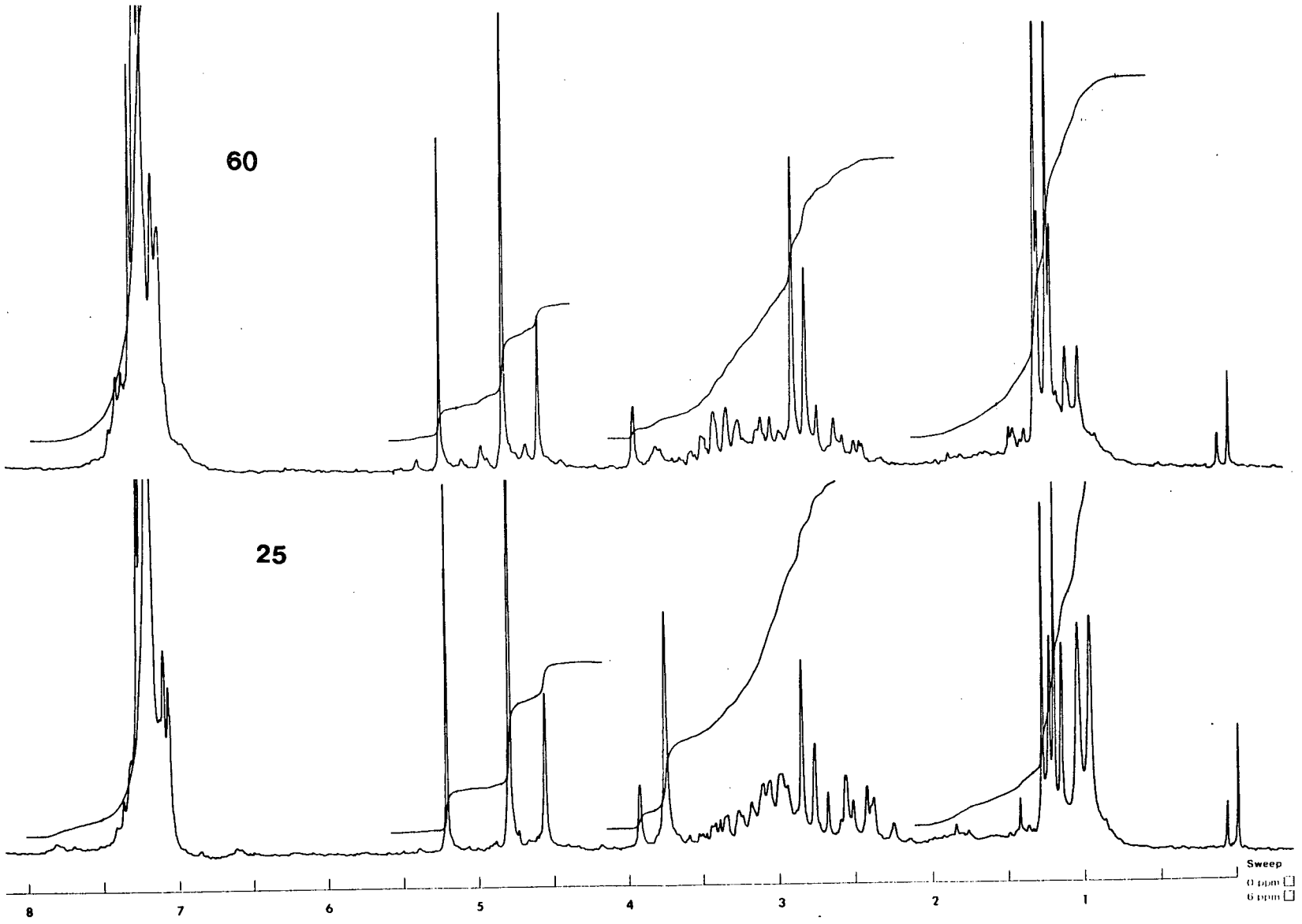
1.64. Mass spectrum of methylene imine (78)



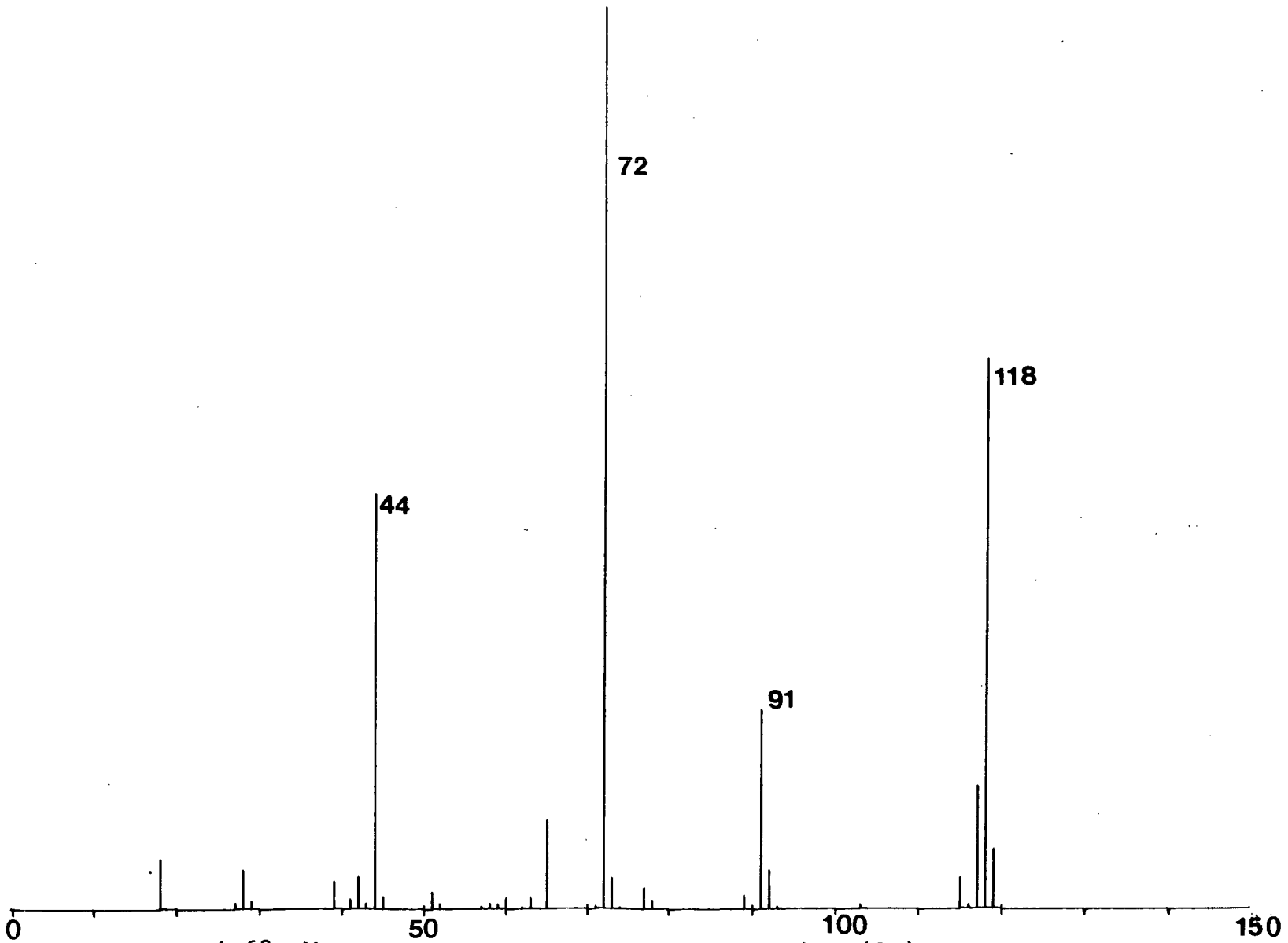
1.65. IR spectrum (film) of methylene imine (78)



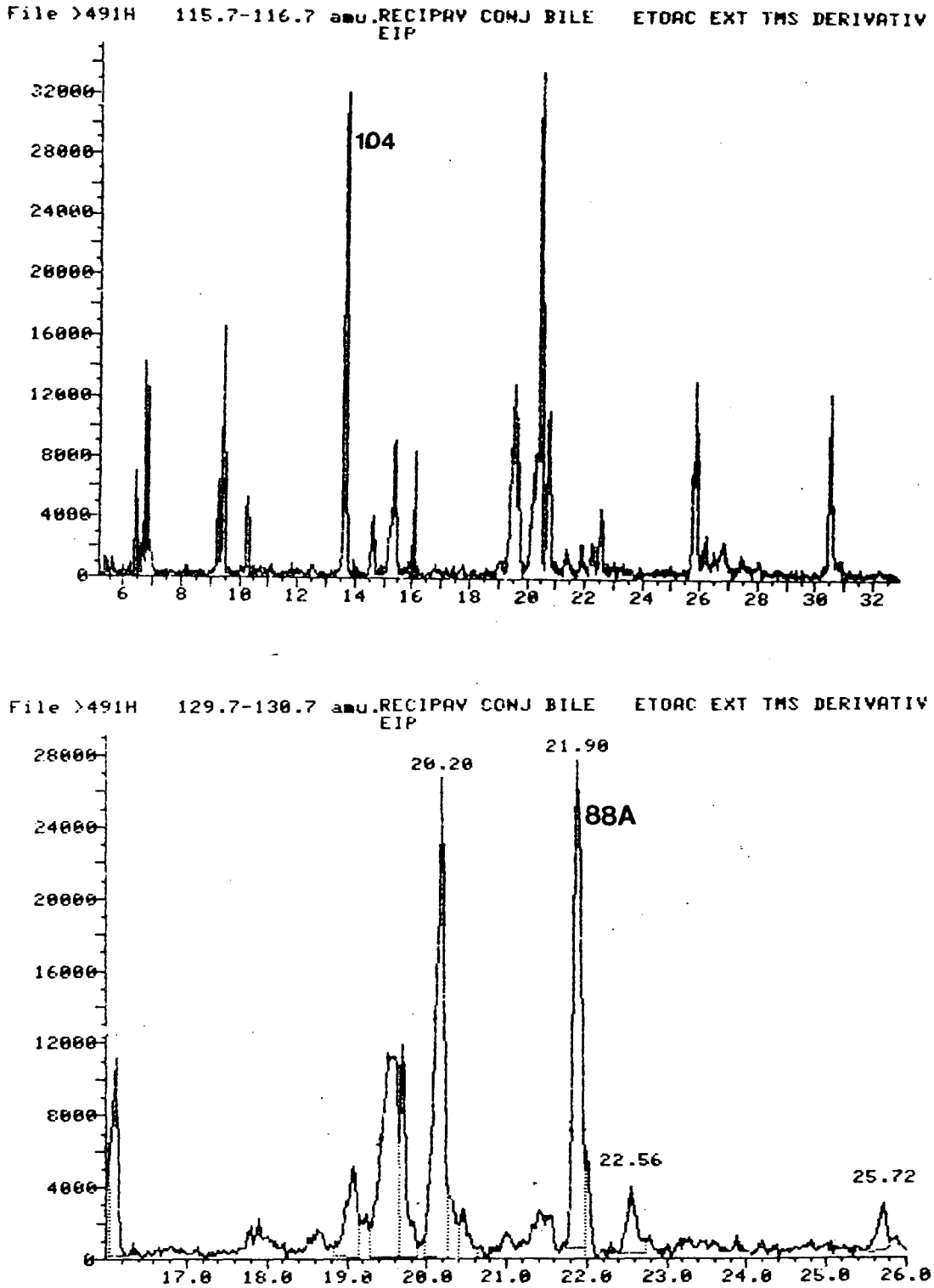
1.66. 80 MHz NMR spectrum of methylene imine (78)(decoupled at arrow)



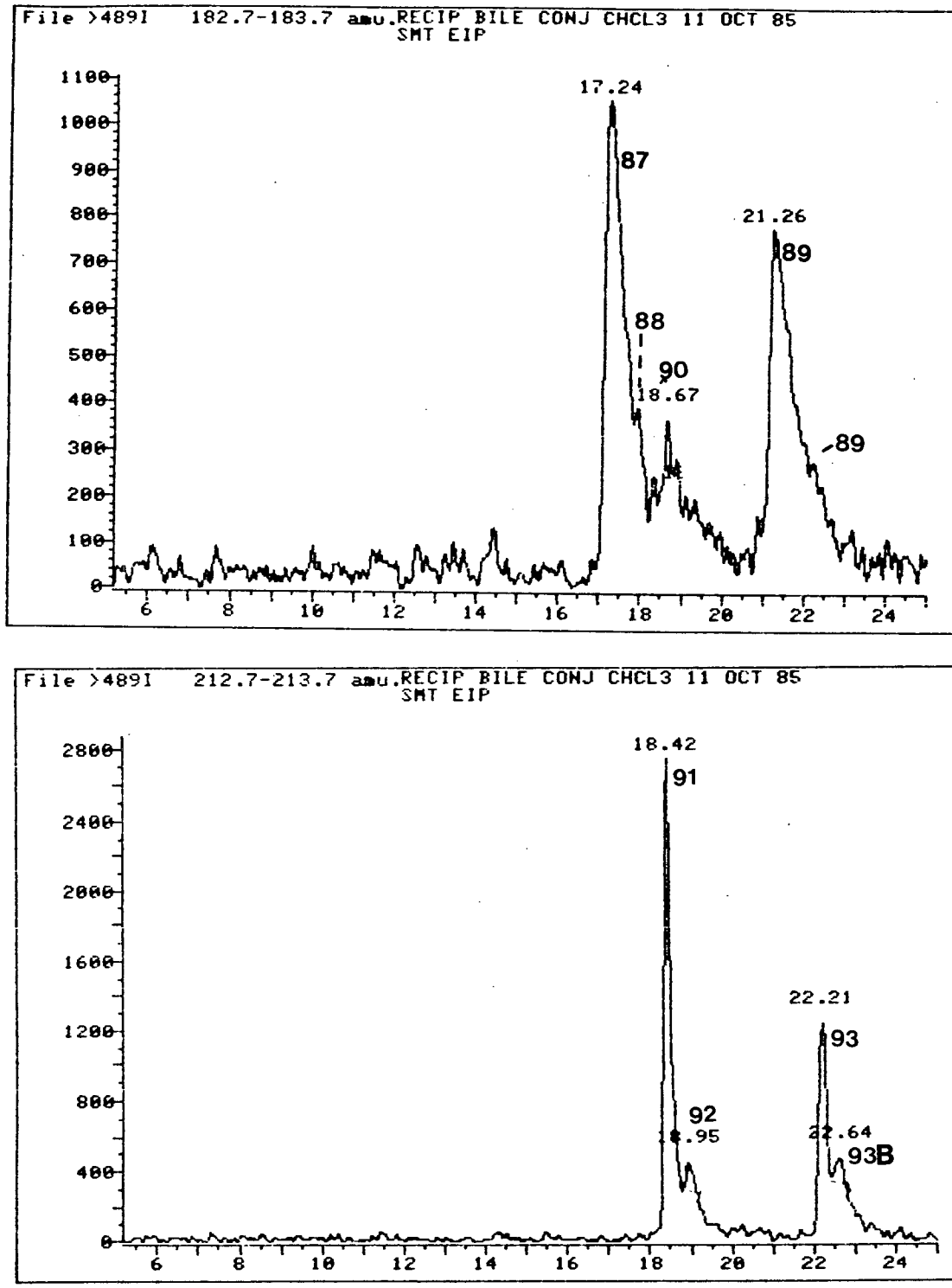
1.67. 400 MHz NMR spectrum of run at 25° (bottom) and at 60°



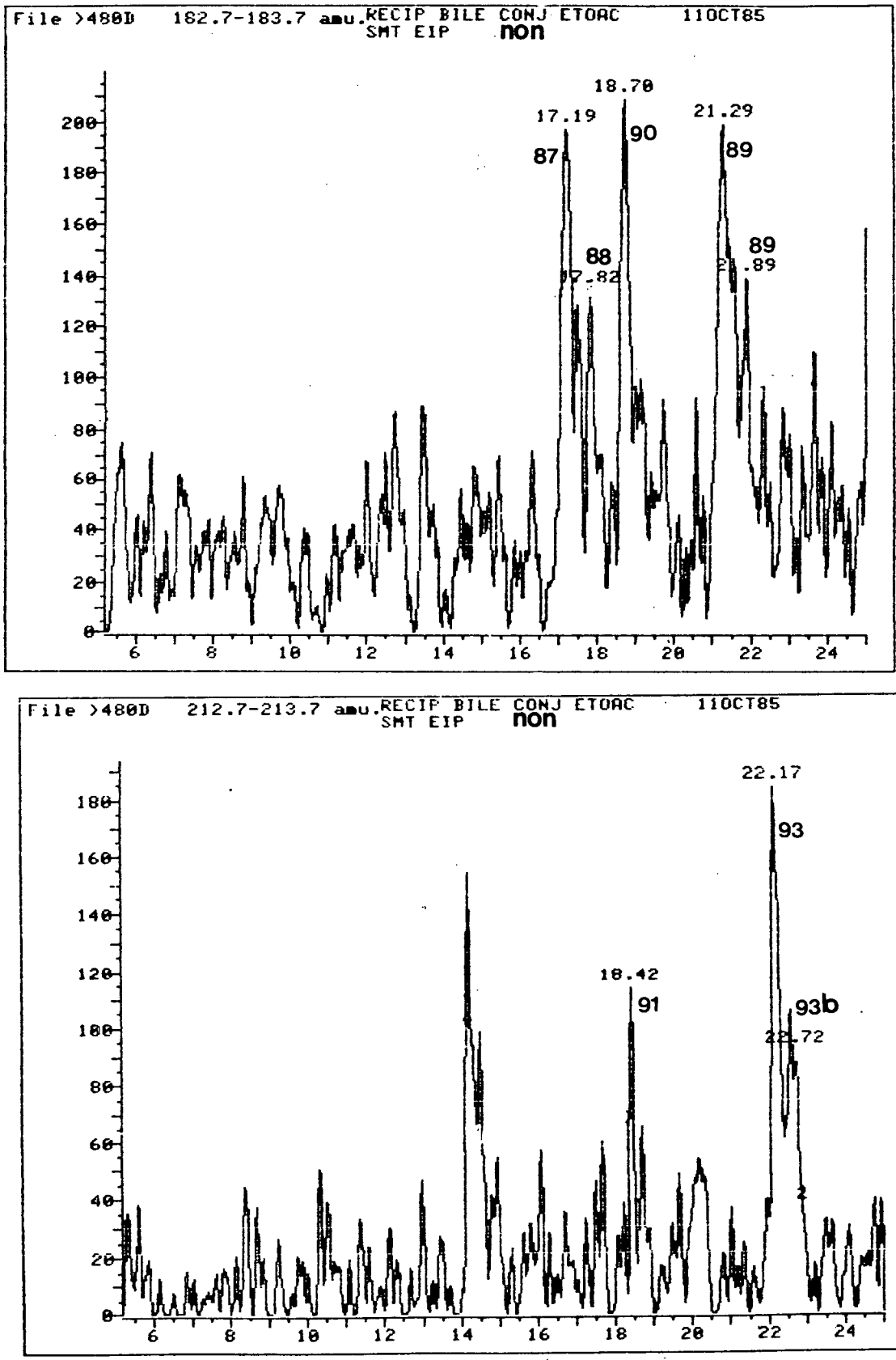
1.68. Mass spectrum of secondary formamide (83)



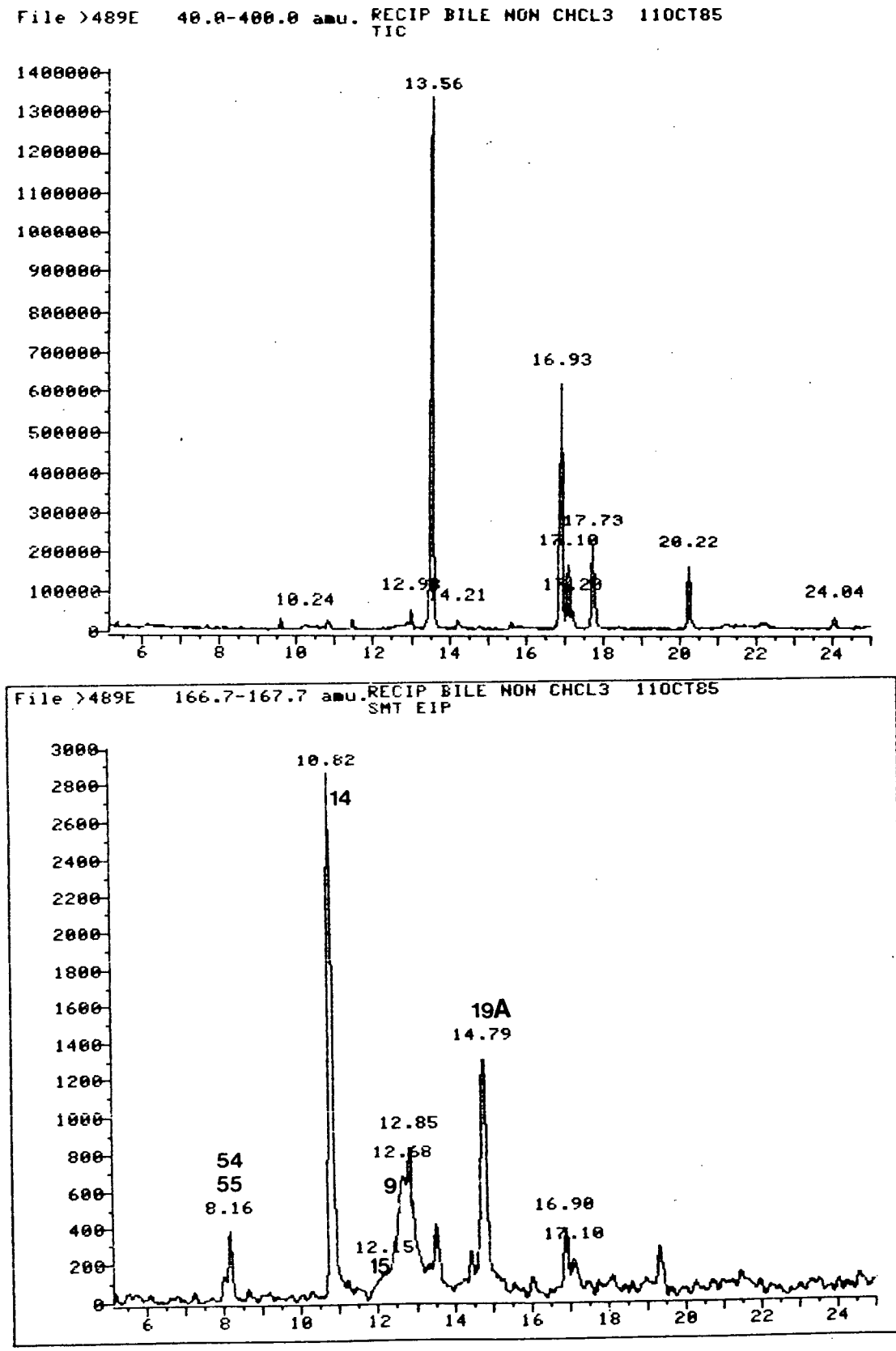
2.1. Mass chromatograms m/z 115 (top) and m/z 130 (bottom) used to locate dinorrecipavrin TMS and norrecipavrin TMS derivatives in the conjugated fraction of bile.



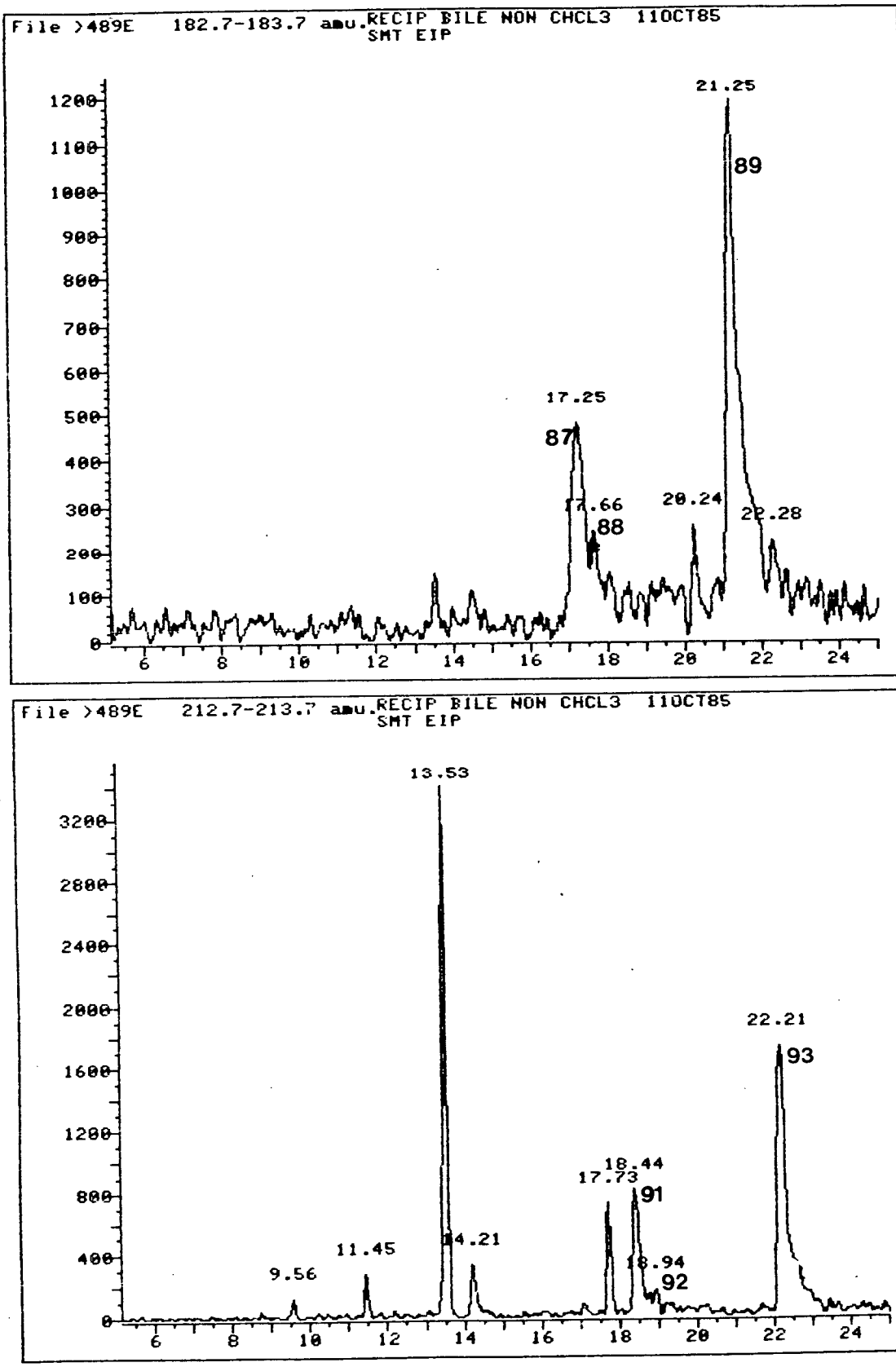
2.2. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the recipavrin conjugated bile chloroform extract.



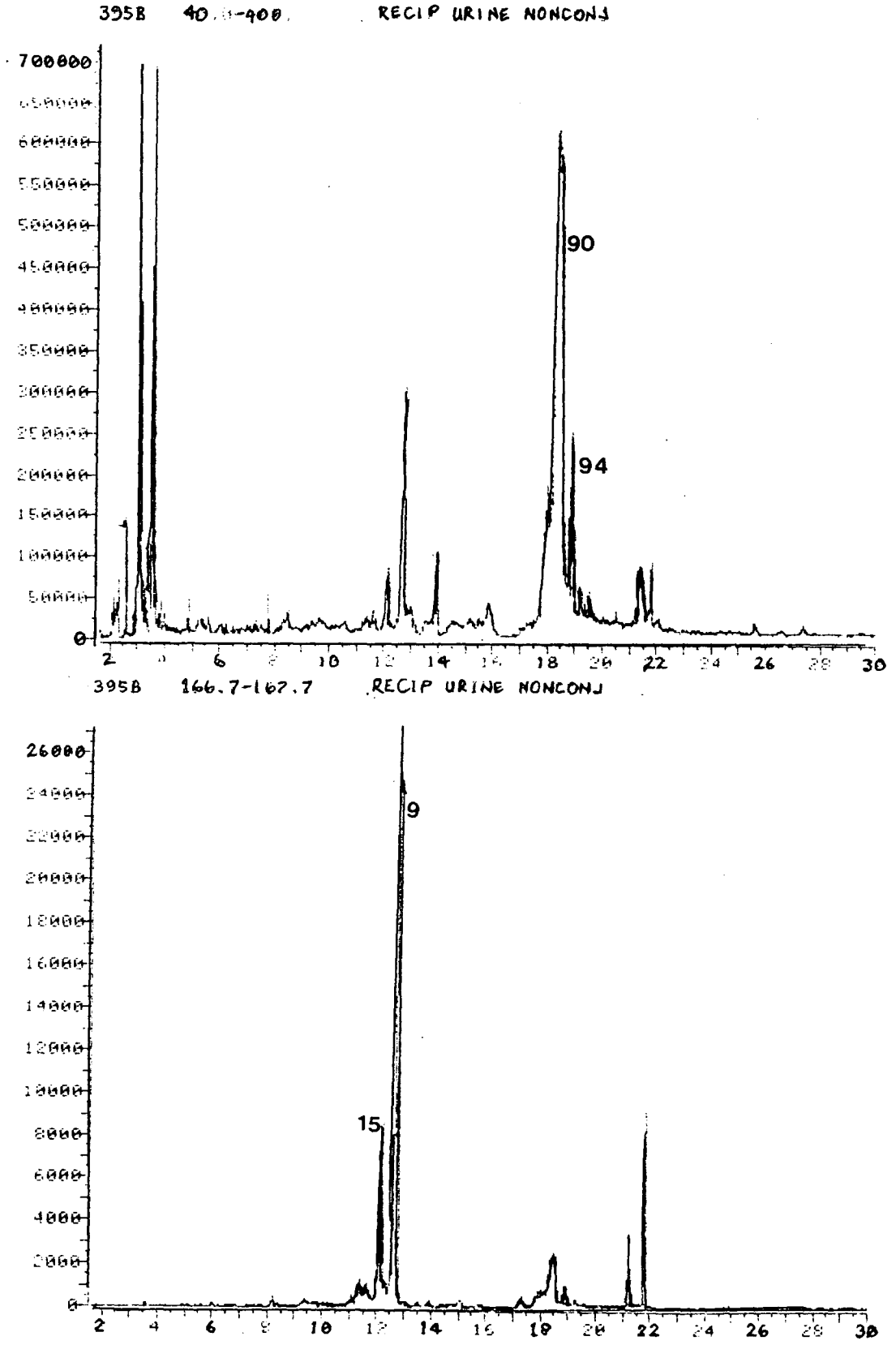
2.3. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the recipavrin nonconjugated bile ethyl acetate extract.



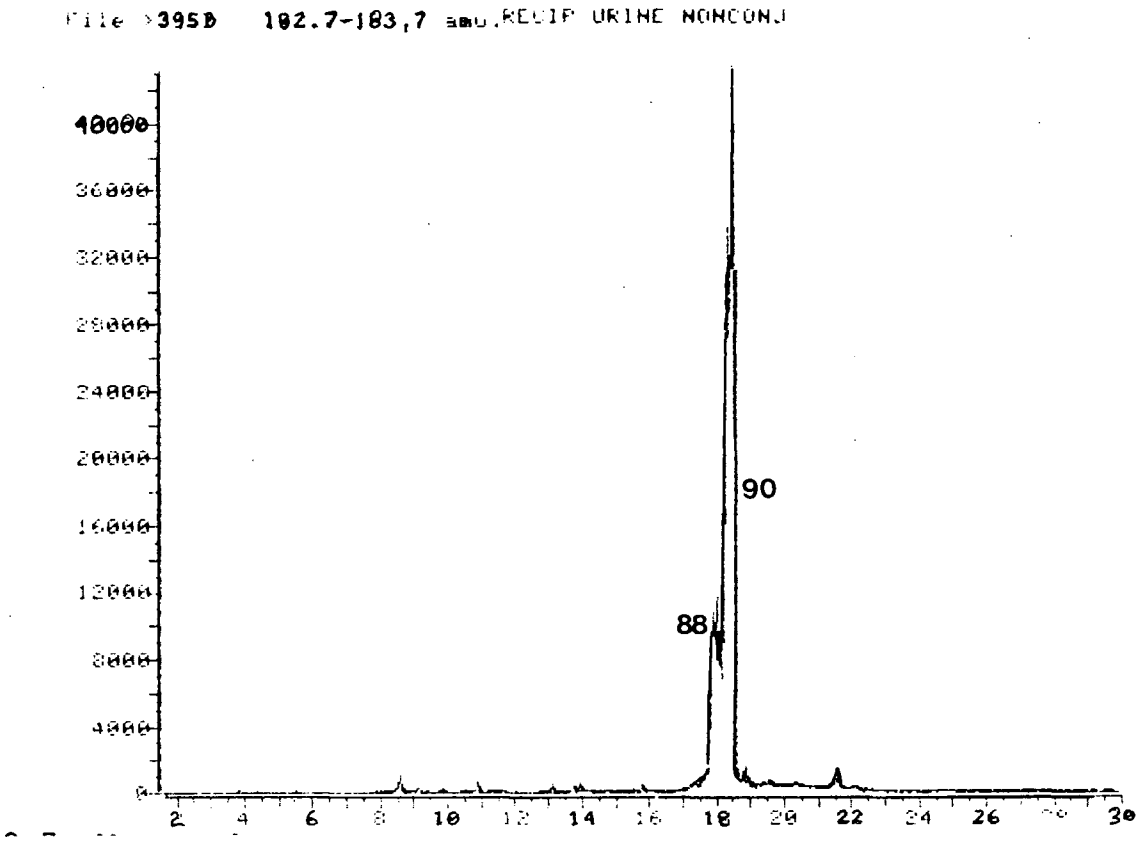
2.4. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the recipavrin nonconjugated bile chloroform extract.



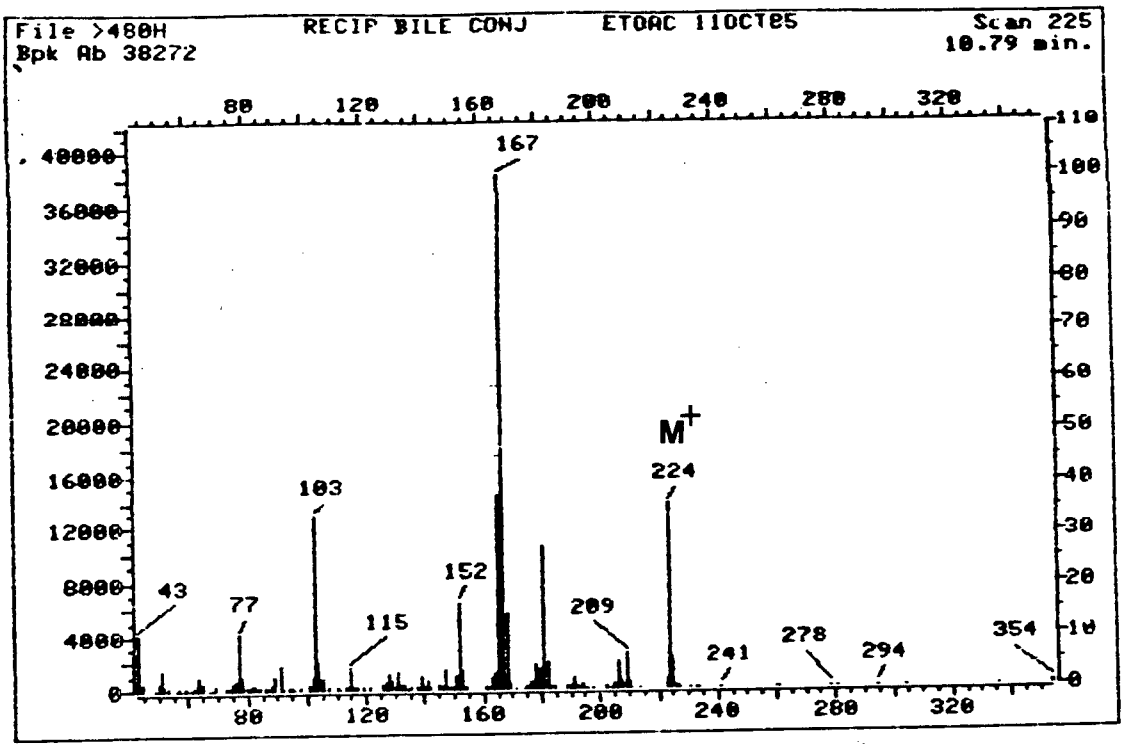
2.5. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the recipavrin nonconjugated bile chloroform extract.

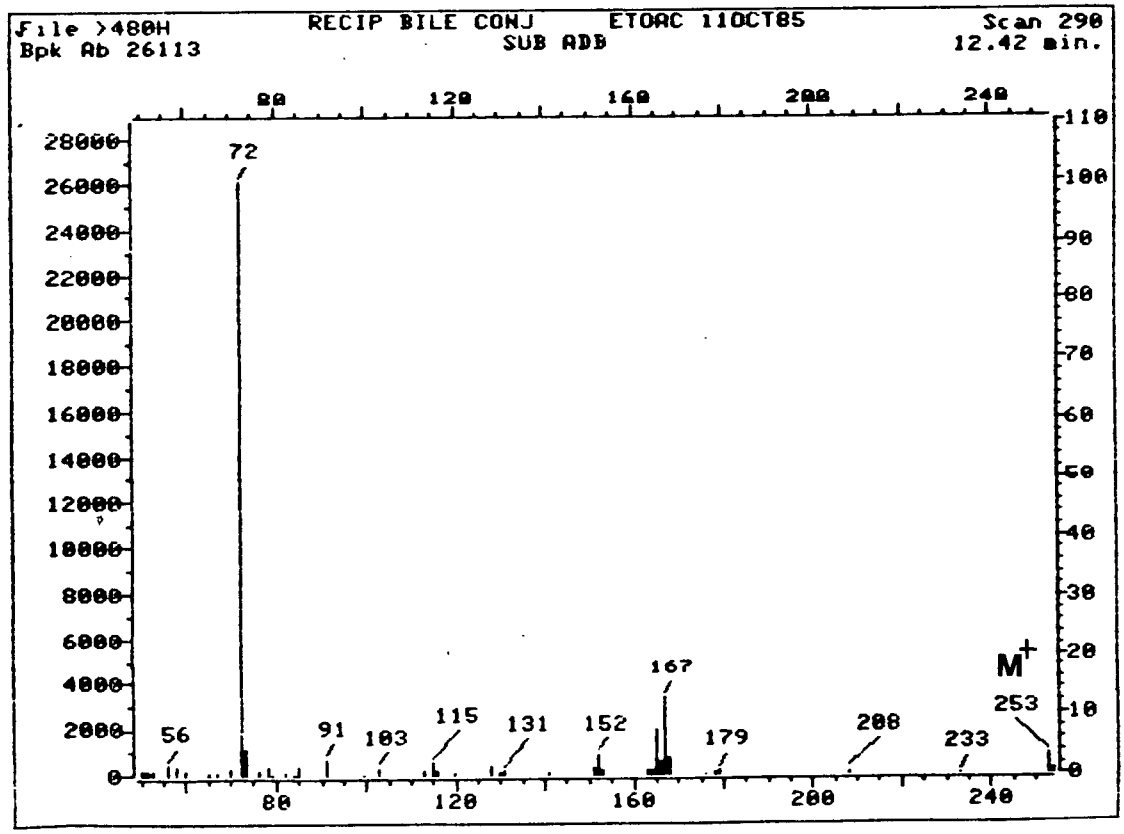


2.6. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the recipavrin nonconjugated urine ethyl acetate extract.

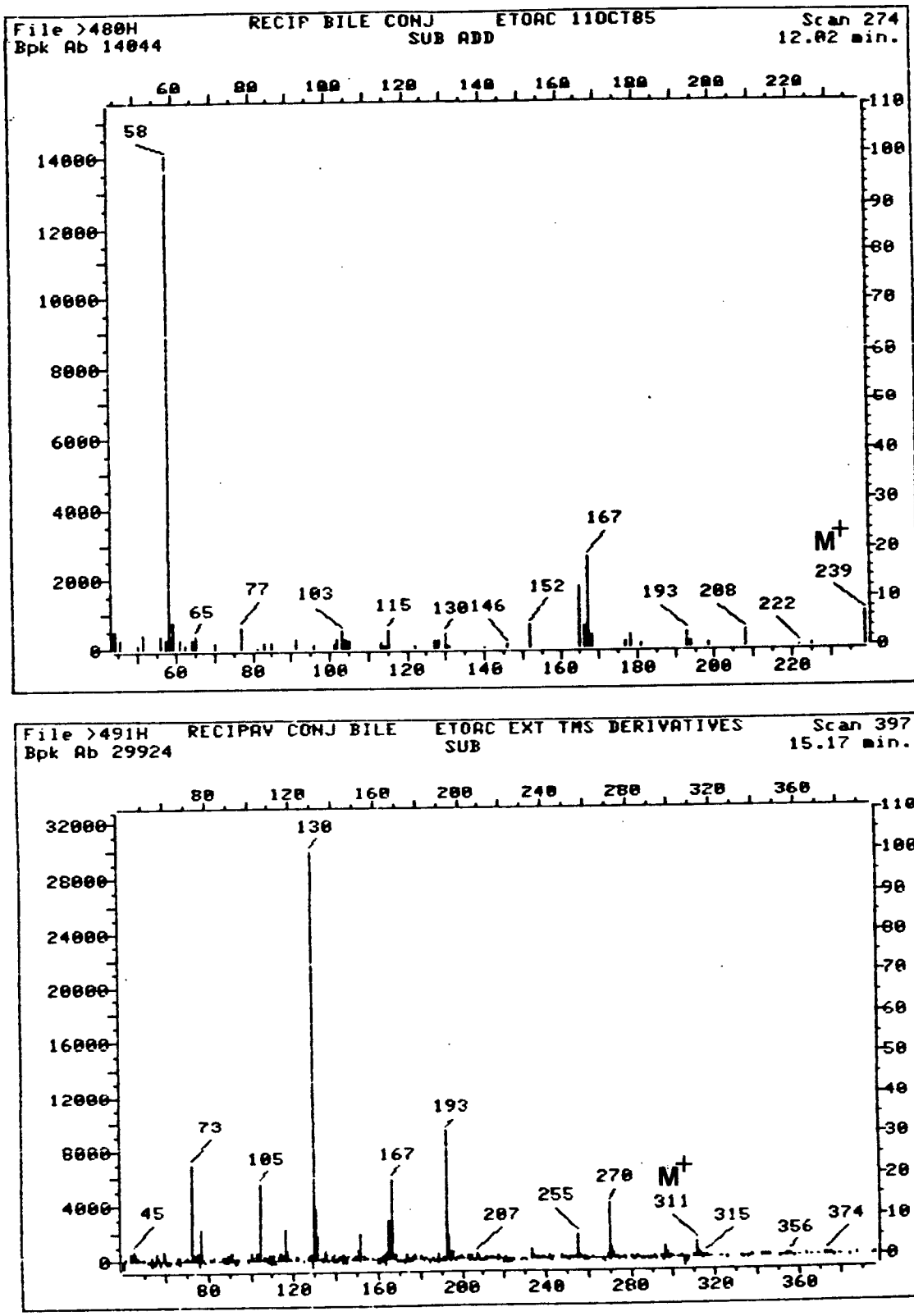


2.7. Mass chromatogram m/z 183 (top) for the recipavrin nonconjugated urine ethyl acetate extract, GC conditions D. No intensity at m/z 213.

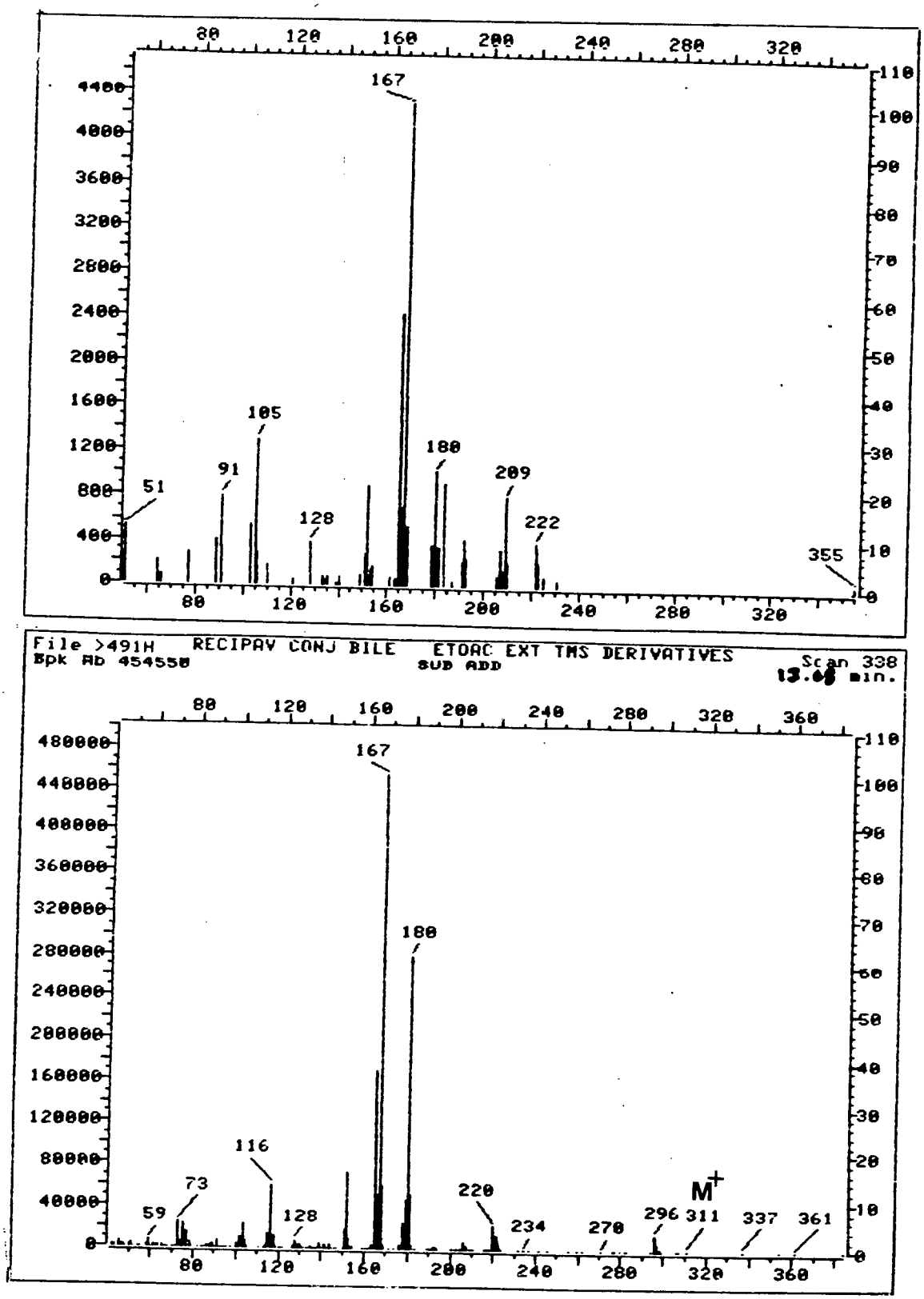




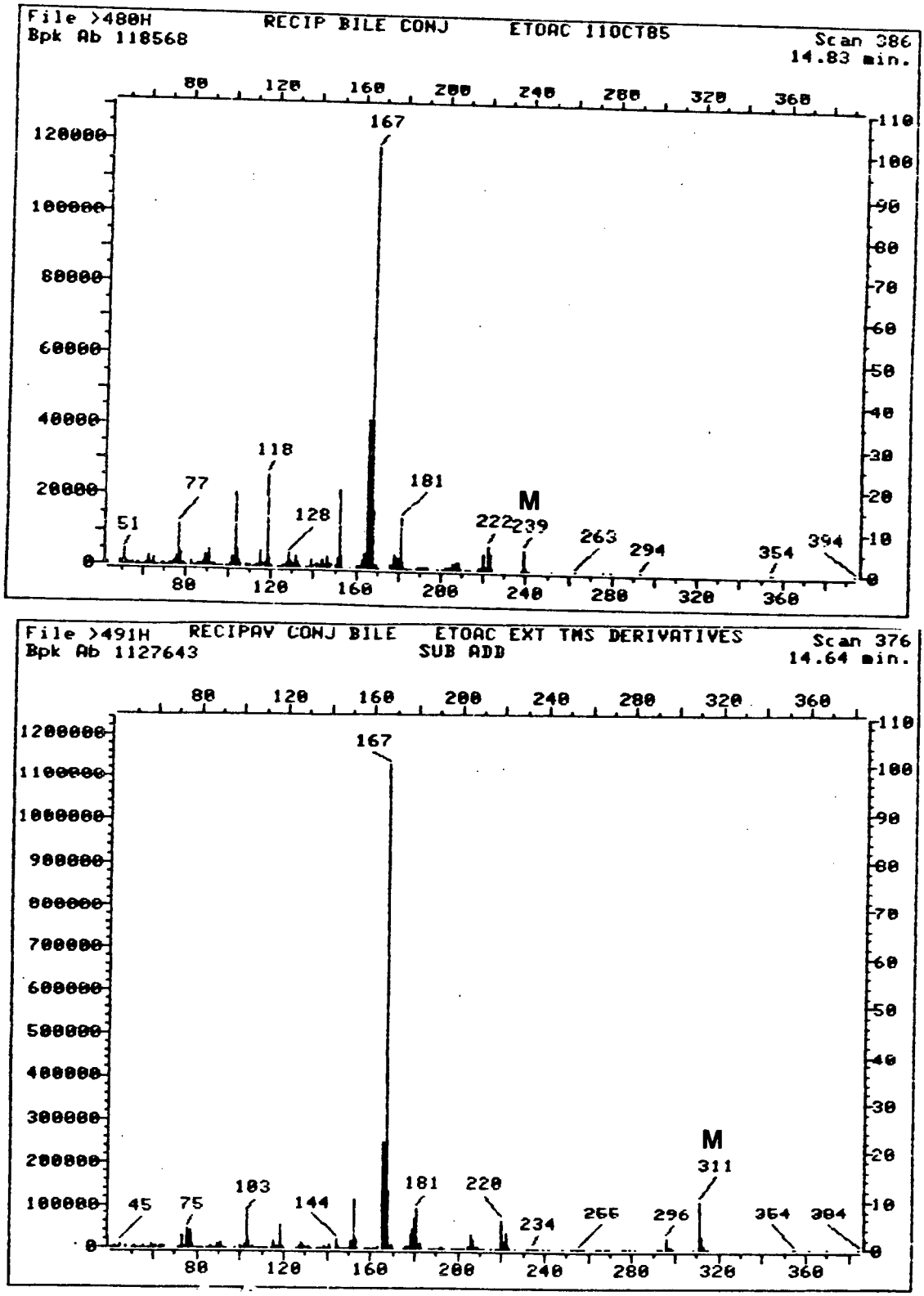
2.8. Mass spectra of recipavrin metabolites and their derivatives: (top) diphenylbutanone (14). (bottom) recipavrin (9).



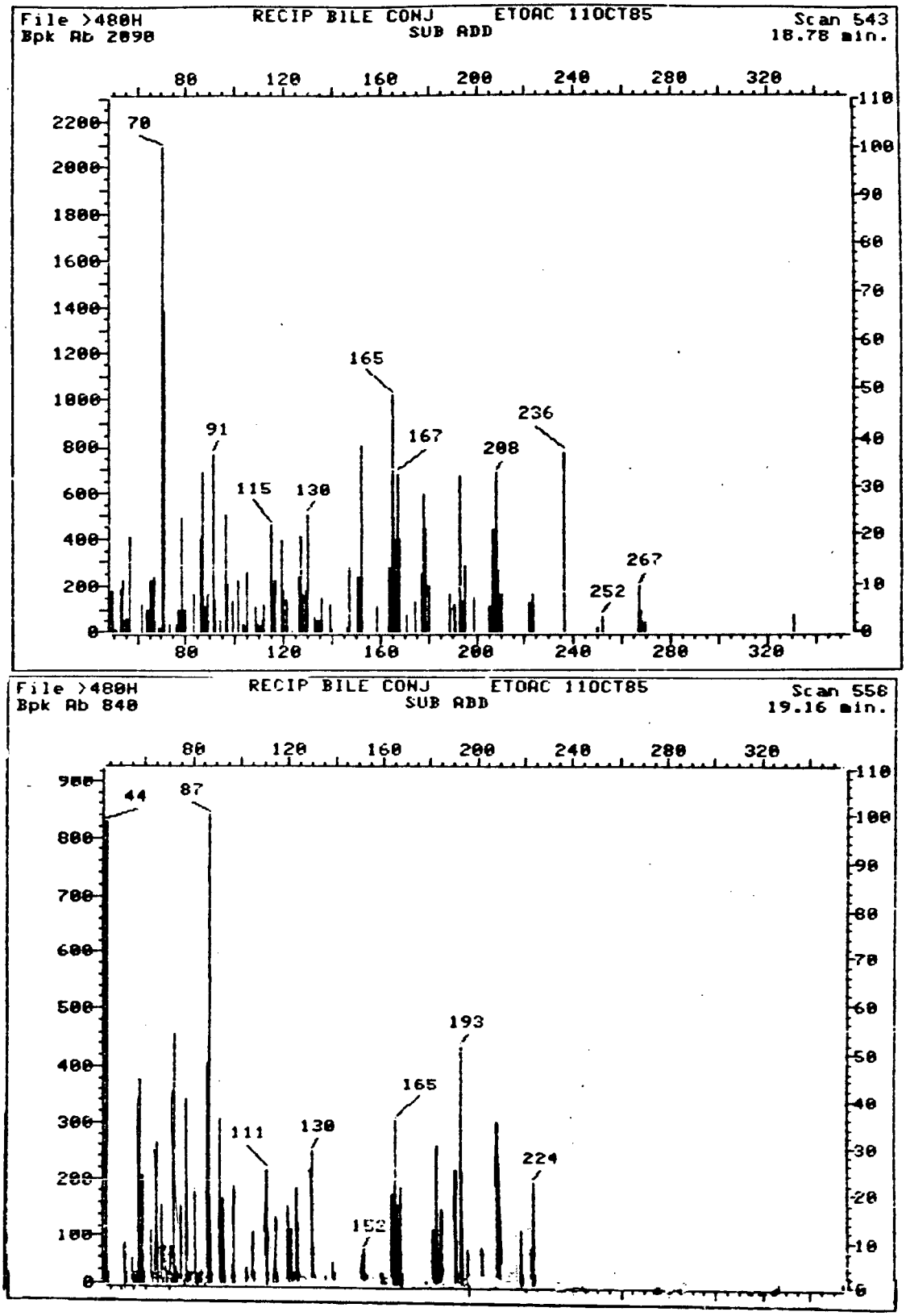
2.9. Mass spectra of recipavrin metabolites and their derivatives: (top) norrecipavrin (15). (bottom) norrecipavrin TMS (114).



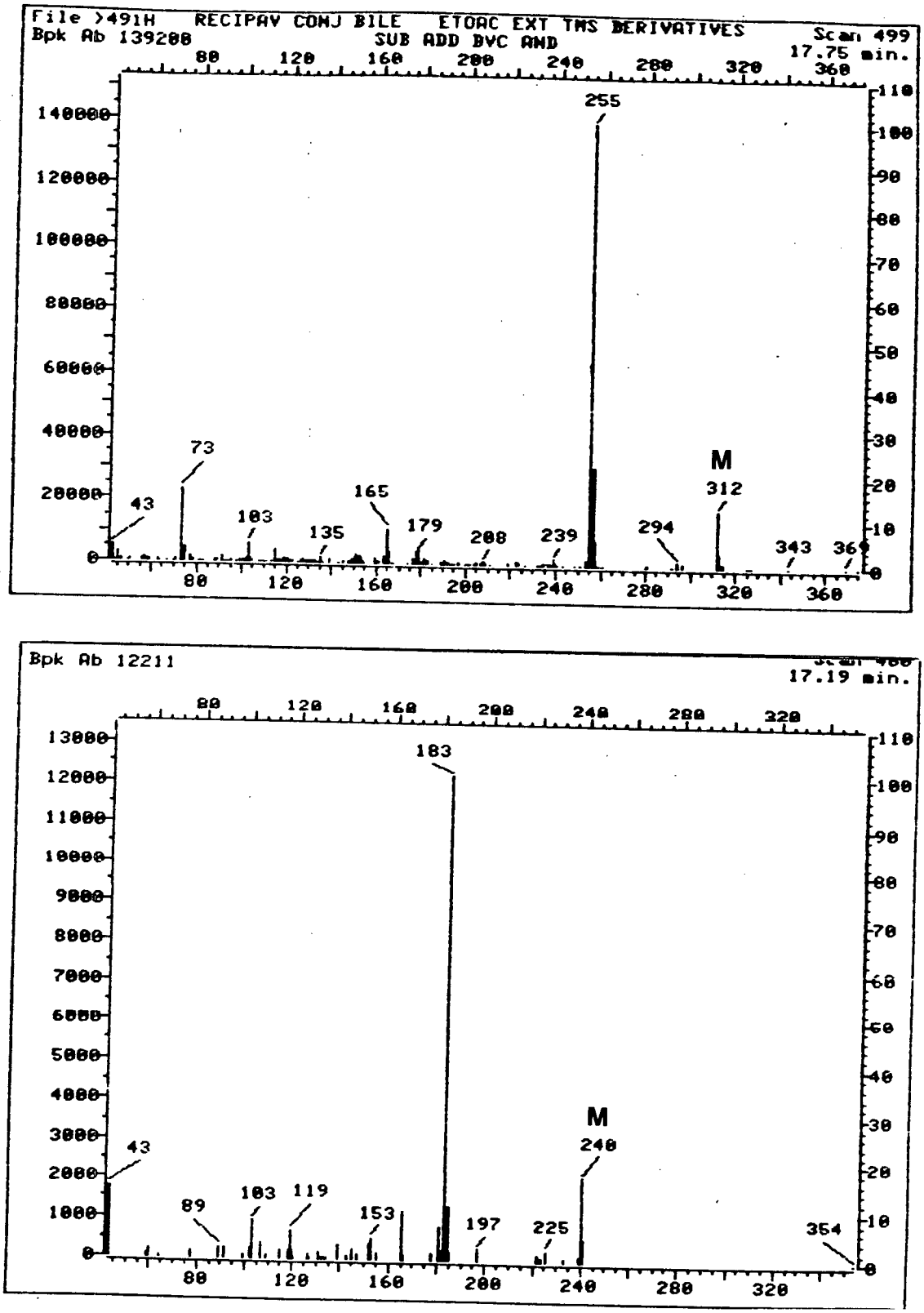
2.10. Mass spectra of recipavrin metabolites and their derivatives: (top) syn-oxime (19). (bottom) syn-oxime TMS (52).



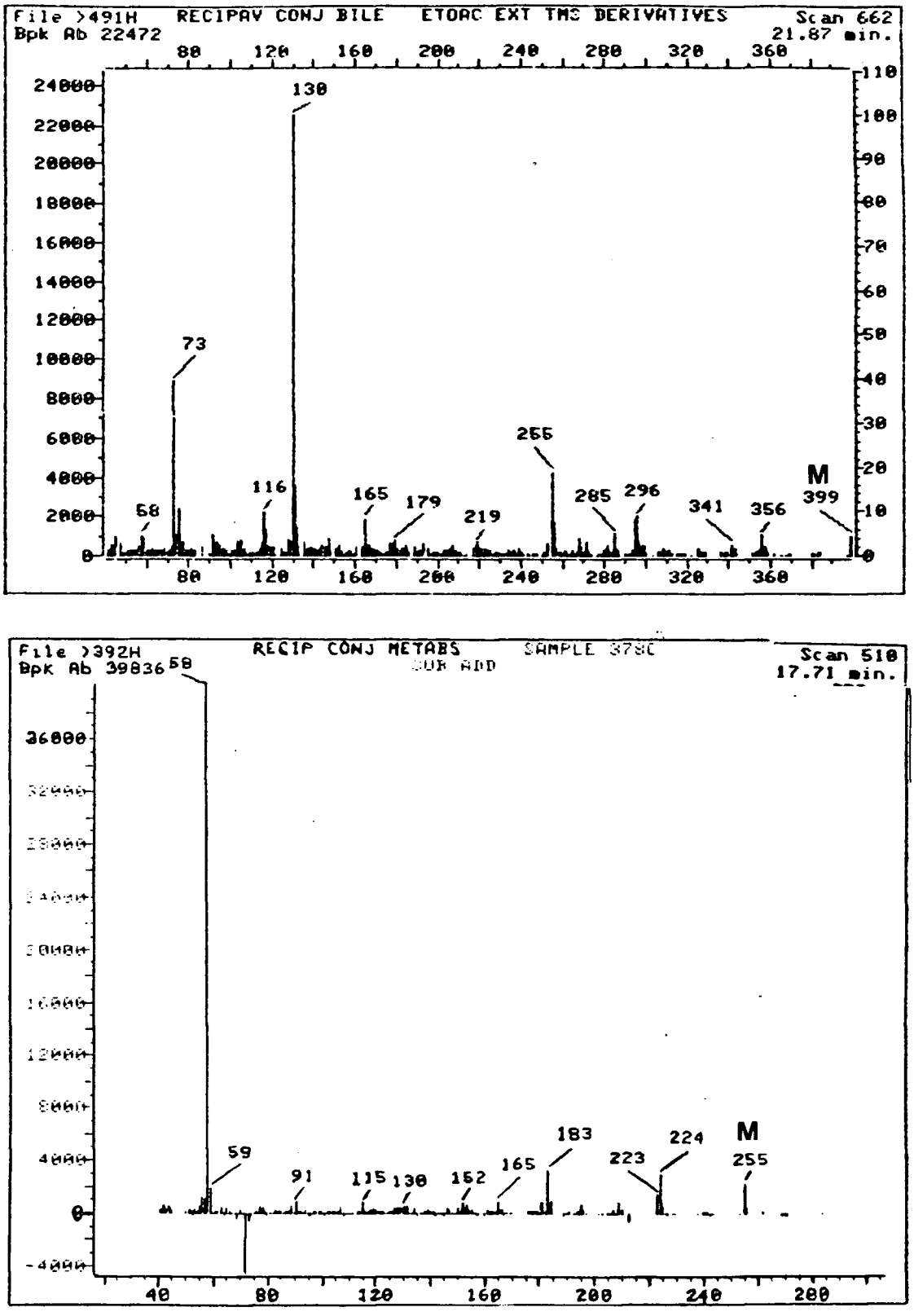
2.11. Mass spectra of recipavrin metabolites and their derivatives: (top) anti-oxime (19a). (bottom) anti-oxime TMS (52a)



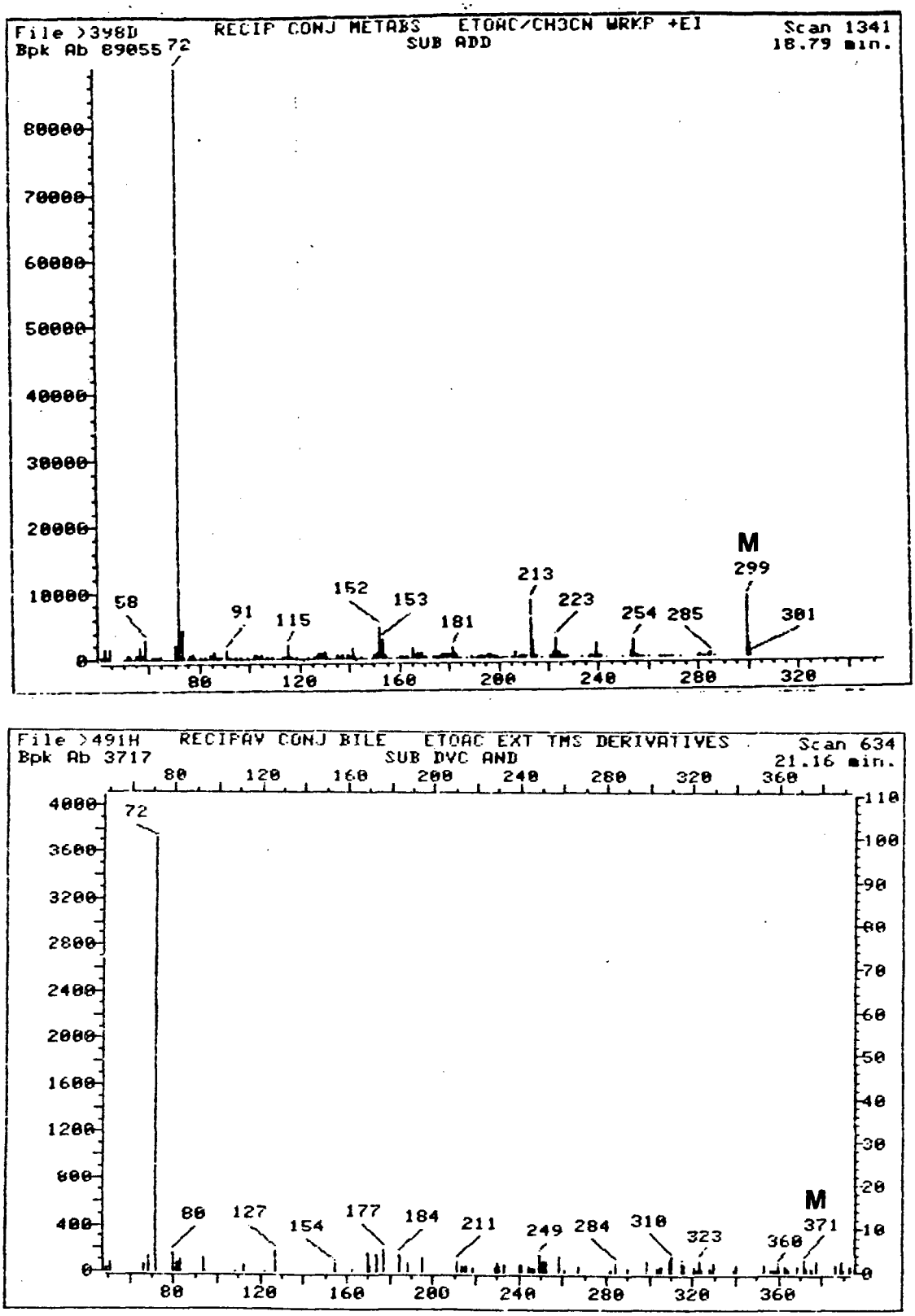
2.12. Mass spectra of recipavrin metabolites and their derivatives: (top) Unidentified metabolite (86). (bottom) acetamide (63).



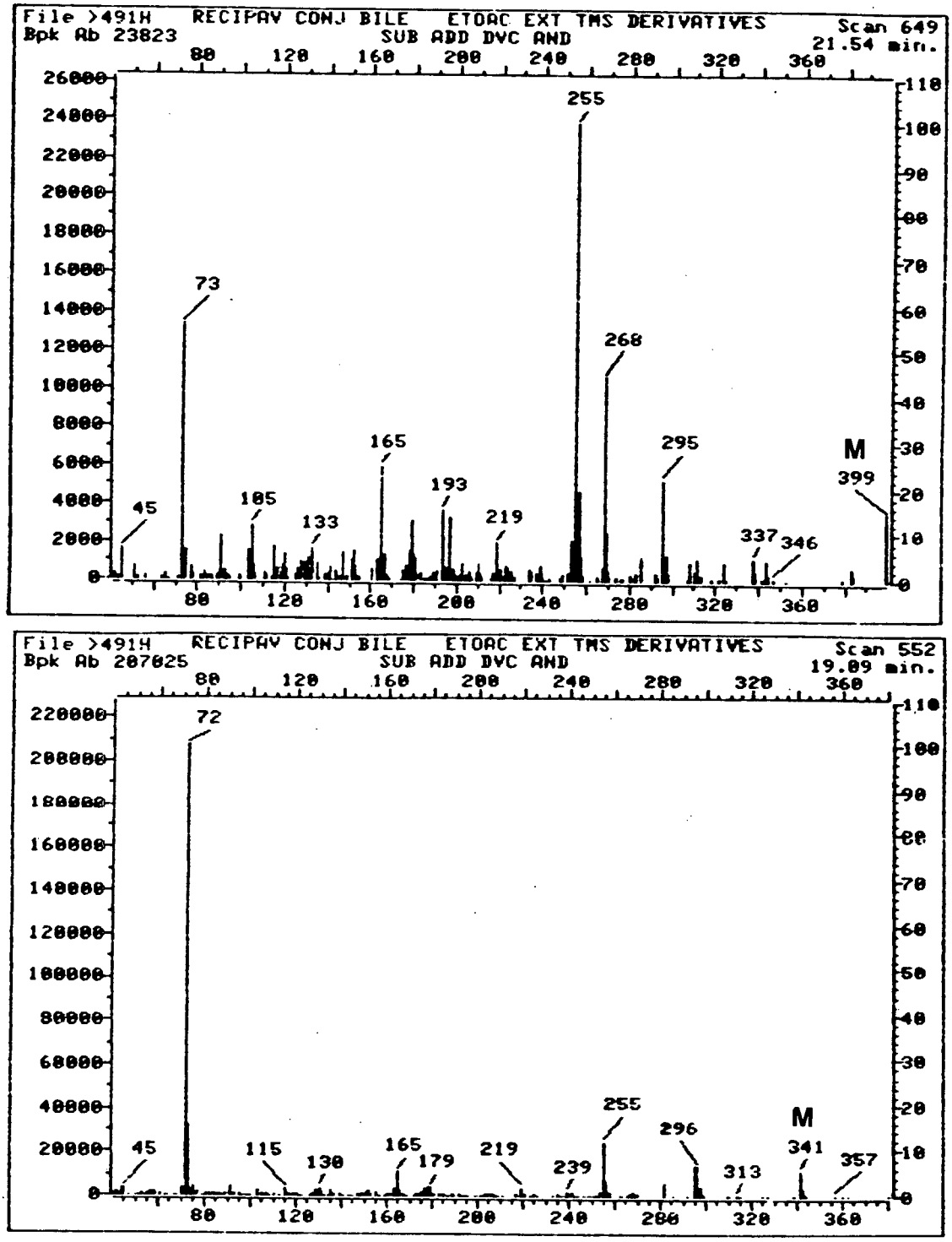
2.13. Mass spectra of recipavrin metabolites and their derivatives: (top) diphenylbutanone phenol TMS (87a). (bottom) diphenylbutanone phenol (87).



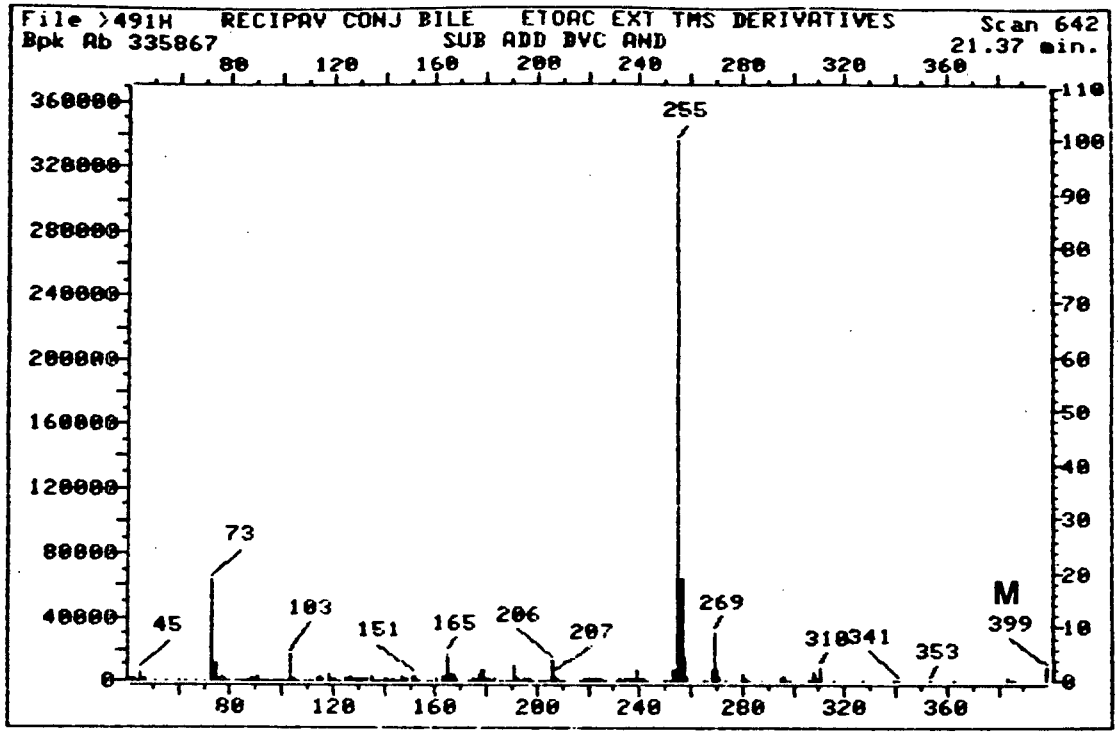
2.14. Mass spectra of recipavrin metabolites and their derivatives: (top) norrecipavrin phenol di-TMS (88a). (bottom) .norrecipavrin phenol (88).

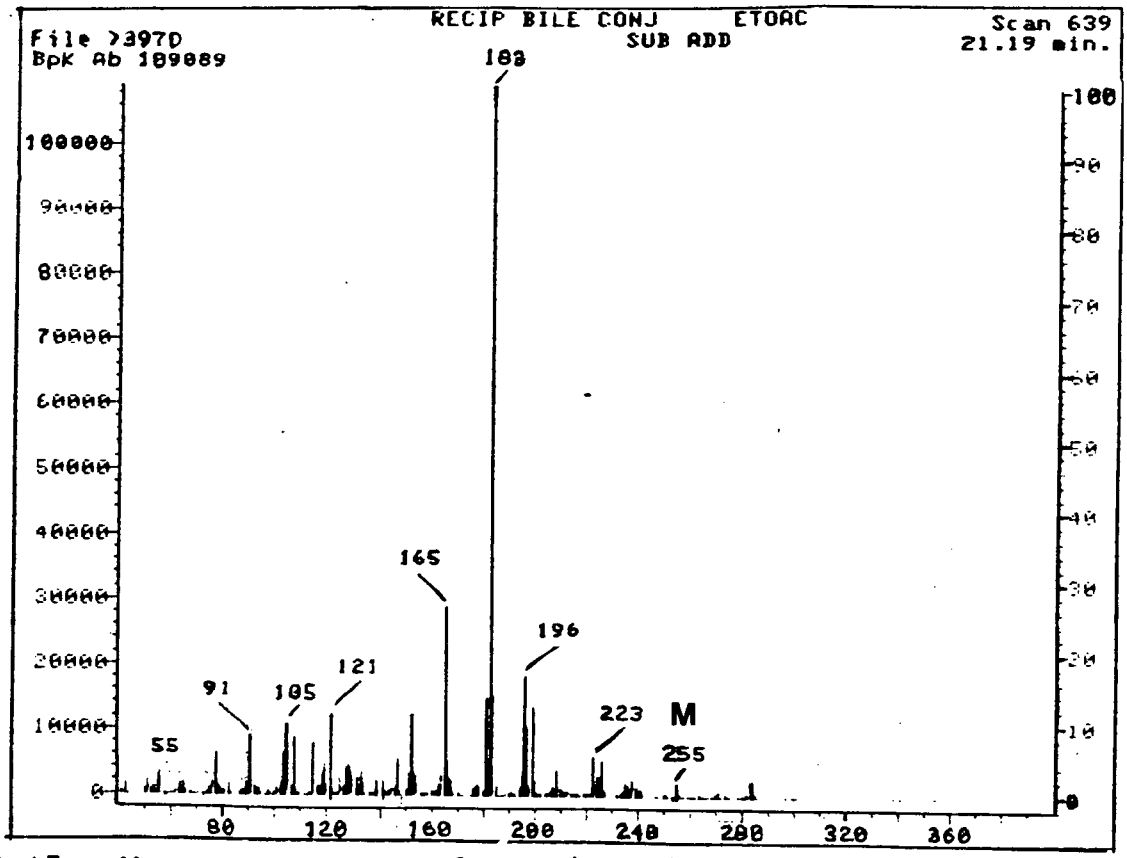


2.15. Mass spectra of recipavrin metabolites and their derivatives: (top) recipavrin O-methylcatechol (94). (bottom) recipavrin O-methylcatechol TMS (94a).

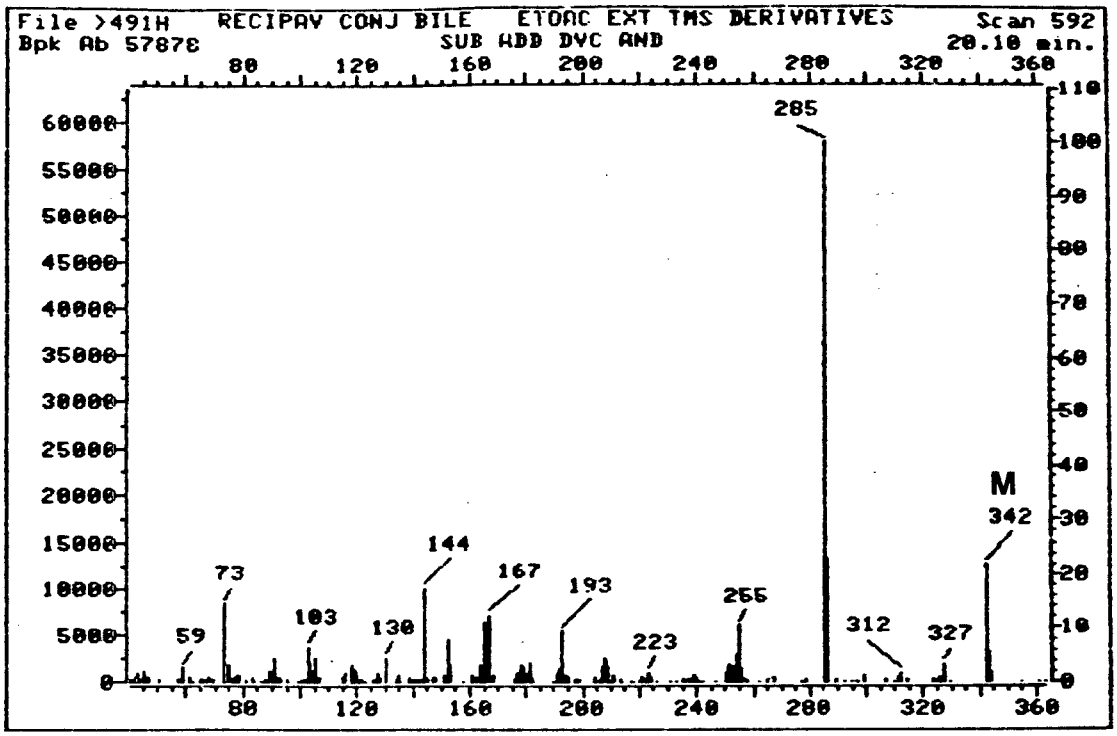


2.16. Mass spectra of recipavrin metabolites and their derivatives: (top) syn-oxime phenol di-TMS (89c). (bottom) recipavrin phenol TMS (90a).

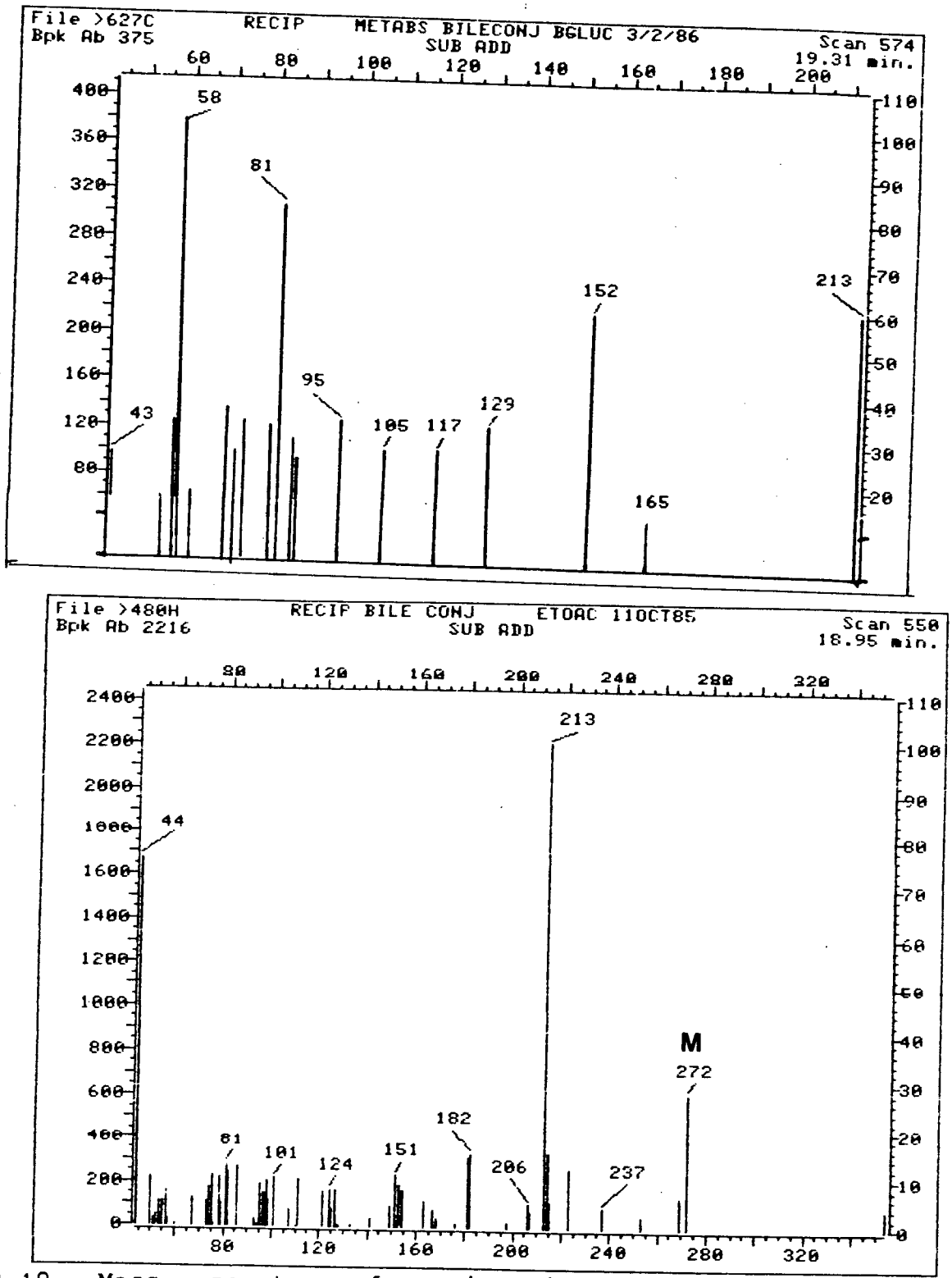




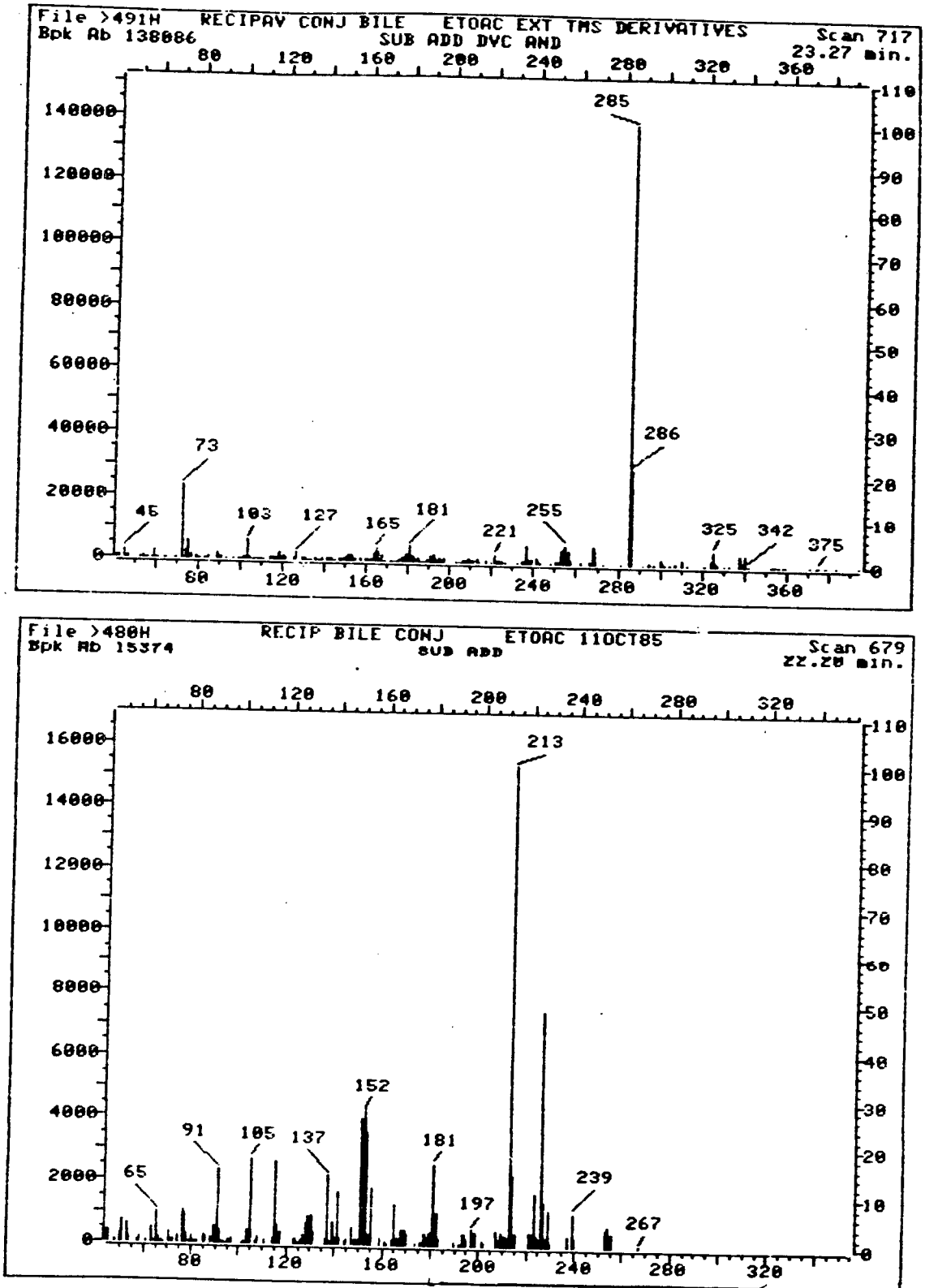
2.17. Mass spectra of recipavrin metabolites and their derivatives: (top) anti-oxime phenol di-TMS (89a). (bottom) anti-oxime phenol (89).



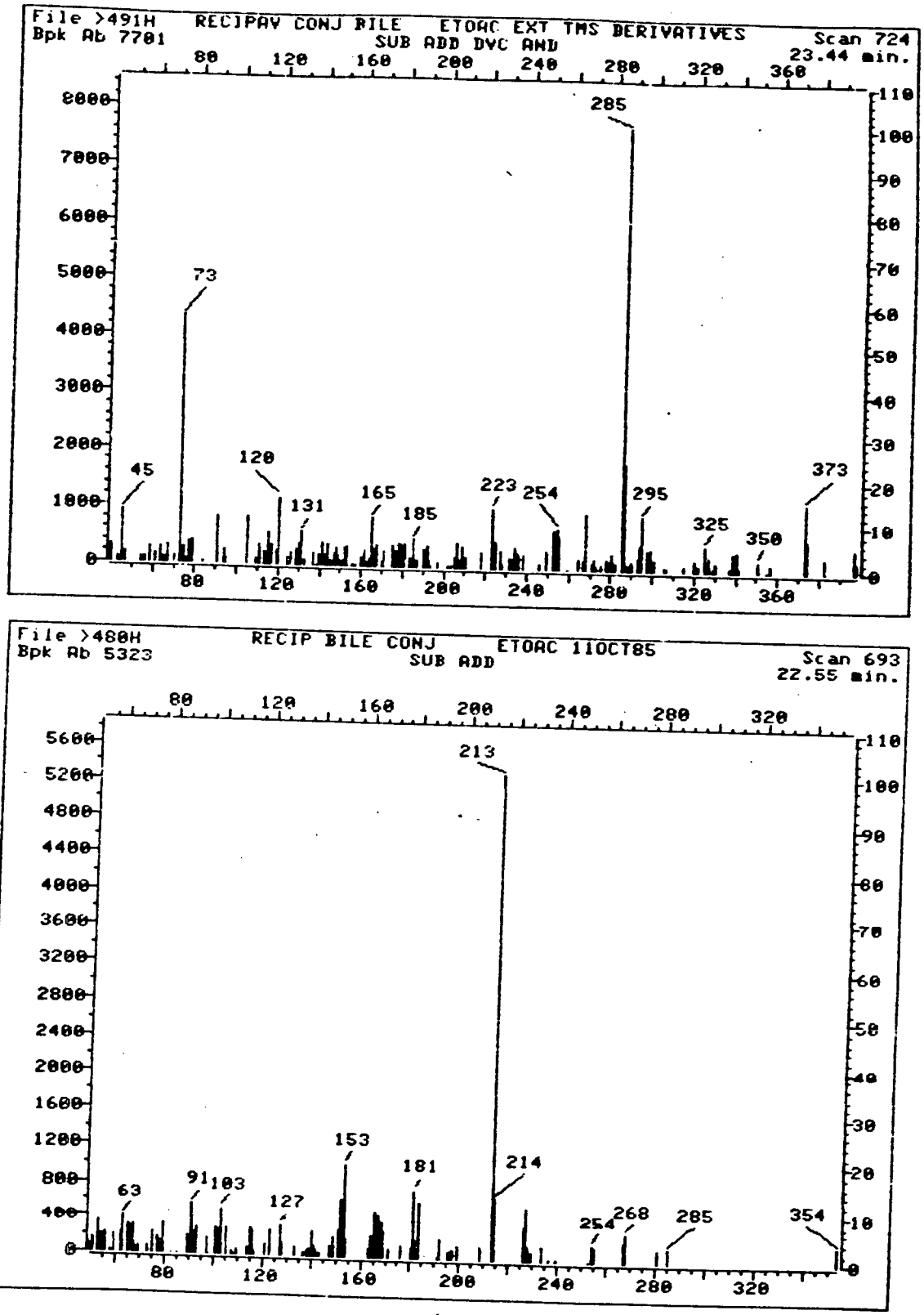
2.18. Mass spectra of recipavrin metabolites and their derivatives: (top) diphenylbutanone O-methylcatechol TMS (91a). (bottom) diphenylbutanone O-methylcatechol (91).



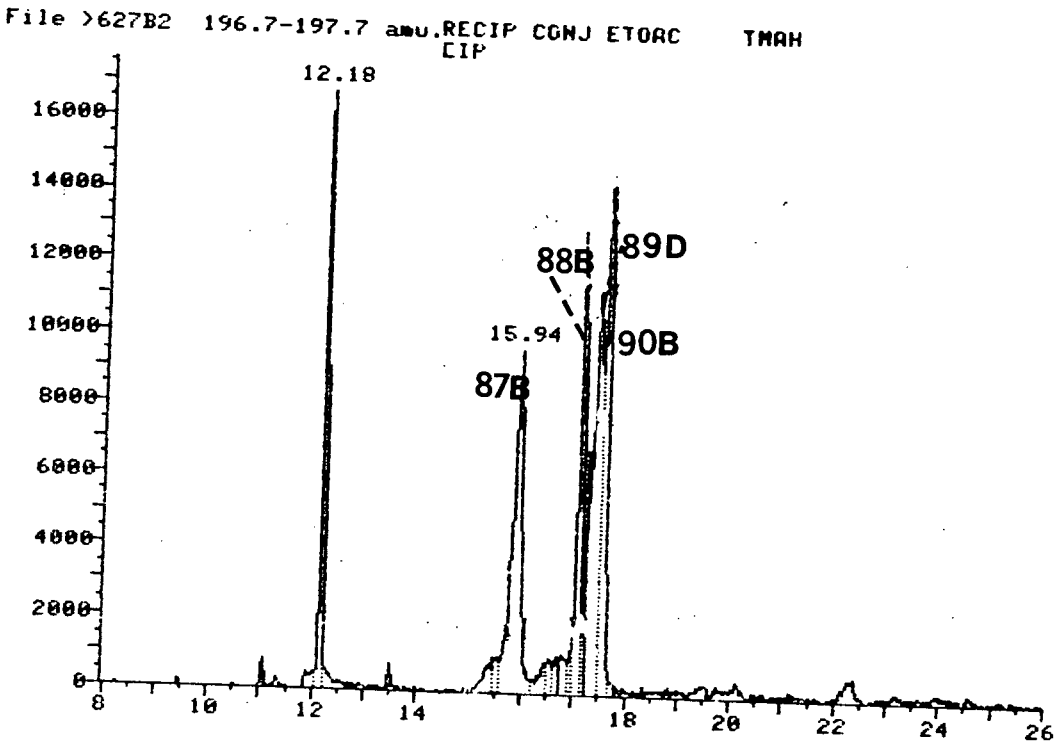
2.19. Mass spectra of recipavrin metabolites and their derivatives: (top) suspected norrecipavrin O-methylcatechol (115).(bottom) diphenylbutanol O-methylcatechol (92).

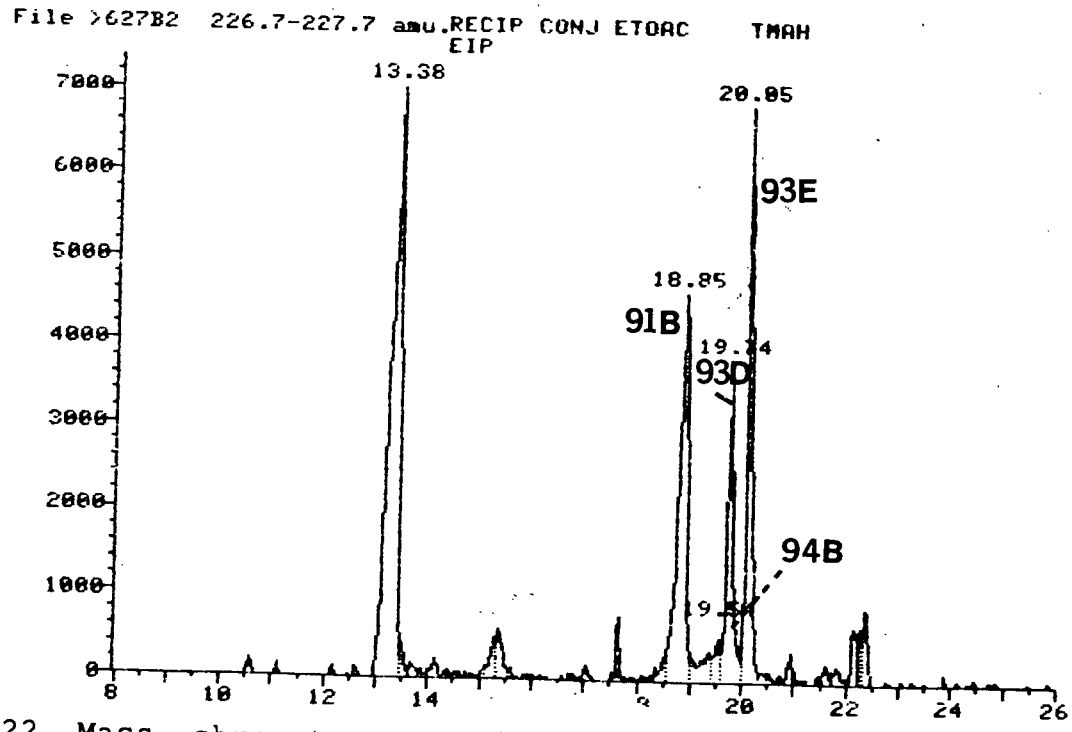


2.20. Mass spectra of recipavrin metabolites and their derivatives: (top) anti-oxime O-methylcatechol di-TMS (93a). (bottom) anti-oxime O-methylcatechol (93)

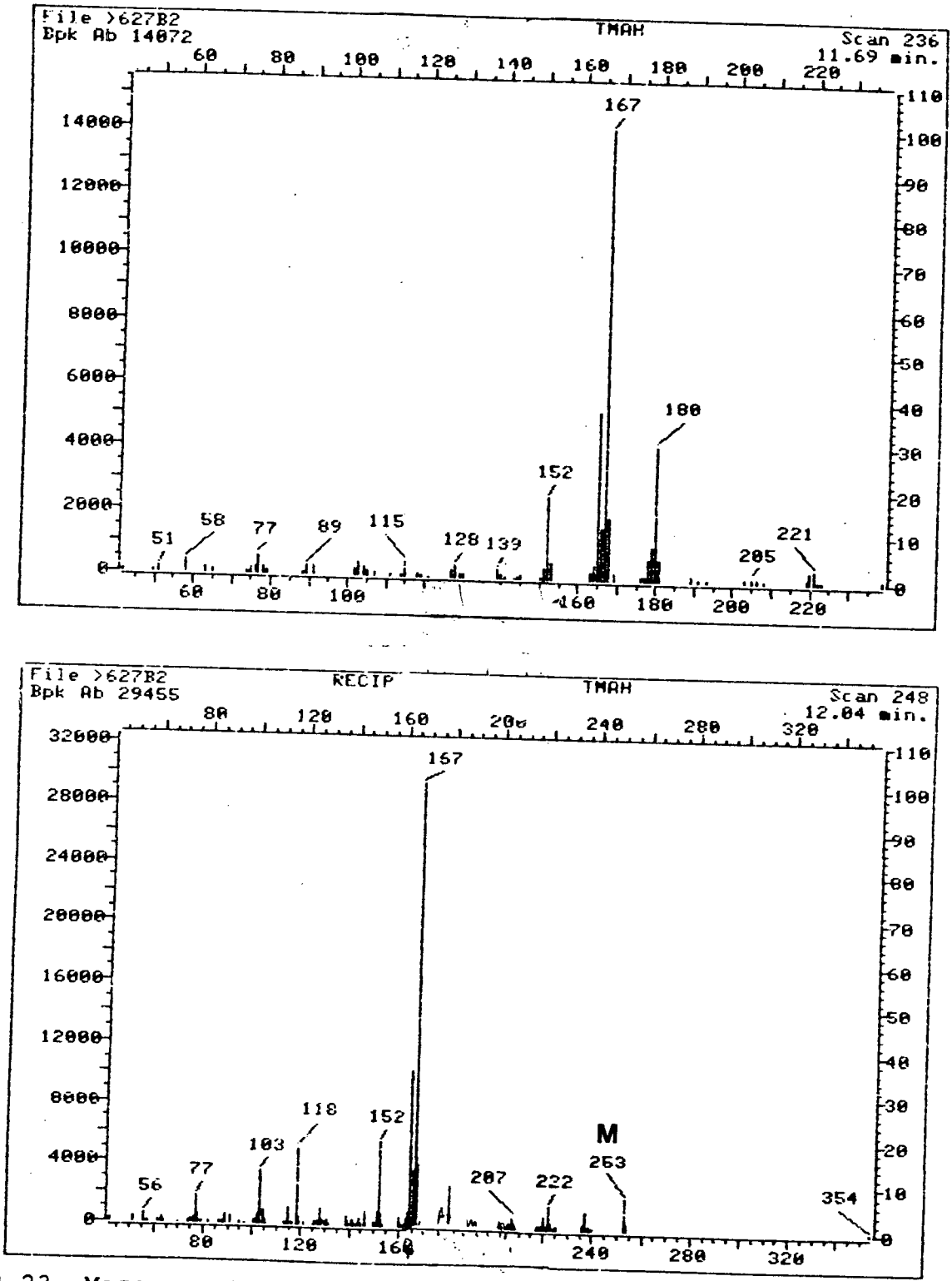


2.21. Mass spectra of recipavrin metabolites and their derivatives: (top) syn-oxime O-methylcatechol di-TMS (93c). (bottom) syn-oxime O-methylcatechol (93b)

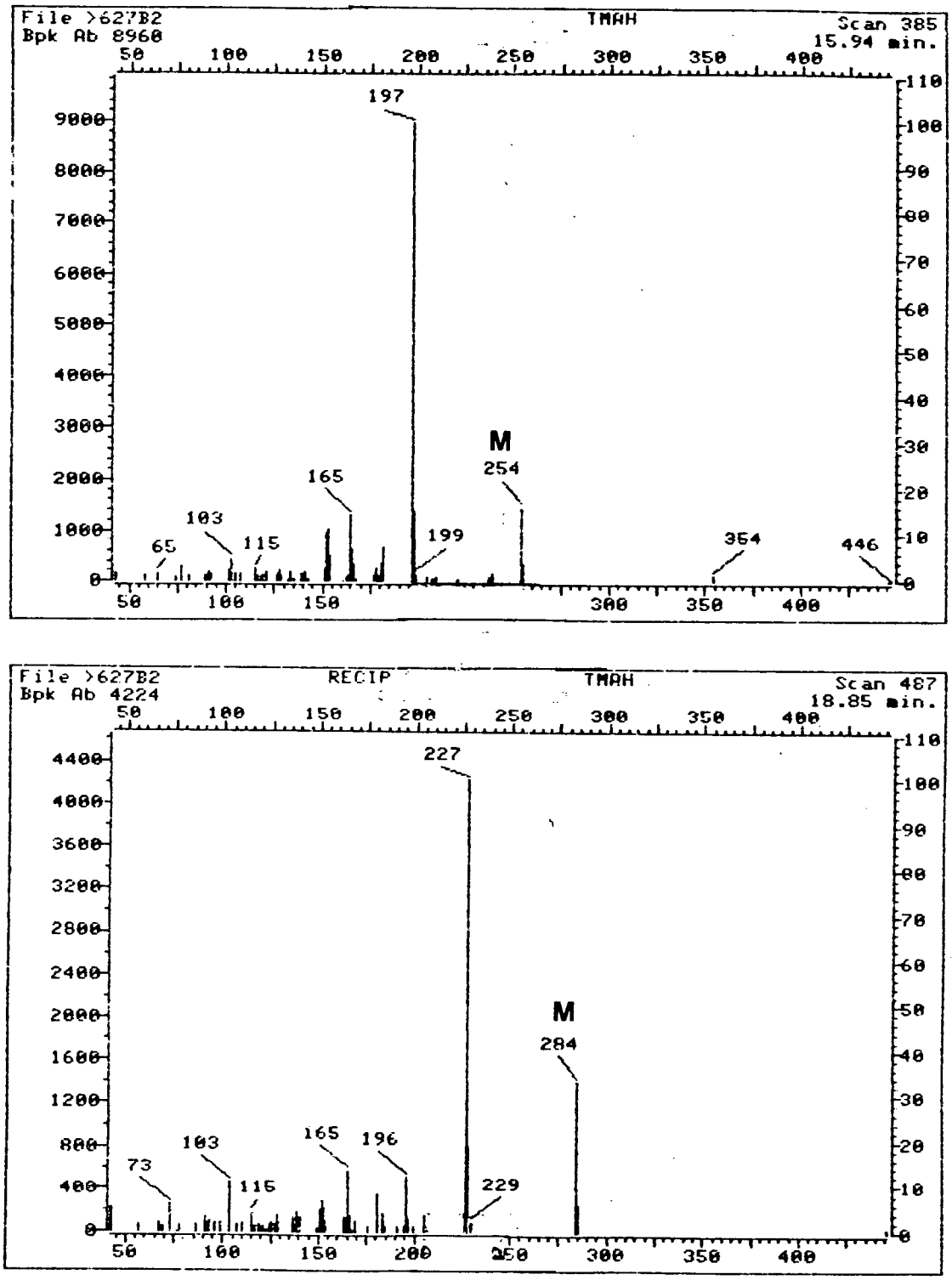




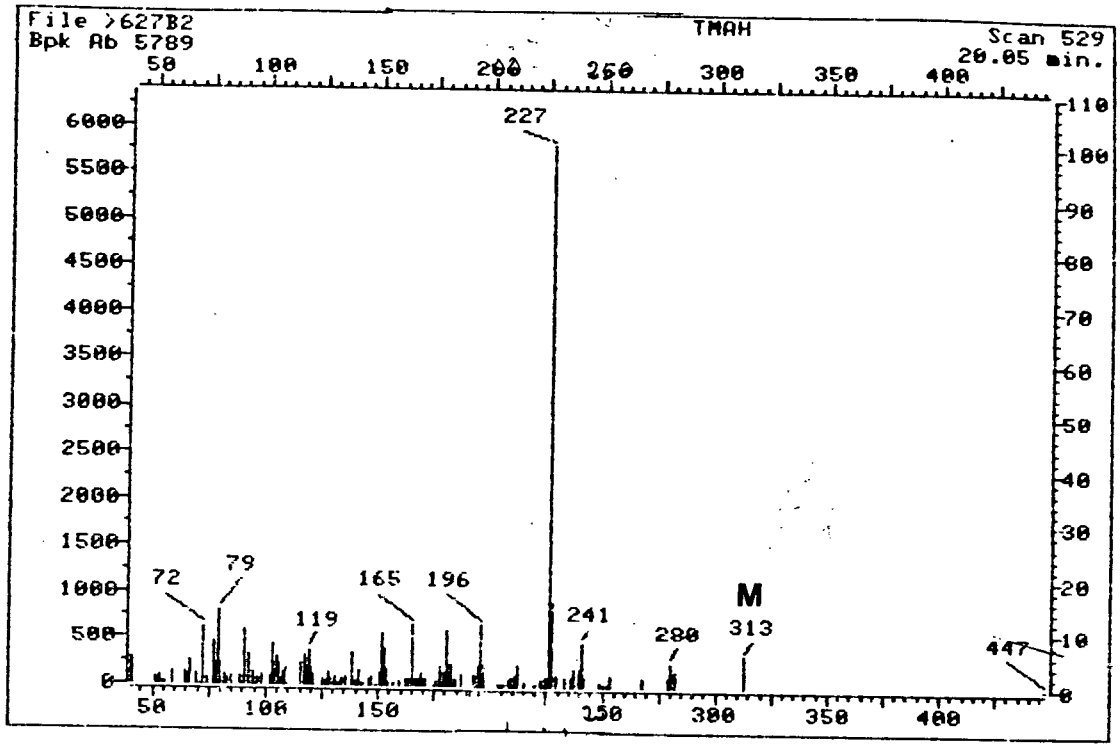
2.22. Mass chromatograms m/z 197 (top) and m/z 227 (bottom) for the recipavrin TMAH derivatized conjugated bile ethyl acetate extract.

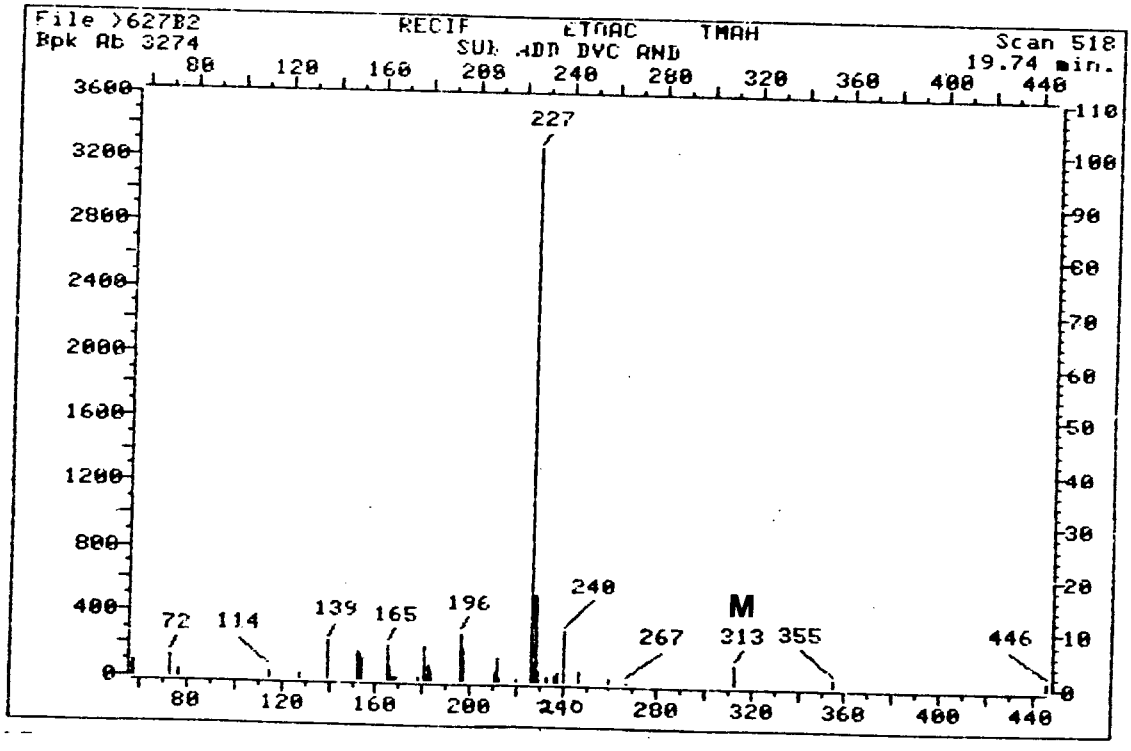


2.23. Mass spectra of TMAH derivatized recipavrin metabolites: (top) anti O-methyl oxime (16).(bottom) syn O-methyl oxime (16a).

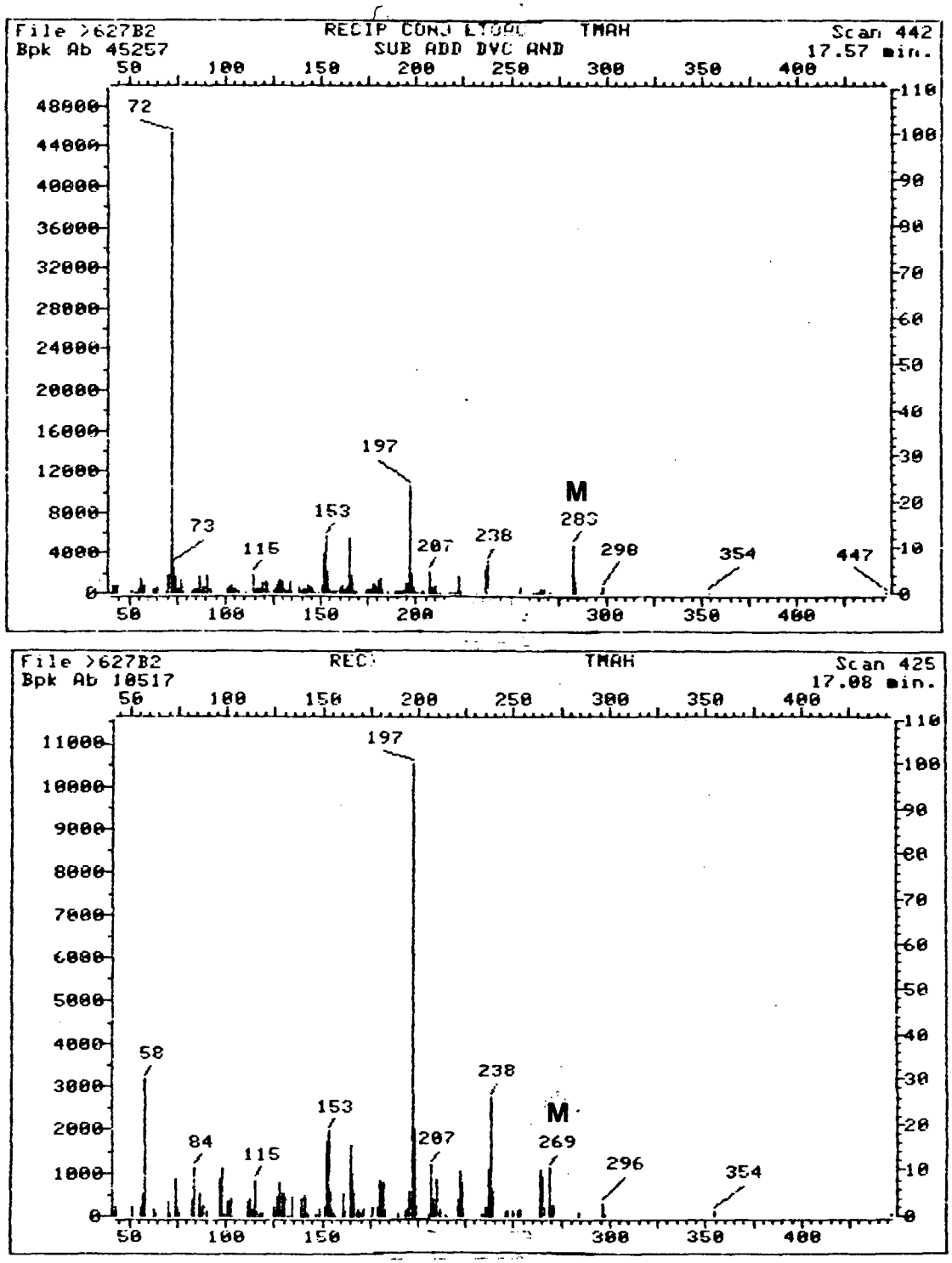


2.24. Mass spectra of TMAH derivatized recipavrin metabolites : (top) diphenylbutanone O-methyl phenol (87b).(bottom) diphenylbutanone dimethylcatechol (91b).

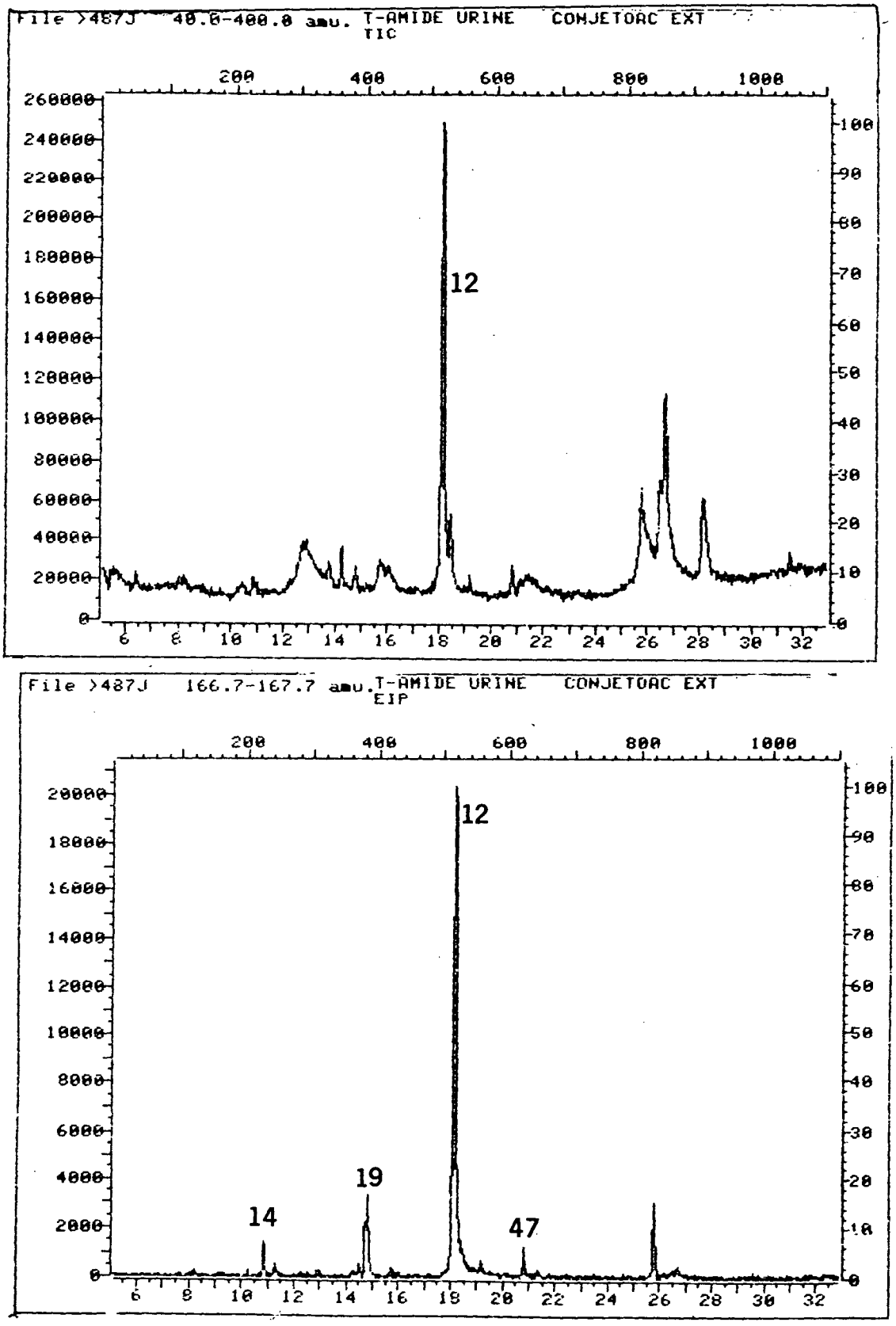




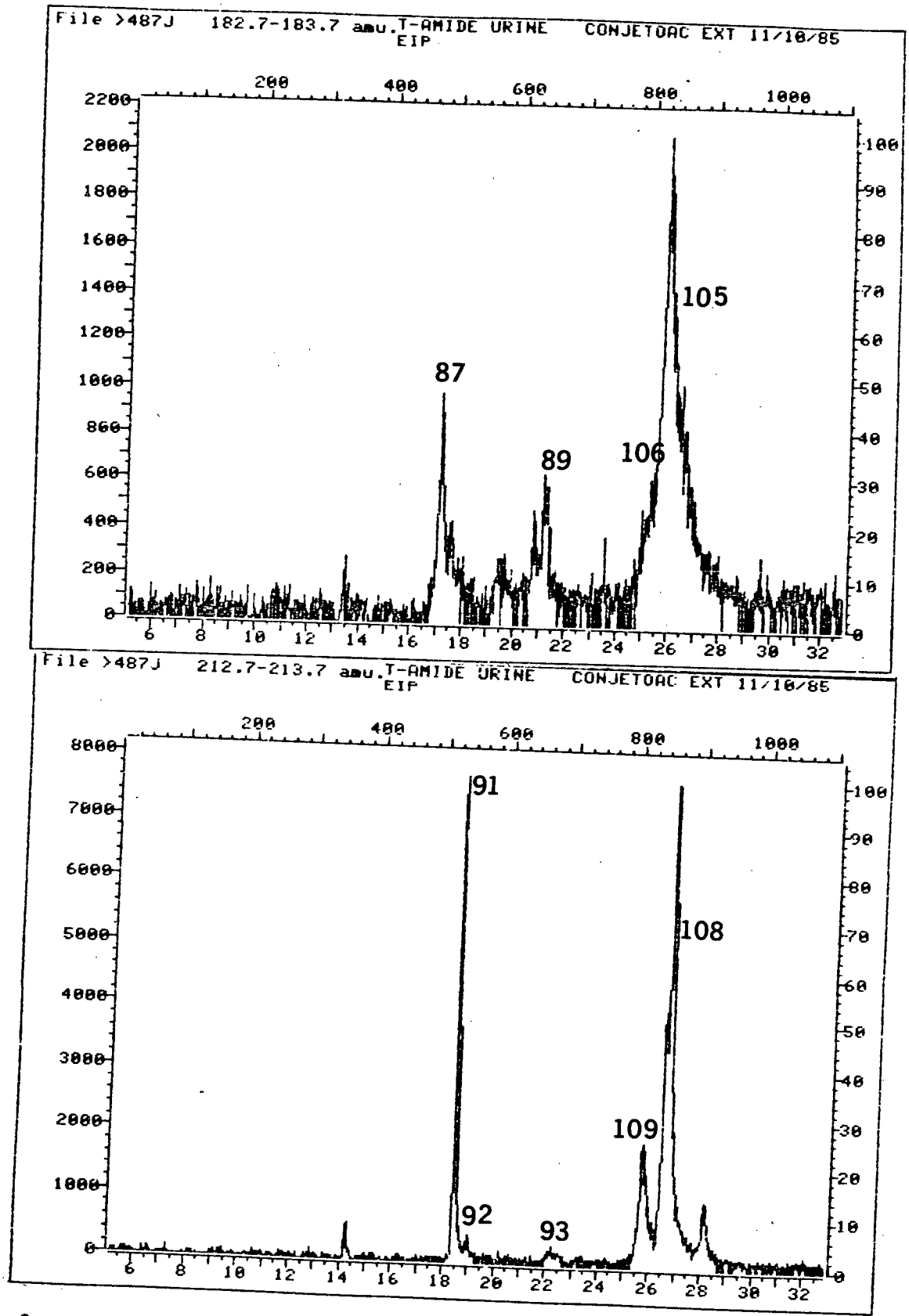
2.25. Mass spectra of TMAH derivatized recipavrin metabolites: (top) anti O-methyl oxime dimethylcatechol (93e). (bottom) syn O-methyl oxime dimethylcatechol (93d).



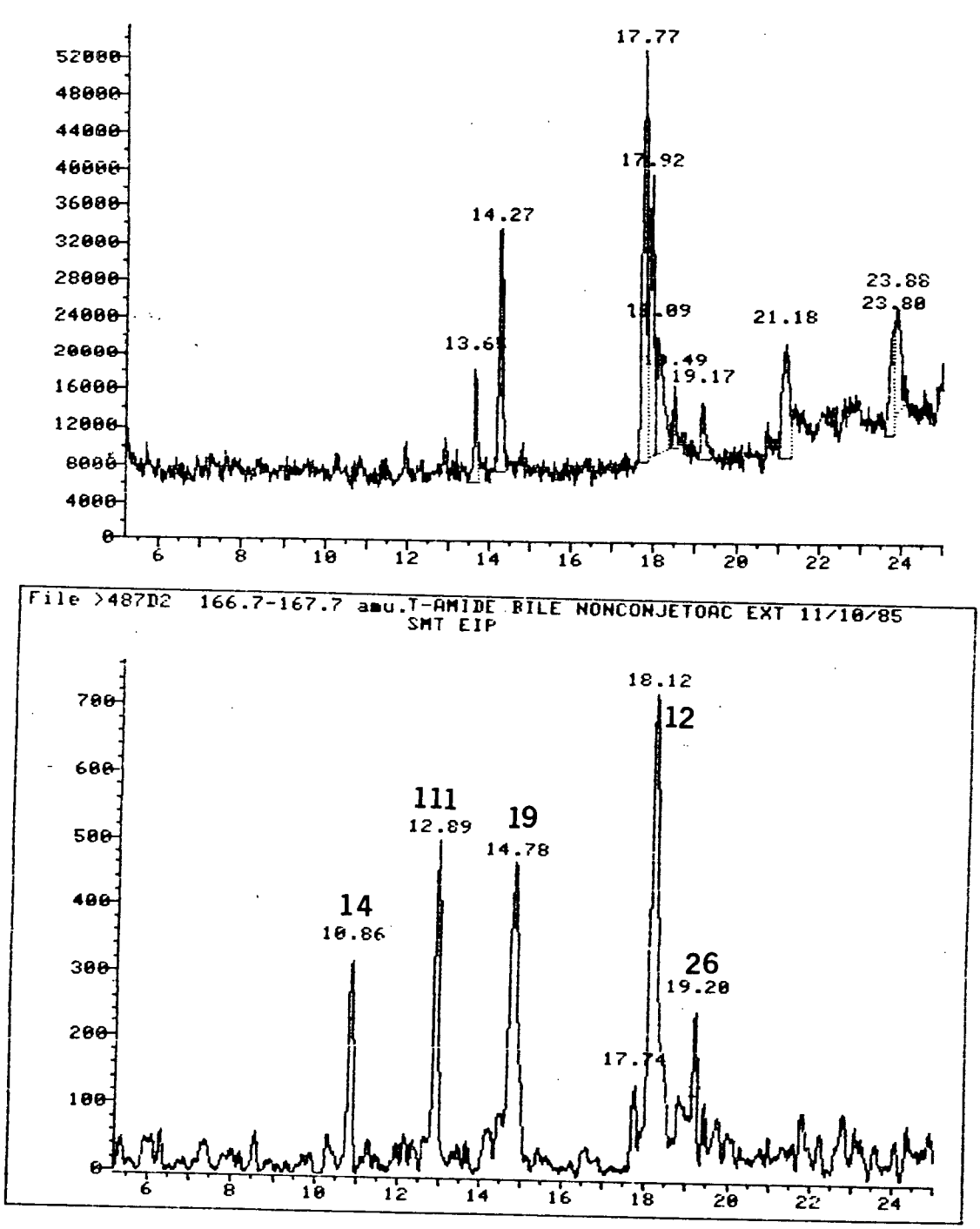
2.26. Mass spectra of TMAH derivatized recipavrin metabolites: (top) recipavrin O-methylphenol (90b). (bottom). Norrecipavrin O-methylphenol (88b).



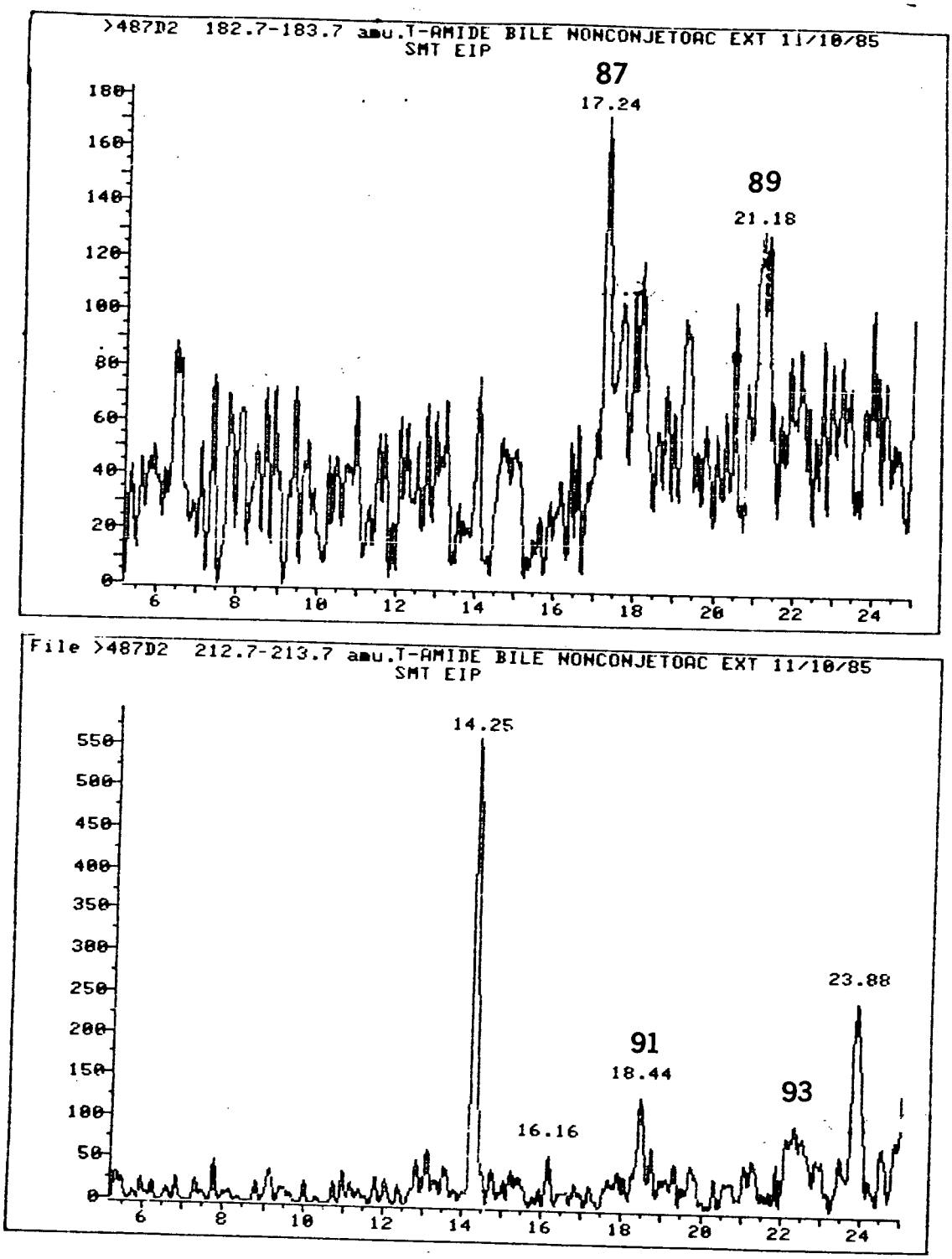
3.1. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the tertiary formamide conjugated urine extract.



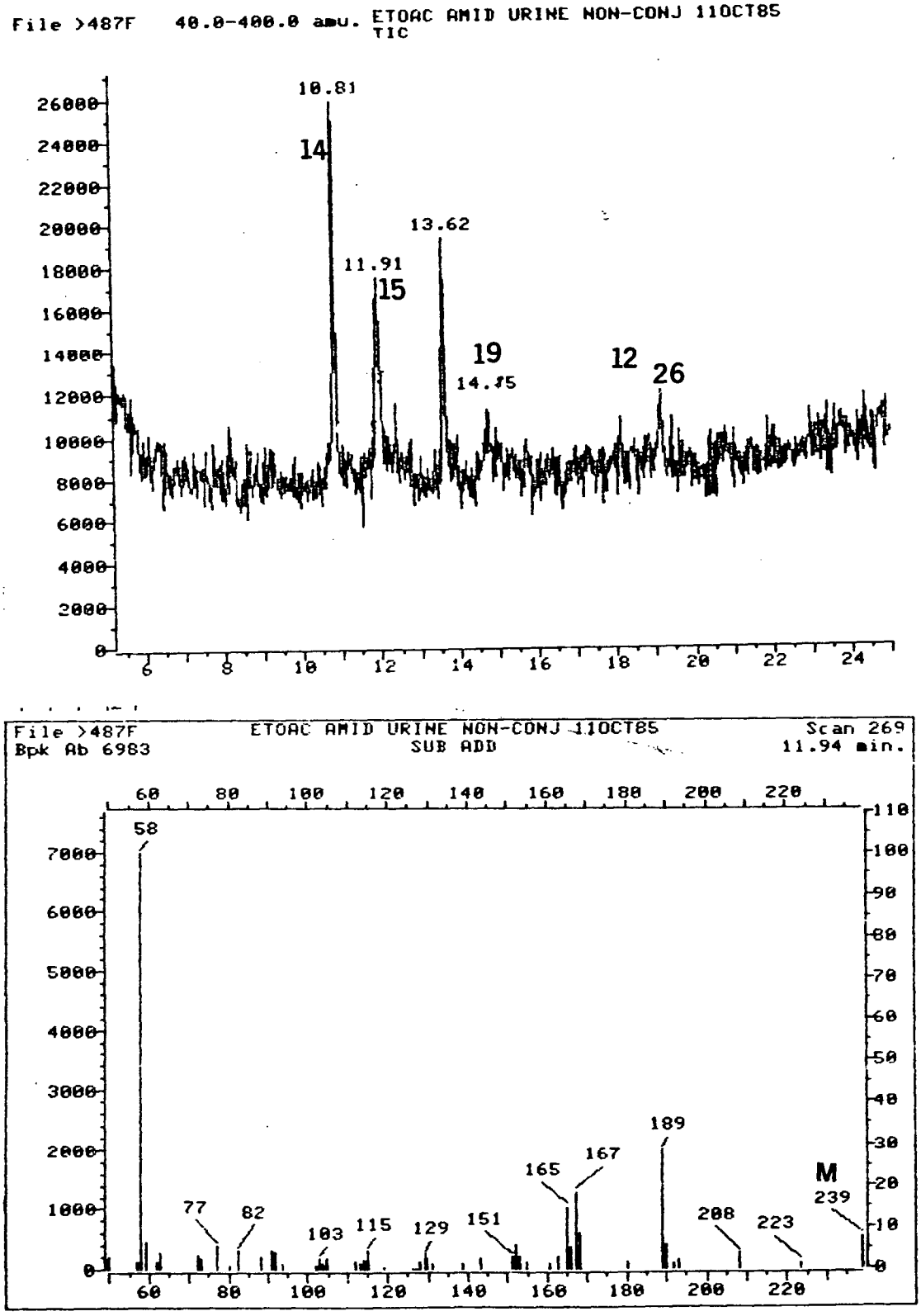
3.2. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the tertiary formamide conjugated urine extract.



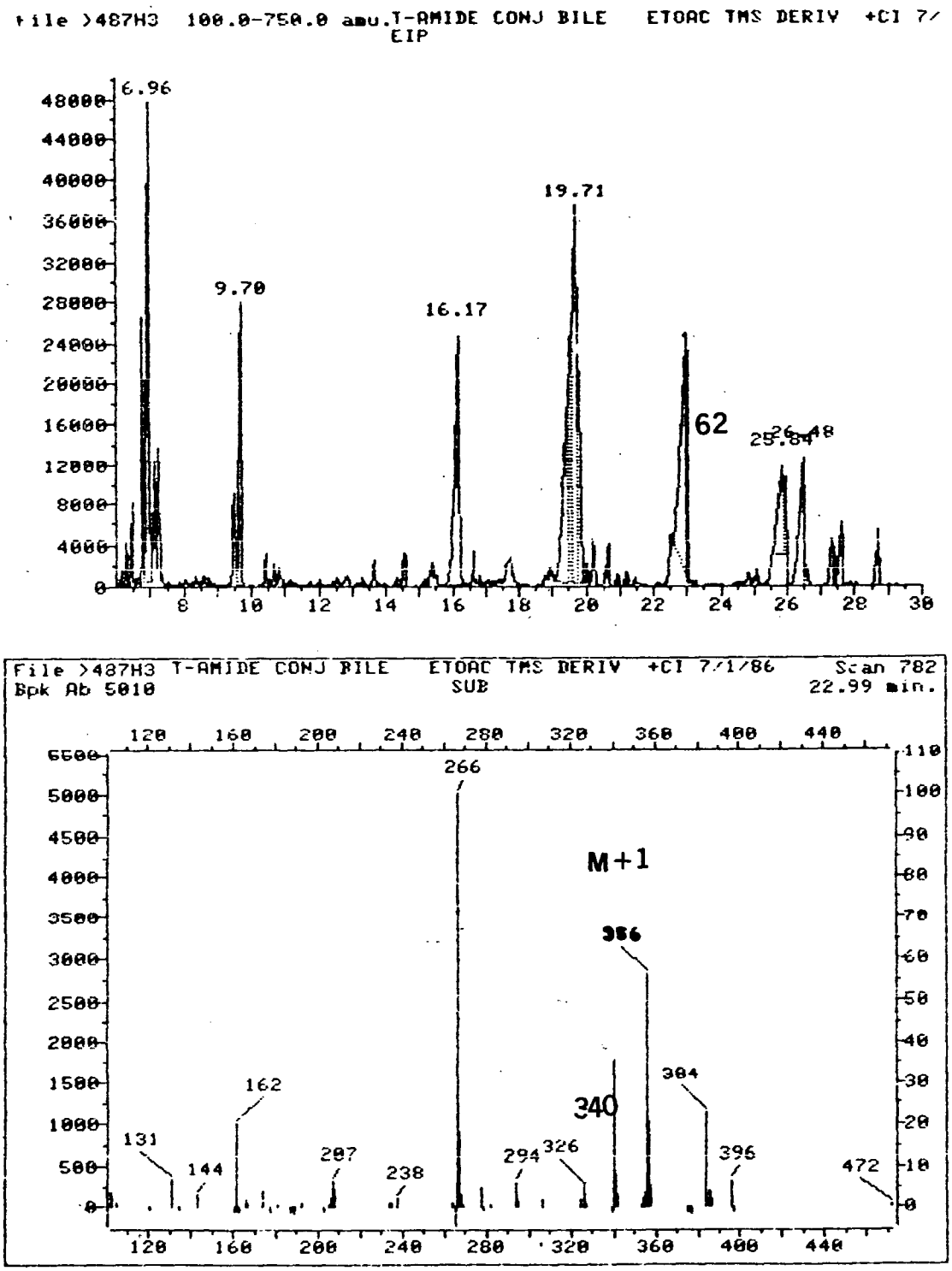
3.3. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the tertiary formamide nonconjugated bile extract.



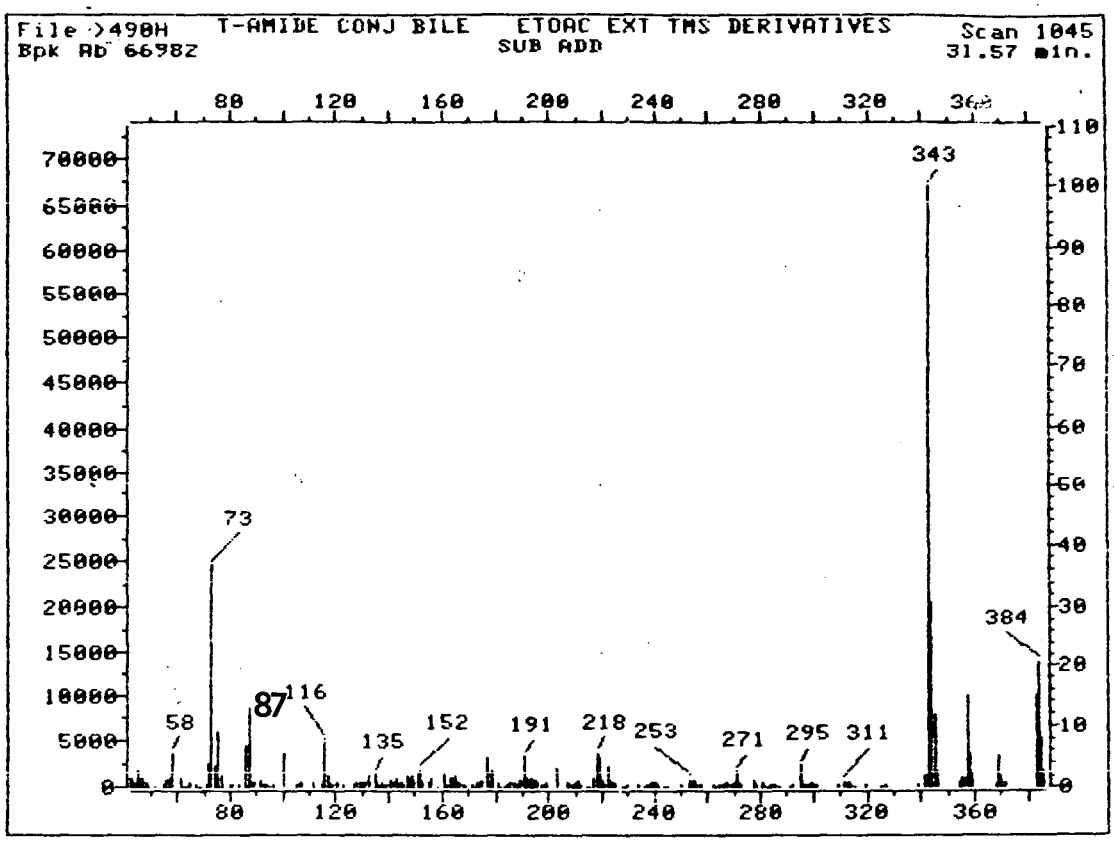
3.4. Mass chromatograms m/z 183 (top) and m/z 213 (bottom) for the tertiary formamide nonconjugated bile extract.

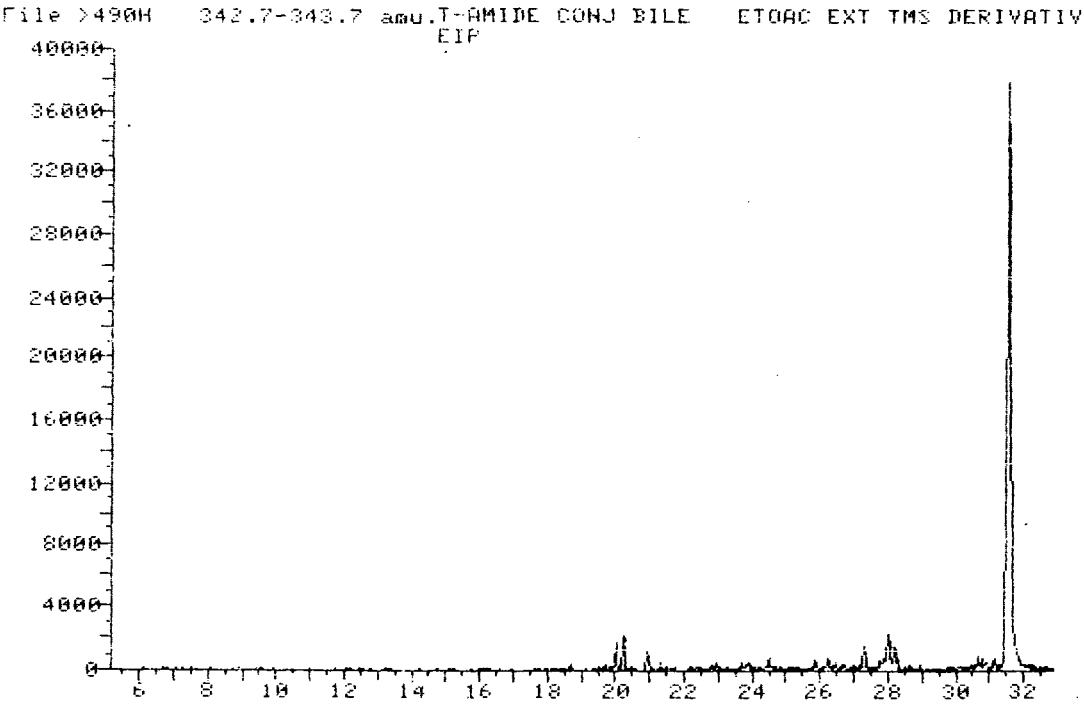


3.5. Total ion current (top) for the tertiary formamide nonconjugated urine extract. (bottom) Mass spectrum of norrecipavrin from the nonconjugated urine extract.

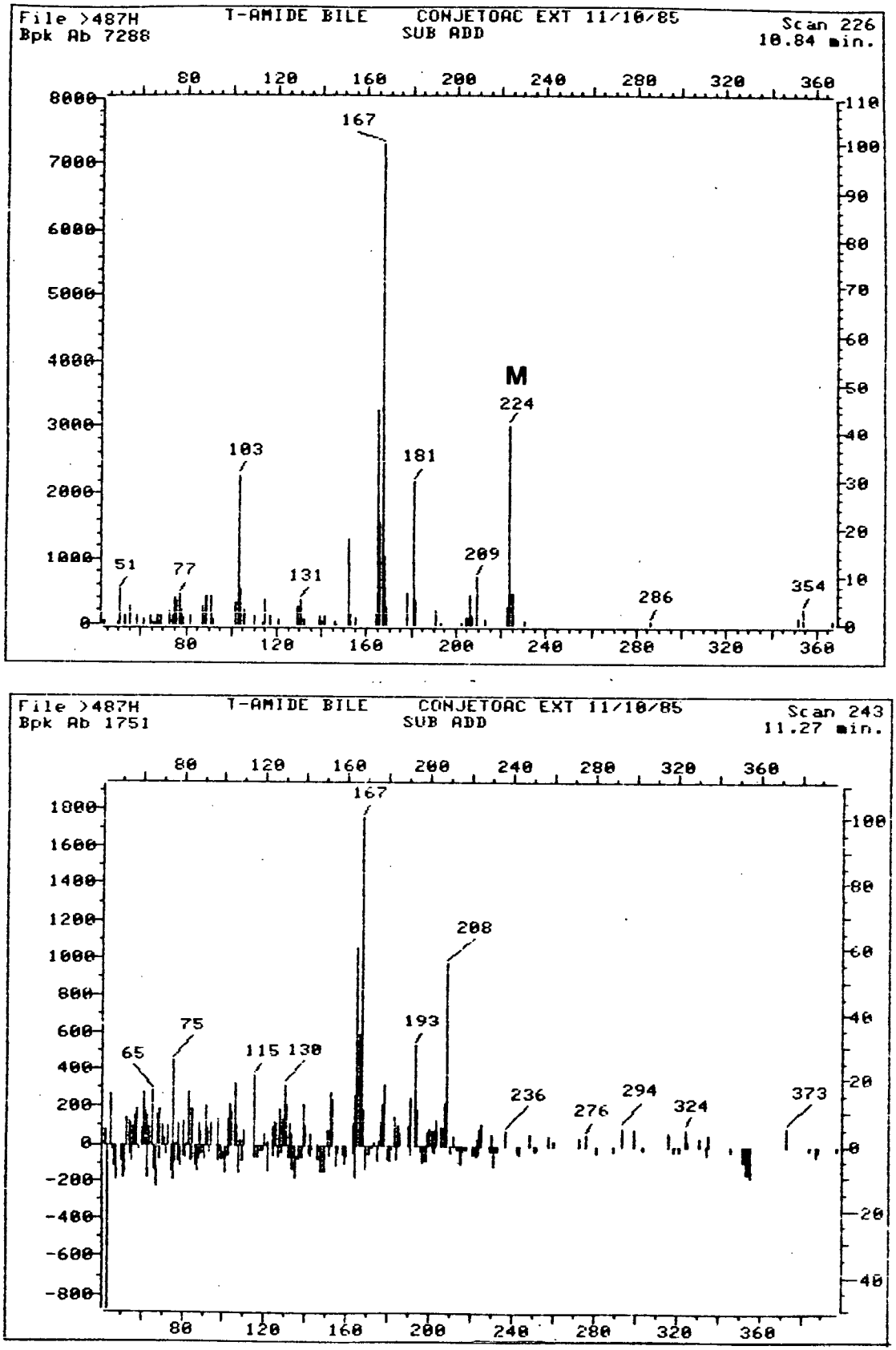


3.6. Total ion current (top) for the tertiary formamide conjugated bile extract (positive chemical ionization using methane reagent gas). (bottom) Positive chemical ionization mass spectrum of the TMS carbinolamide (62) from the conjugated bile extract.

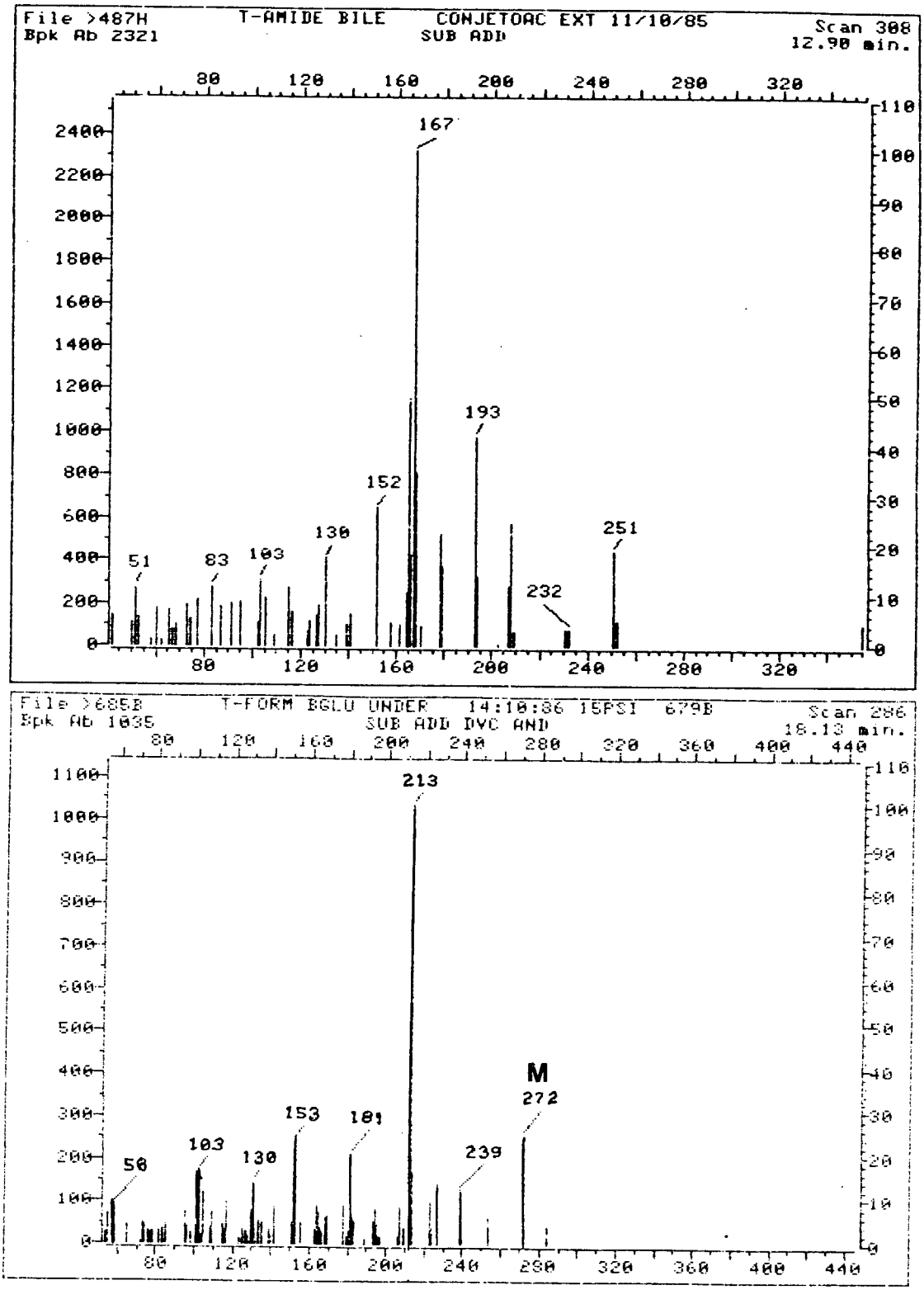




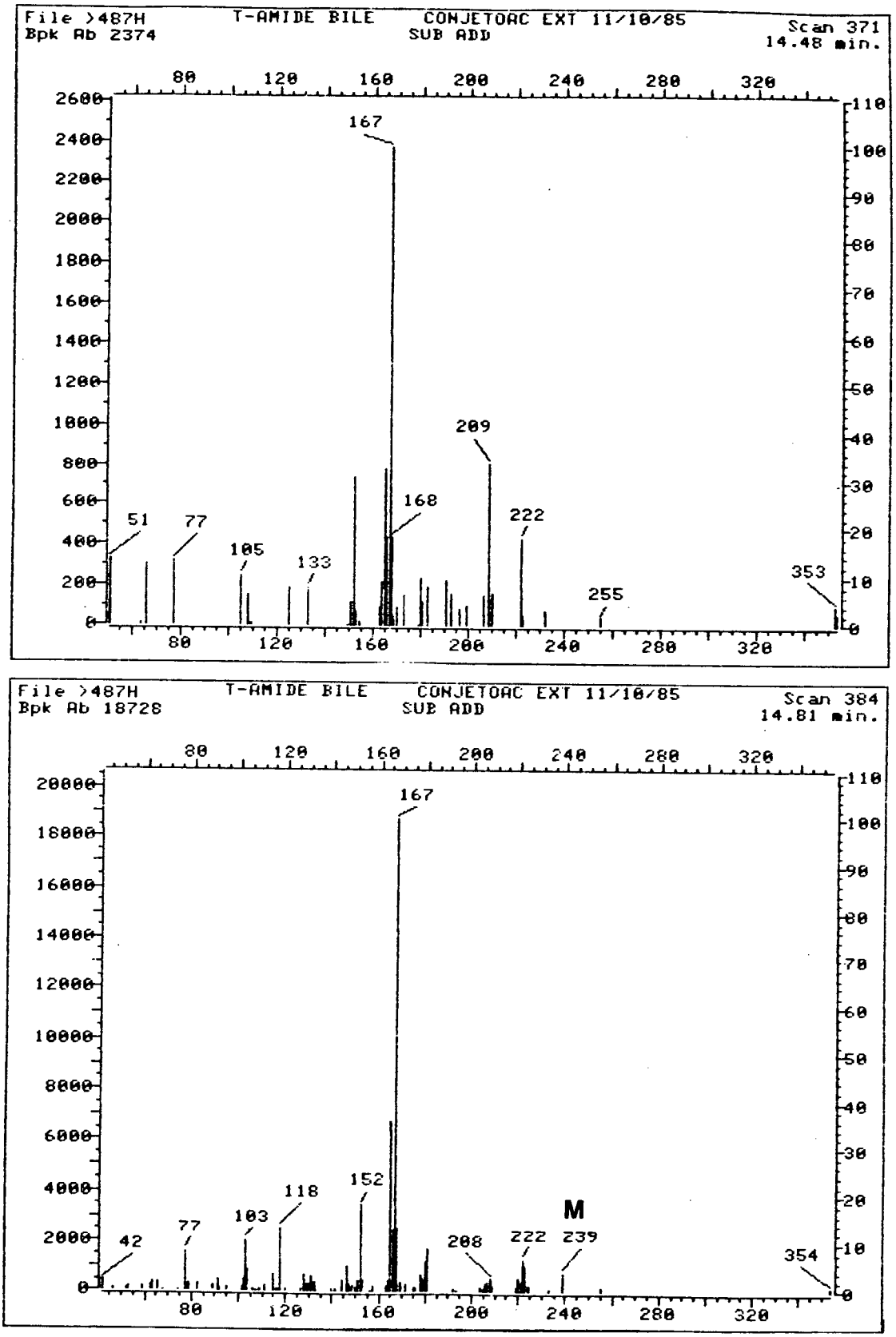
3.7. Mass spectrum of a suspected tertiary formamide catechol metabolite. Mass chromatogram m/z 343 used to locate underivatized catechol metabolites



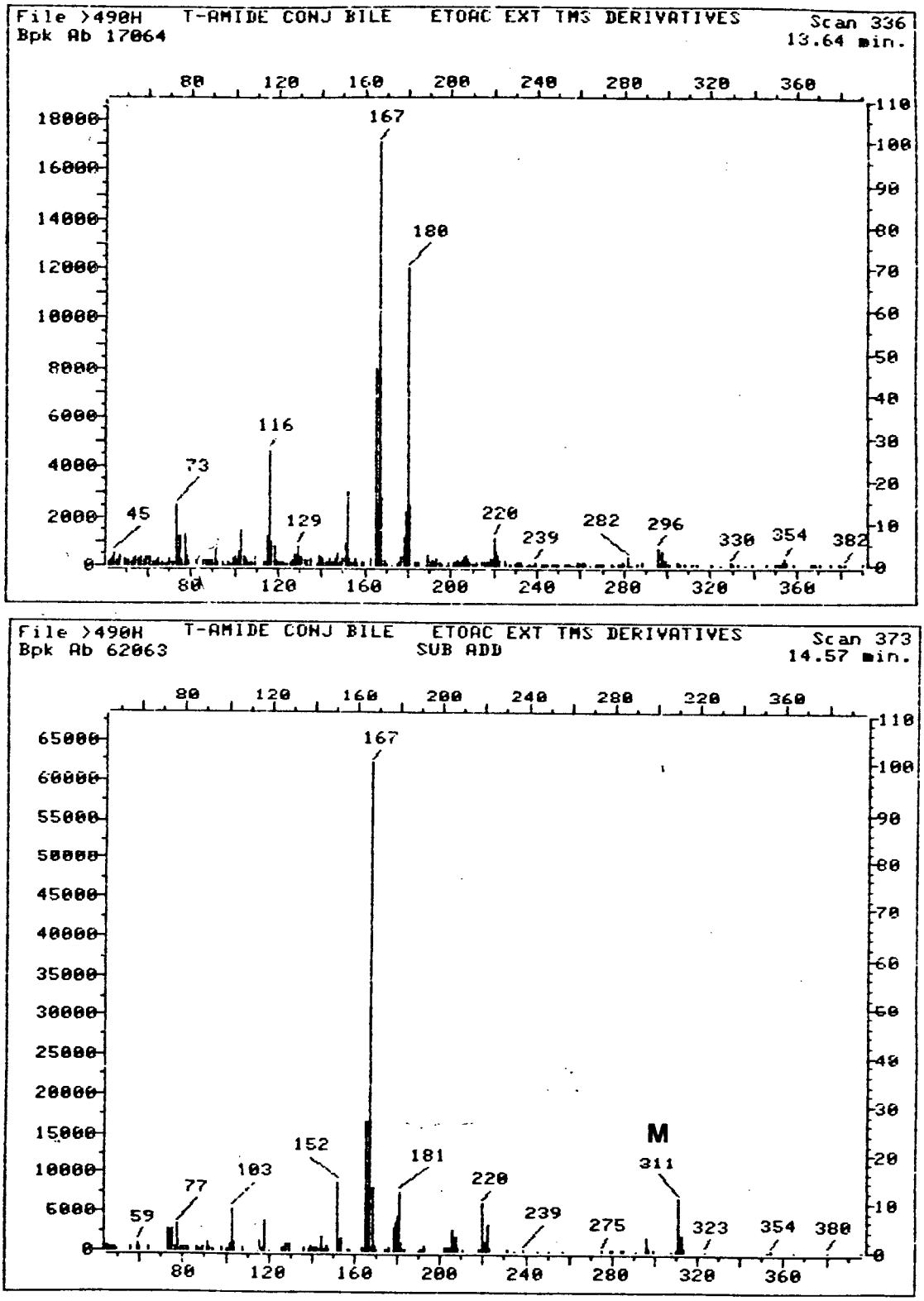
3.8. Mass spectra of tertiary formamide metabolites and their derivatives: (top) diphenylbutanone (14). (bottom) diphenylbutanol (97).



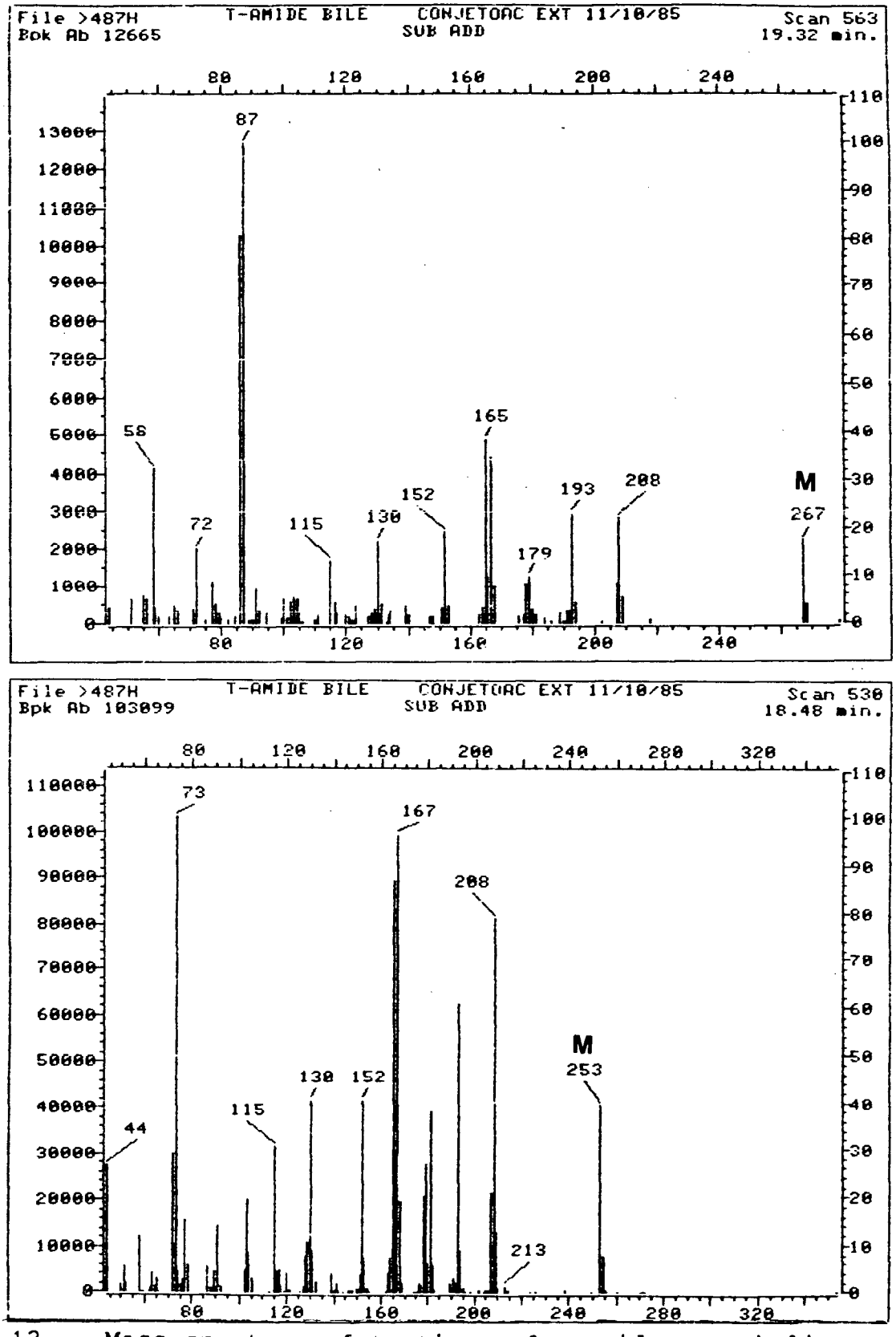
3.9. Mass spectra of tertiary formamide metabolites and their derivatives: (top) carbinolamide (111). (bottom) diphenylbutanol O-methylcatechol (92).



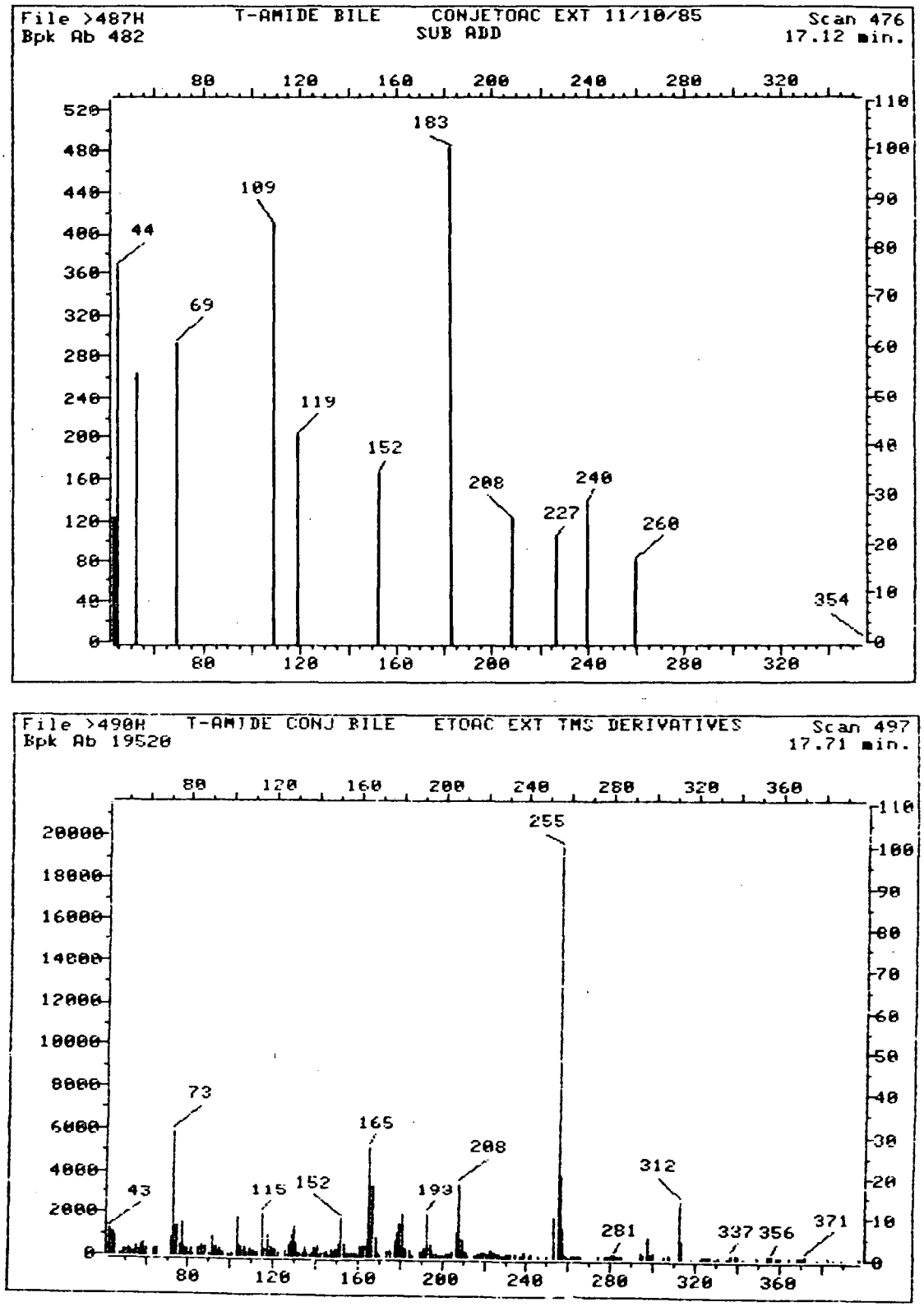
3.10. Mass spectra of tertiary formamide metabolites and their derivatives: (top) syn-oxime (19). (bottom) anti-oxime (19a).



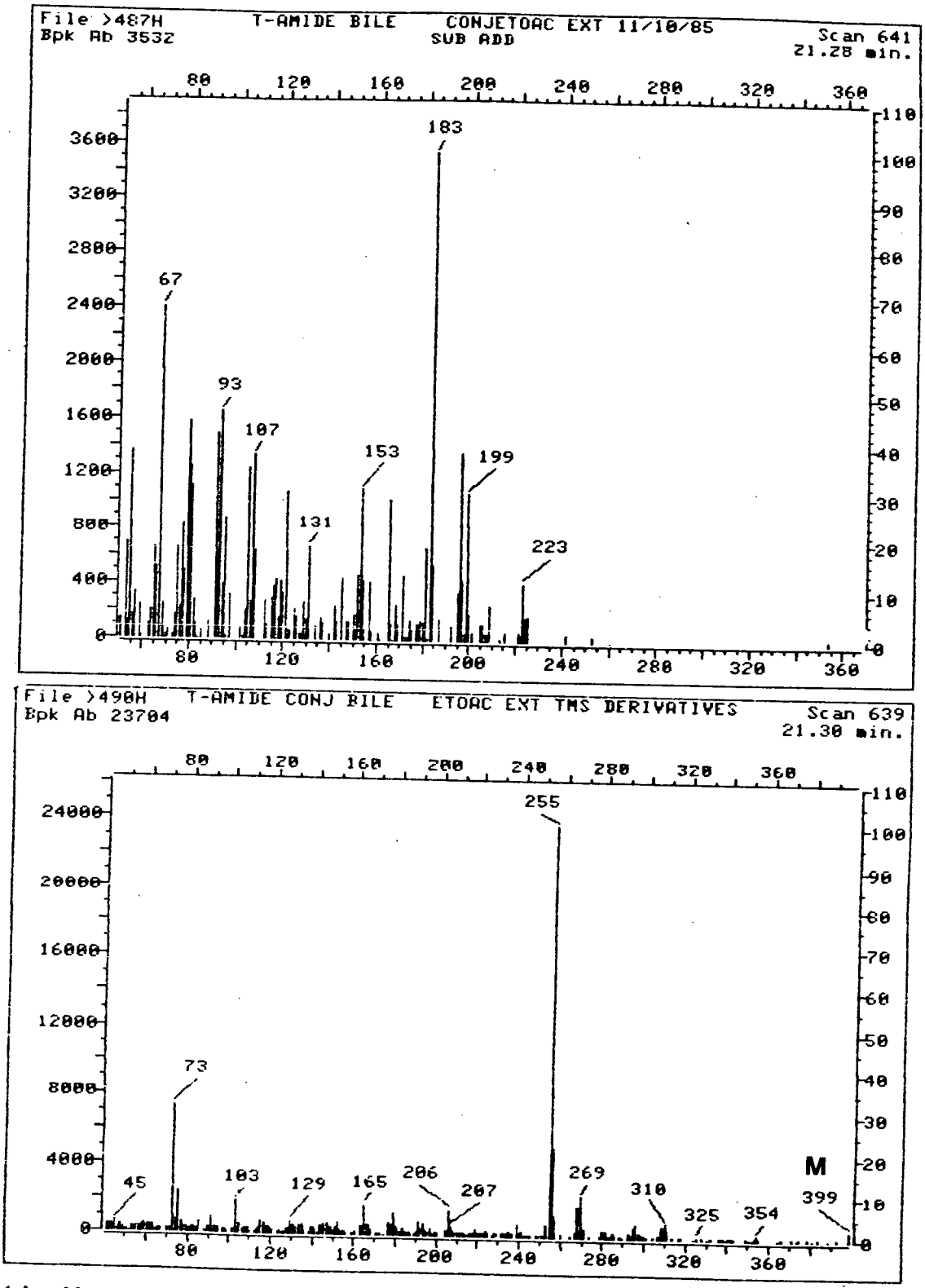
3.11. Mass spectra of tertiary formamide metabolites and their derivatives: (top) syn-oxime TMS (103). (bottom) anti-oxime TMS (103a).



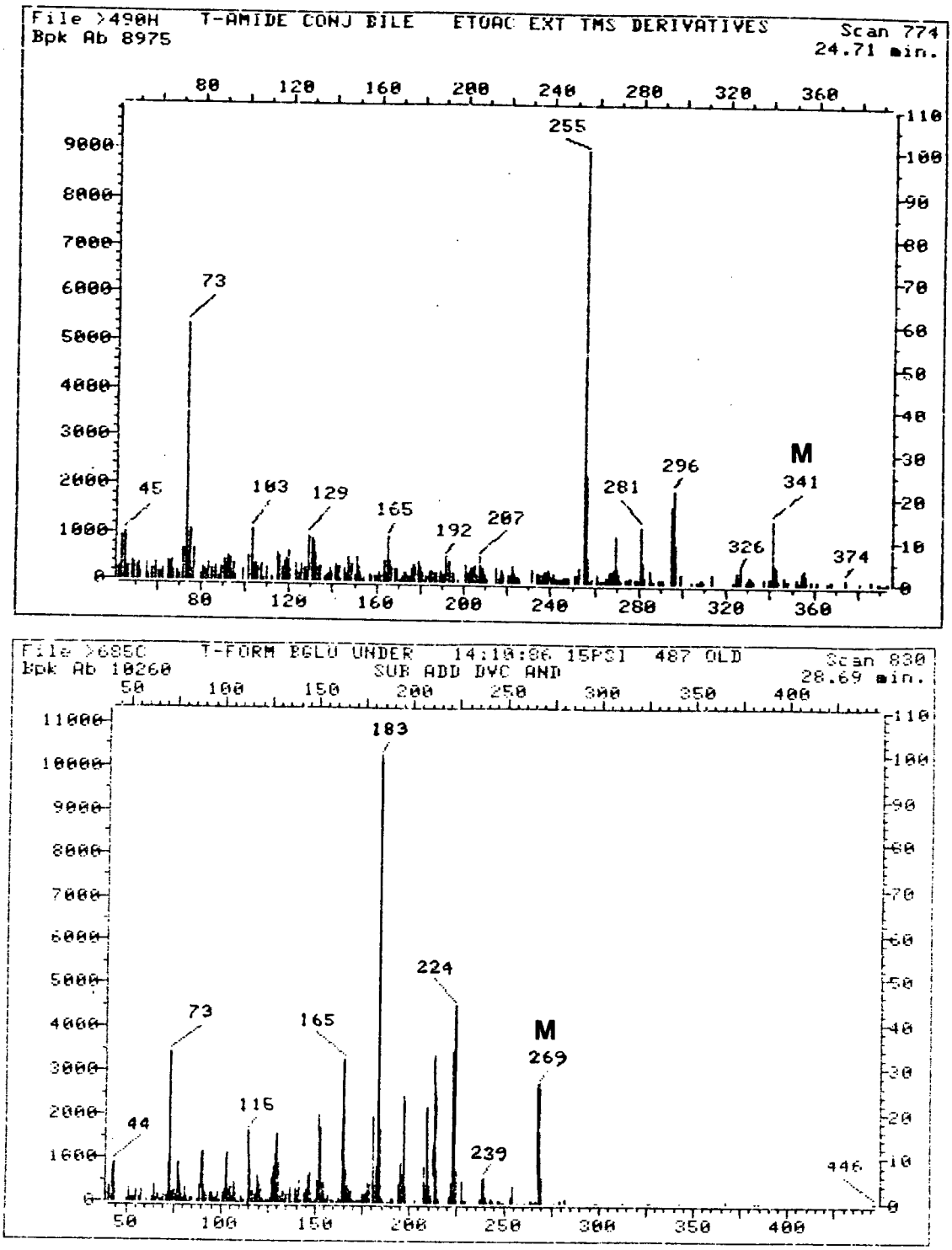
3.12 . Mass spectra of tertiary formamide metabolites and their derivatives: (top) tertiary formamide (26). (bottom) secondary formamide (12).



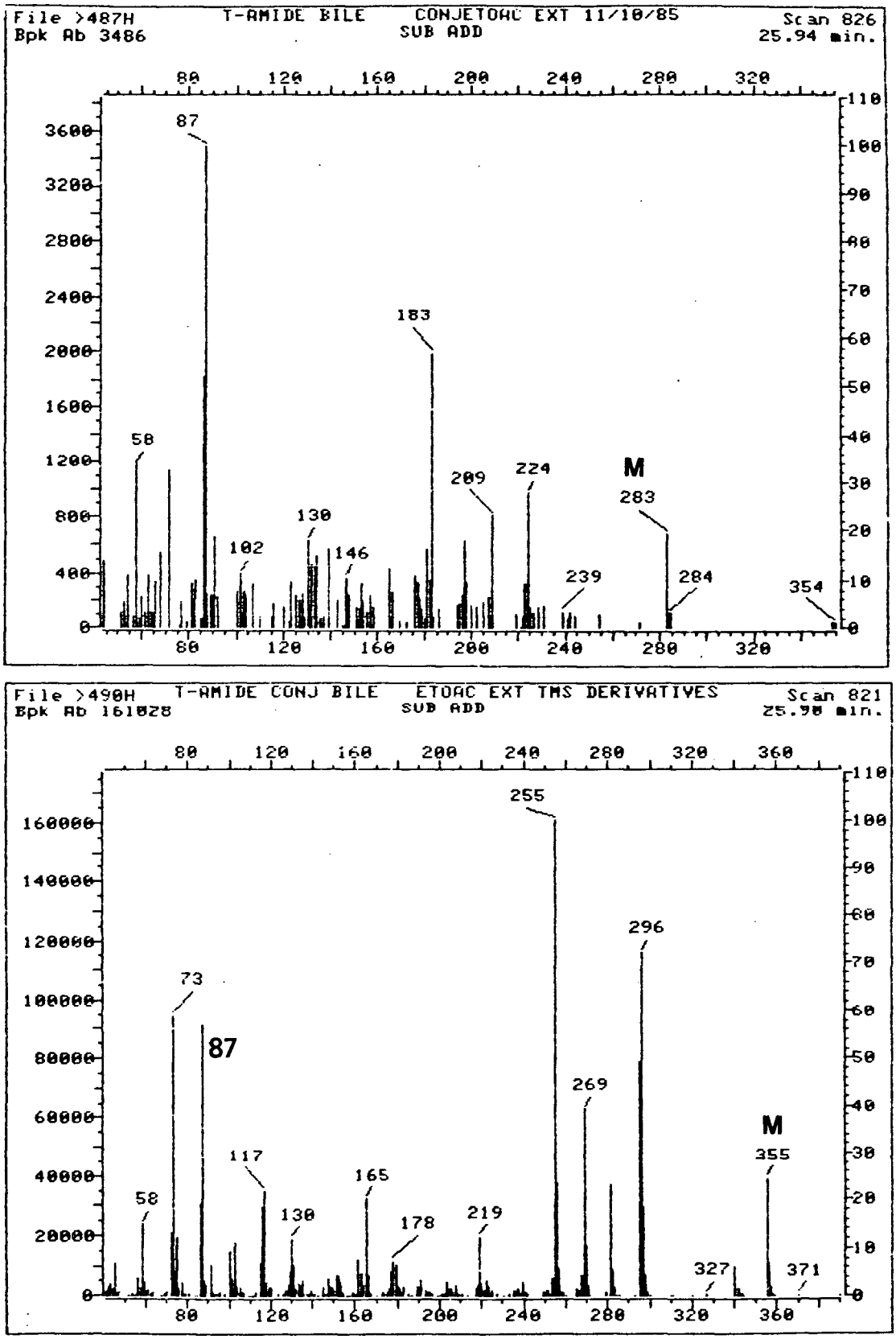
3.13. Mass spectra of tertiary formamide metabolites and their derivatives: (top) diphenylbutanone phenol (87). (bottom) diphenylbutanone phenol TMS (87a).



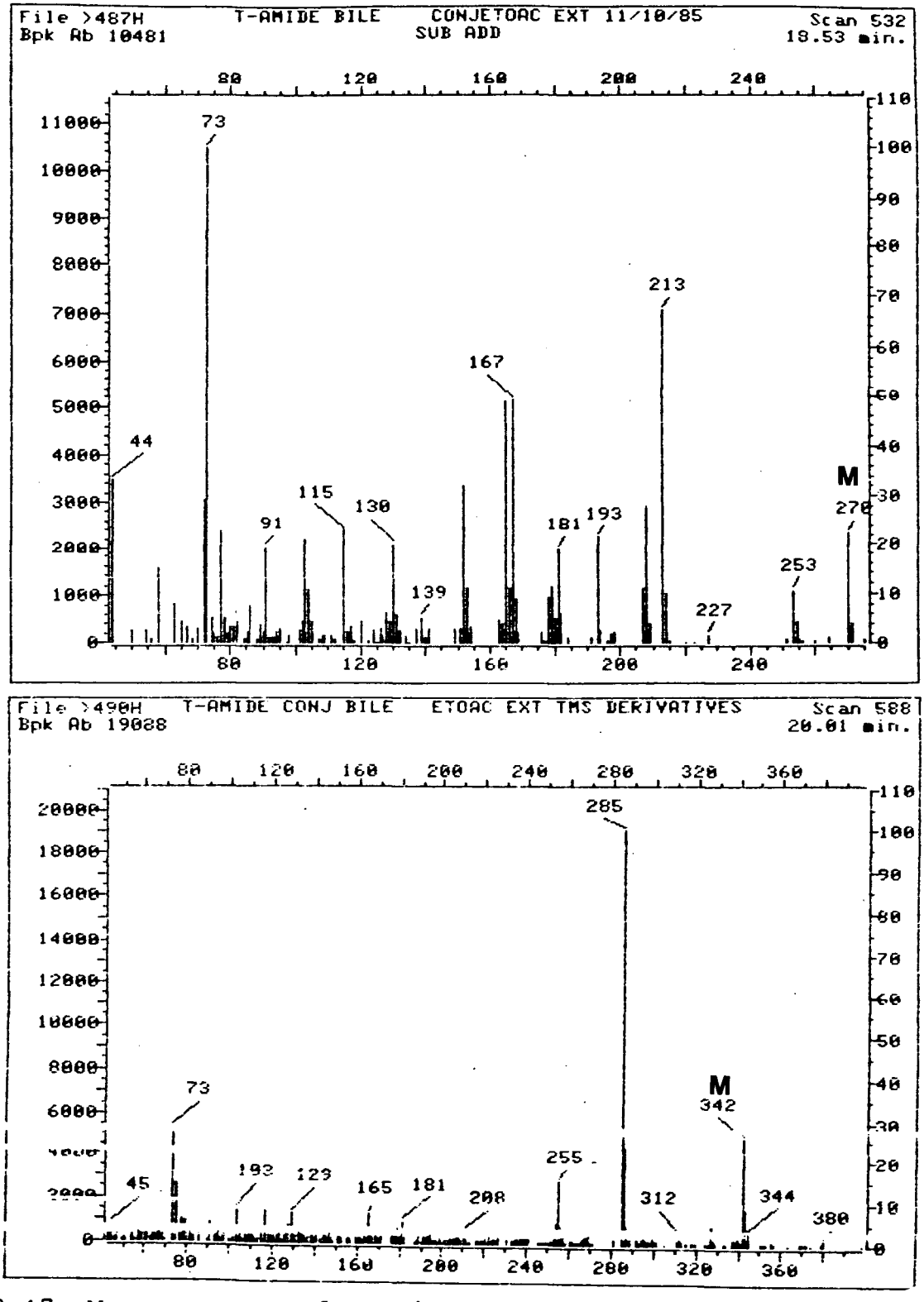
3.14. Mass spectra of tertiary formamide metabolites and their derivatives: (top) oxime phenol (89). (bottom) oxime phenol di-TMS (89a).



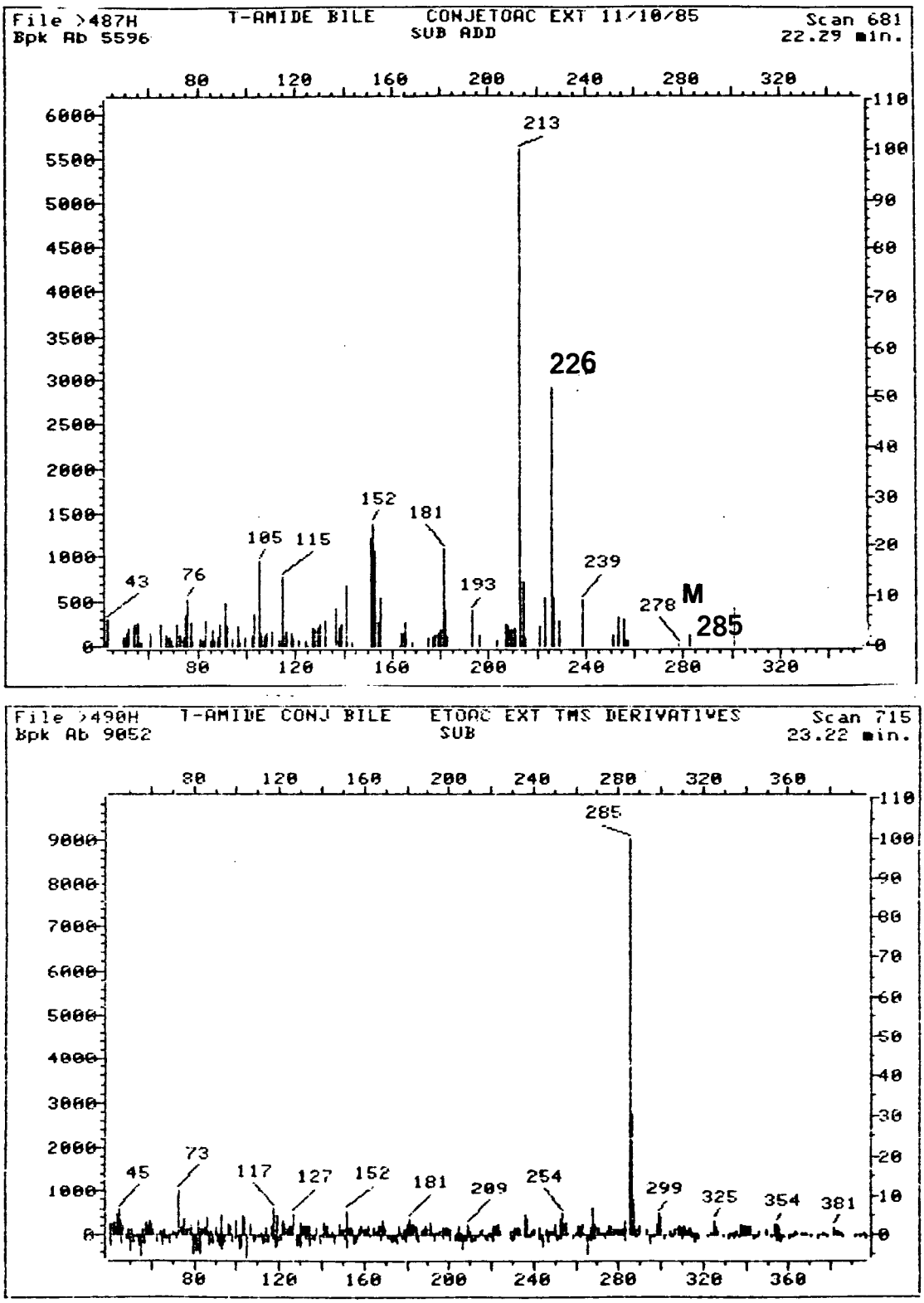
3.15. Mass spectra of tertiary formamide metabolites and their derivatives: (top) secondary formamide phenol TMS (106a). (bottom) secondary formamide phenol (106).



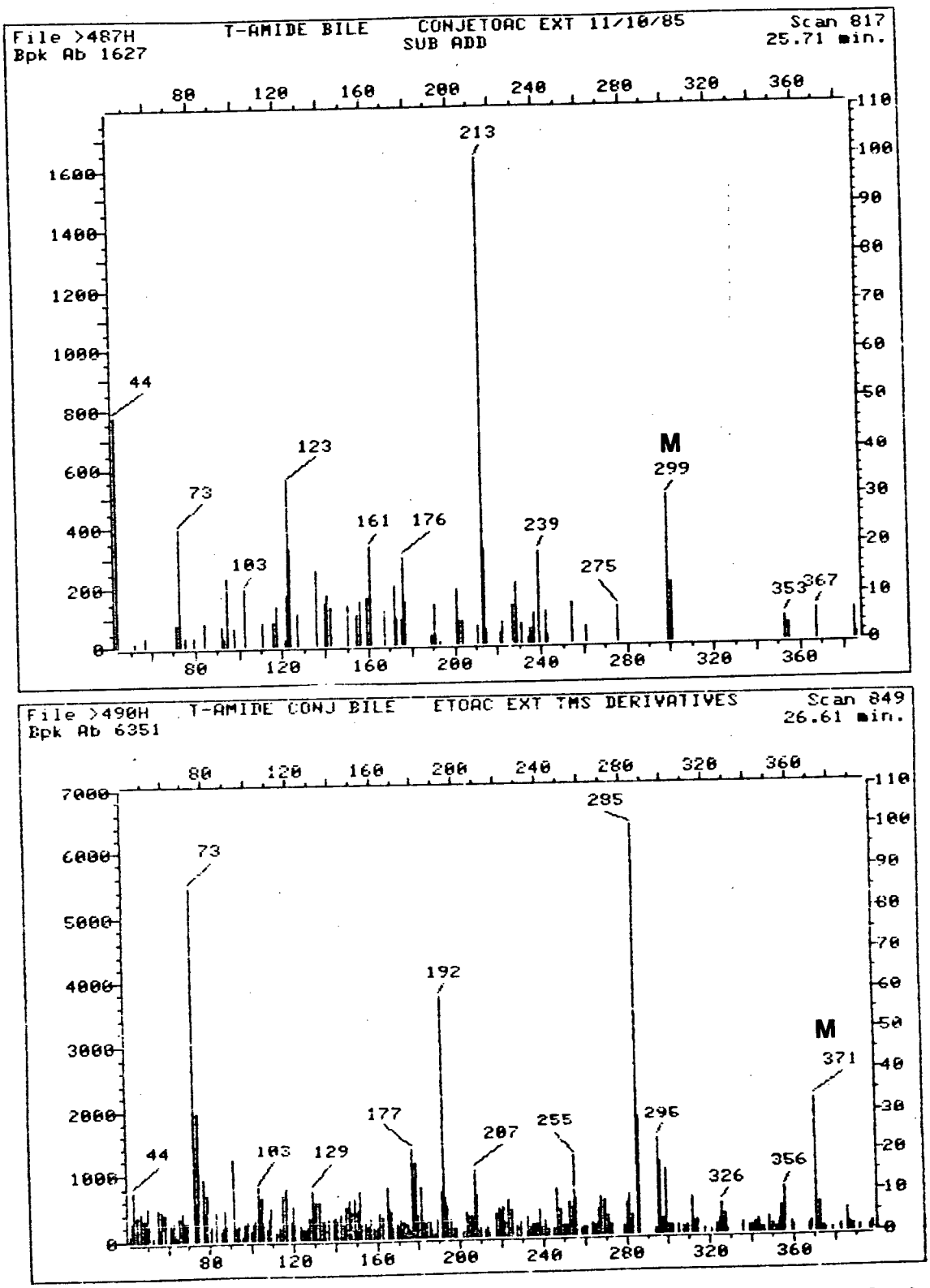
3.16. Mass spectra of tertiary formamide metabolites and their derivatives: (top) tertiary formamide phenol (105). (bottom) tertiary formamide phenol TMS (105a).



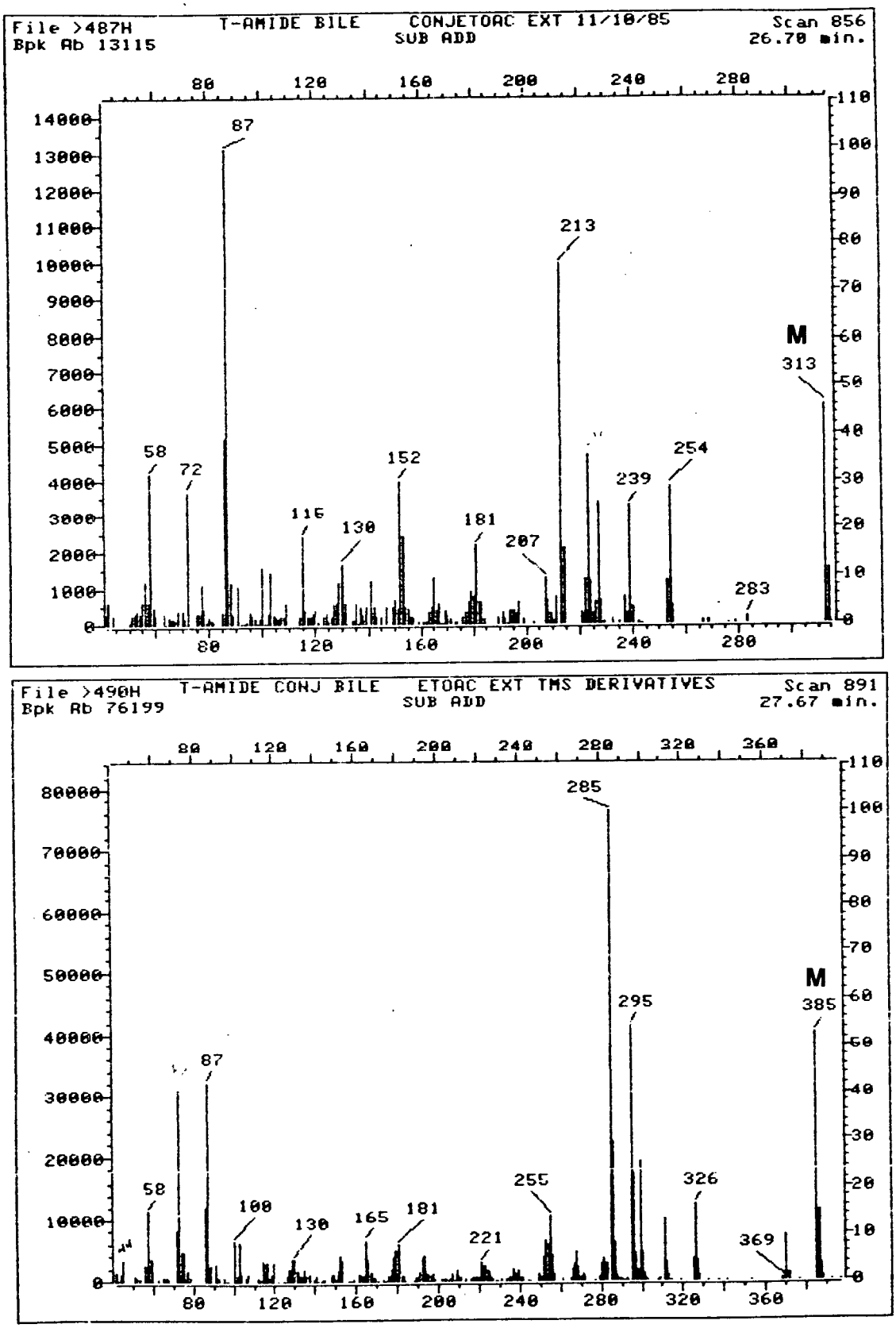
3.17. Mass spectra of tertiary formamide metabolites and their derivatives: (top) diphenylbutanone O-methylcatechol (91) (some peak overlap with the secondary formamide (12)). (bottom) diphenylbutanone O-methylcatechol TMS (91a).



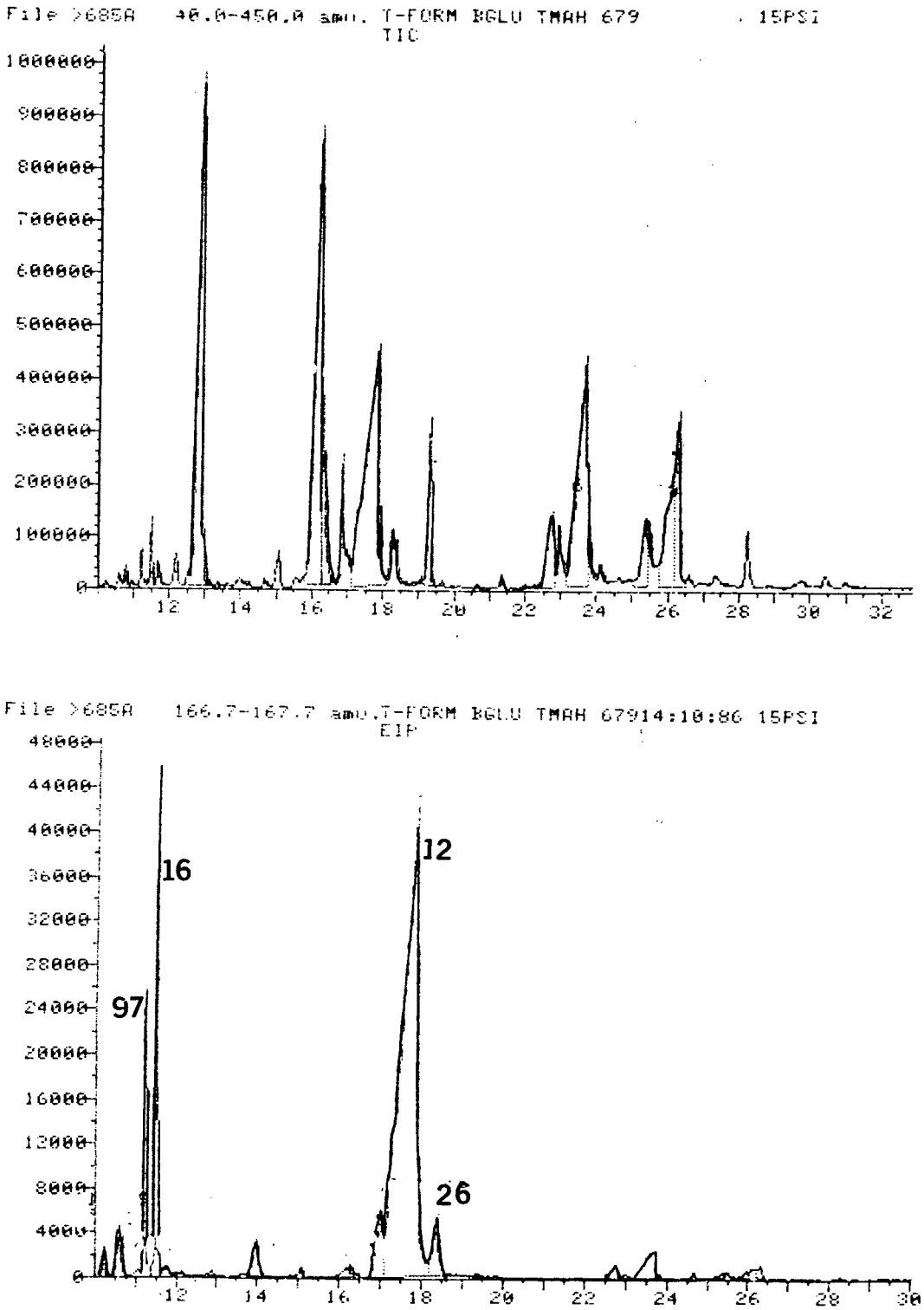
3.18. Mass spectra of tertiary formamide metabolites and their derivatives: (top) oxime O-methylcatechol (93). (bottom) oxime O-methylcatechol TMS (93a).



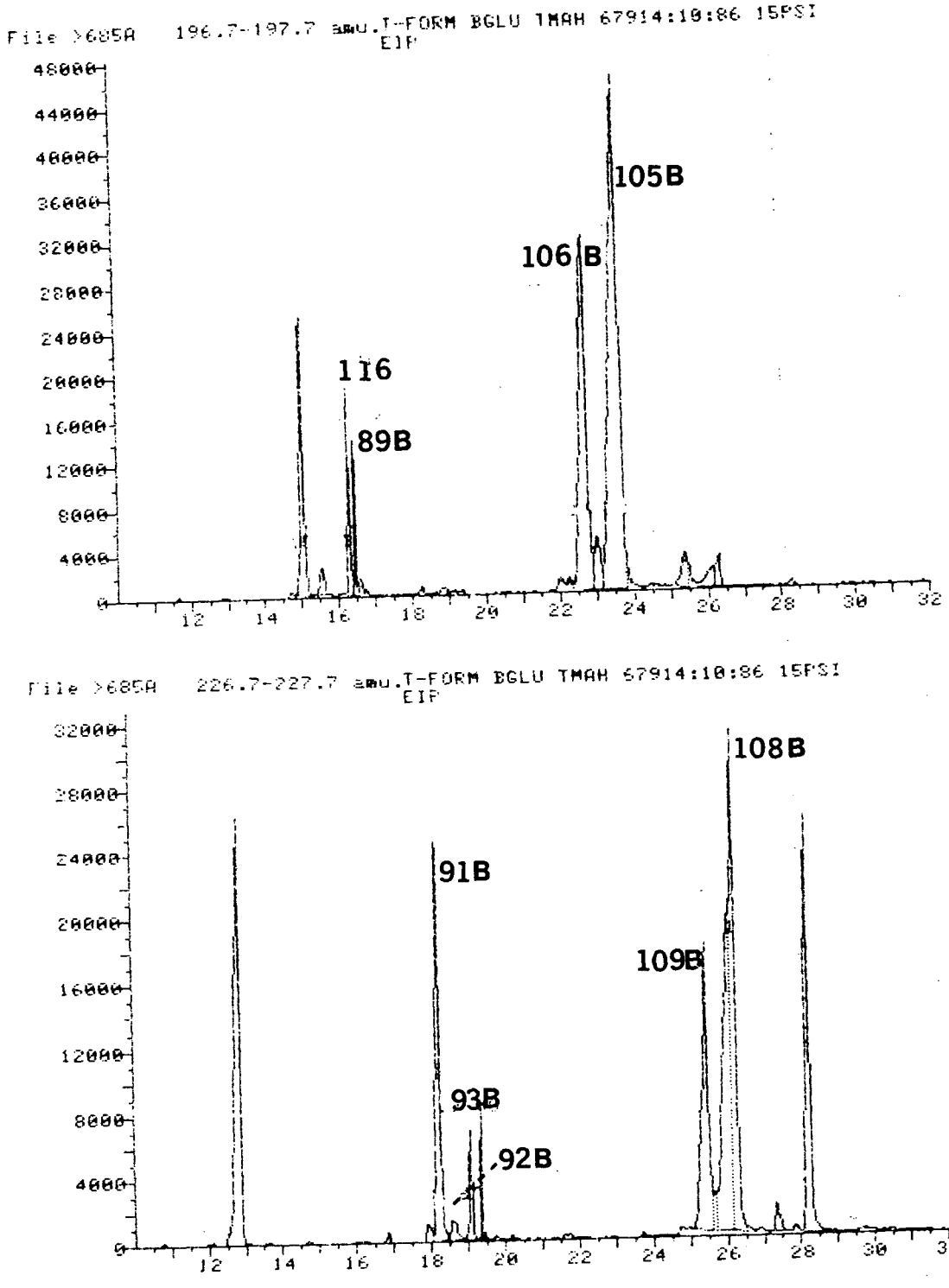
3.19. Mass spectra of tertiary formamide metabolites and their derivatives: (top) secondary formamide O-methylcatechol (109). (bottom) secondary formamide O-methylcatechol TMS (109a).



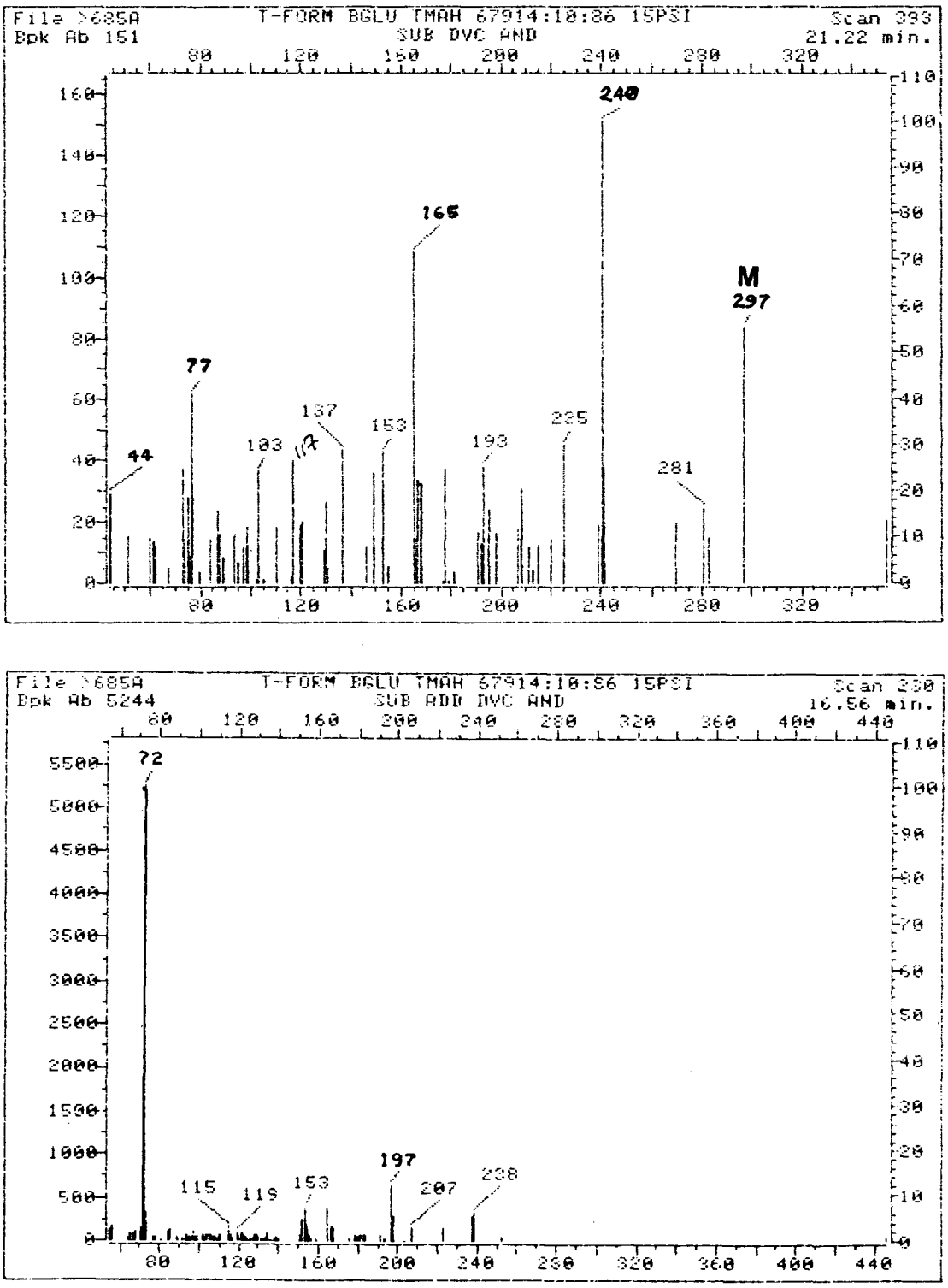
3.20. Mass spectra of tertiary formamide metabolites and their derivatives: (top) tertiary formamide O-methylcatechol (108). (bottom) tertiary formamide O-methylcatechol TMS (108a).



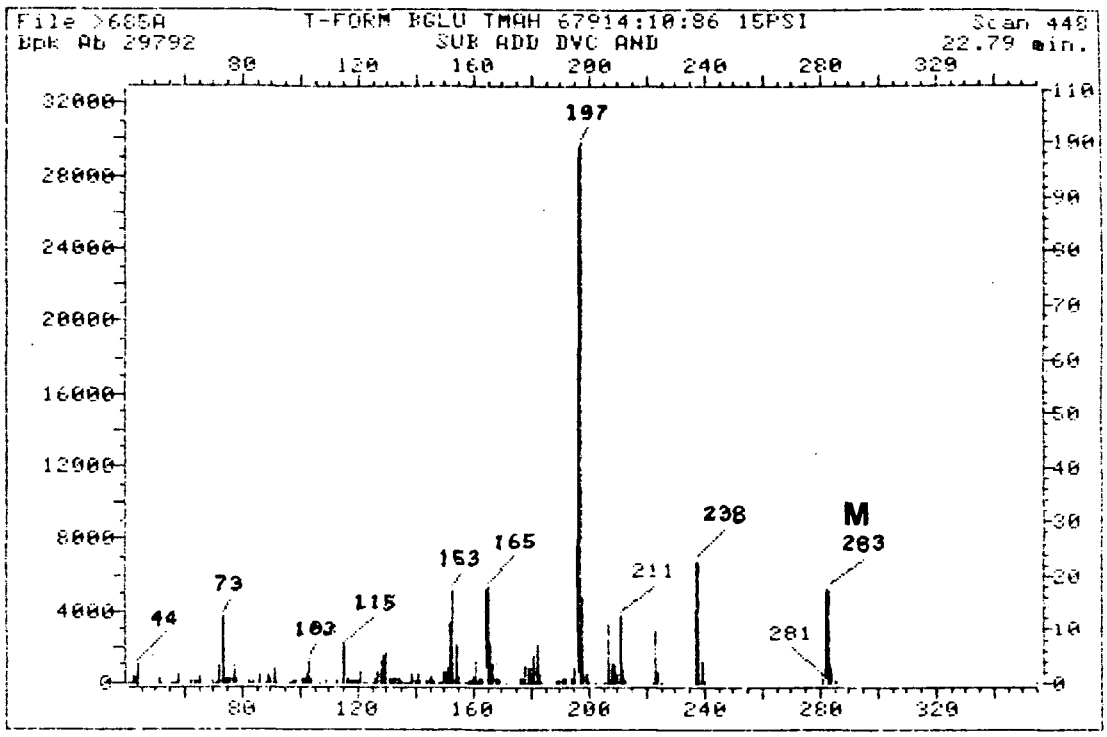
3.21. Total ion current (top) and mass chromatogram m/z 167 (bottom) for the tertiary formamide TMAH derivatized conjugated bile extract. (Retention times not reliable, subtract approx 0.5 min.)

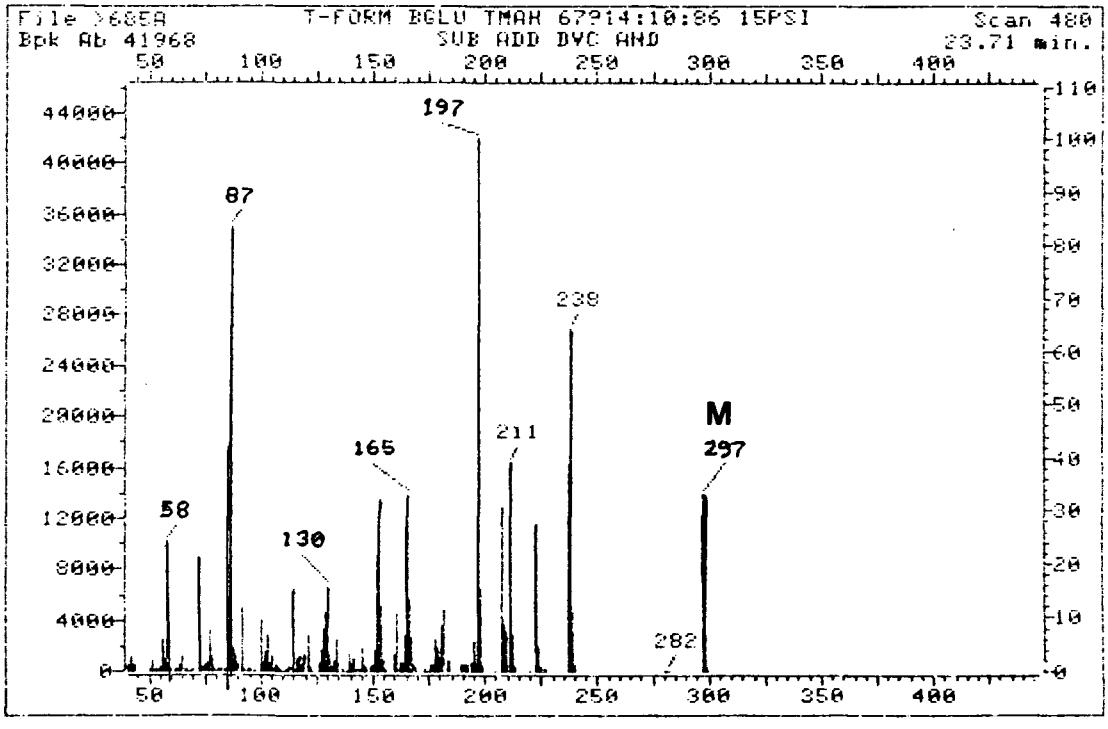


3.22. Mass chromatograms m/z 197 (top) and m/z 227 (bottom) for the tertiary formamide TMAH derivatized conjugated bile ethyl acetate extract. (Retention times not reliable, subtract approx 0.5 min.)

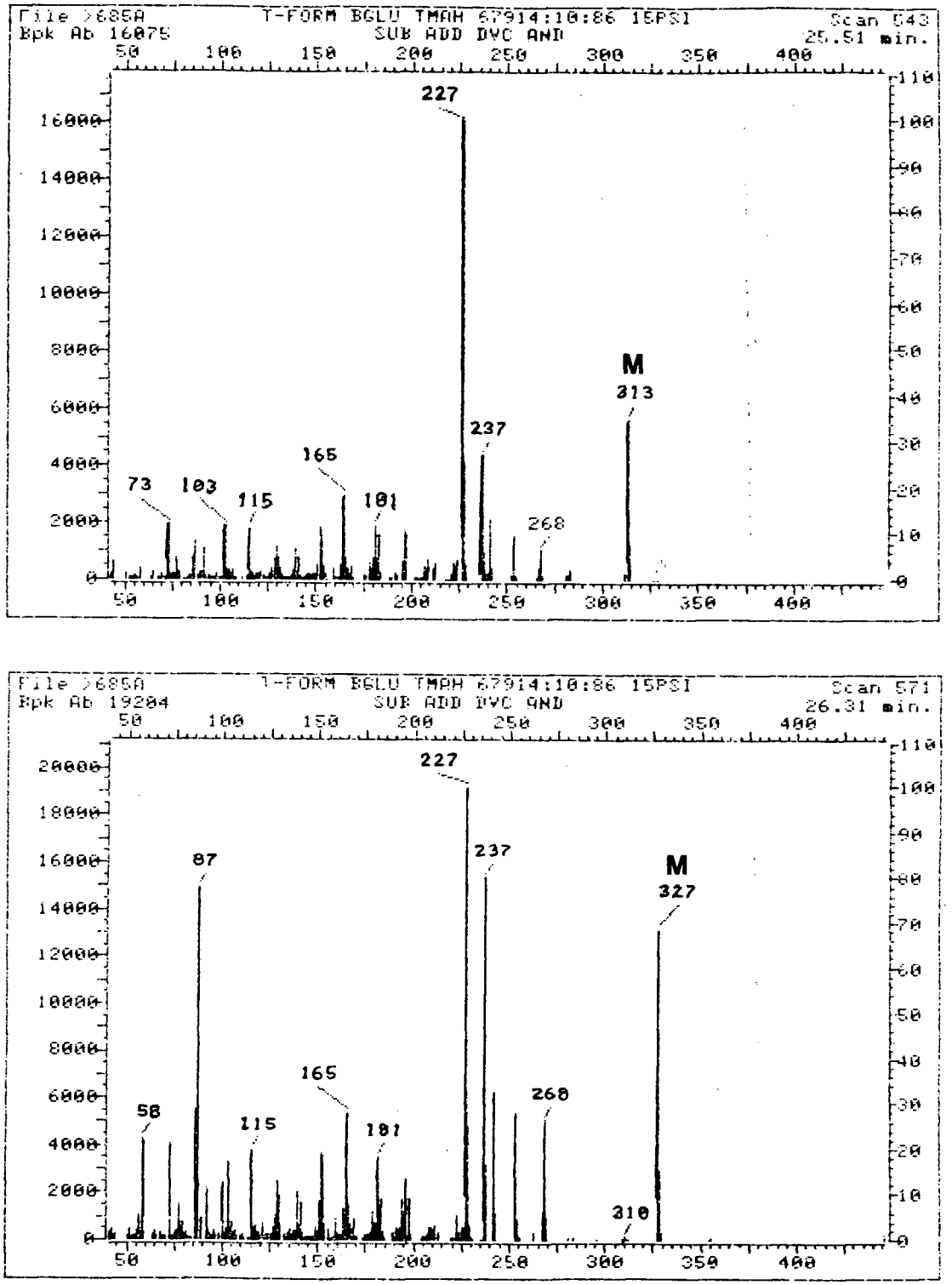


3.23. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) Suspected O-methyl derivative of carbinolamide (47). (bottom) Suspected recipavrin O-methyl phenol (116)(a norrecipavrin phenol TMAH derivative).

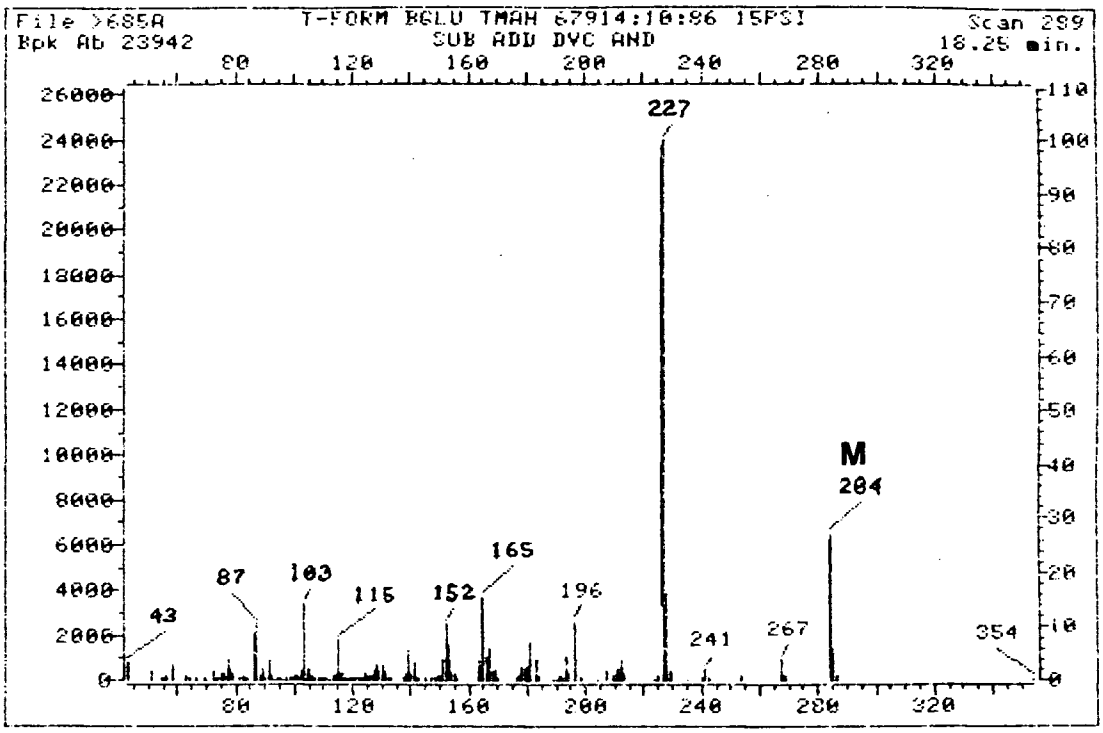


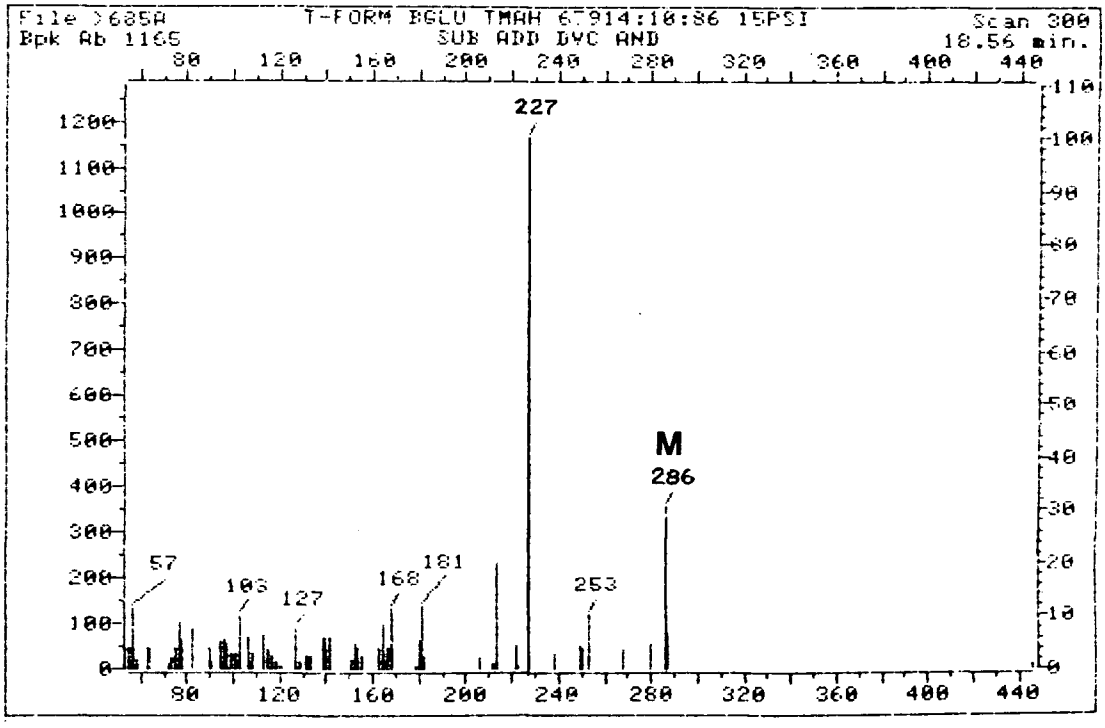


3.24. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) secondary formamide O-methyl phenol (106b). (bottom) tertiary formamide O-methyl phenol (105b).

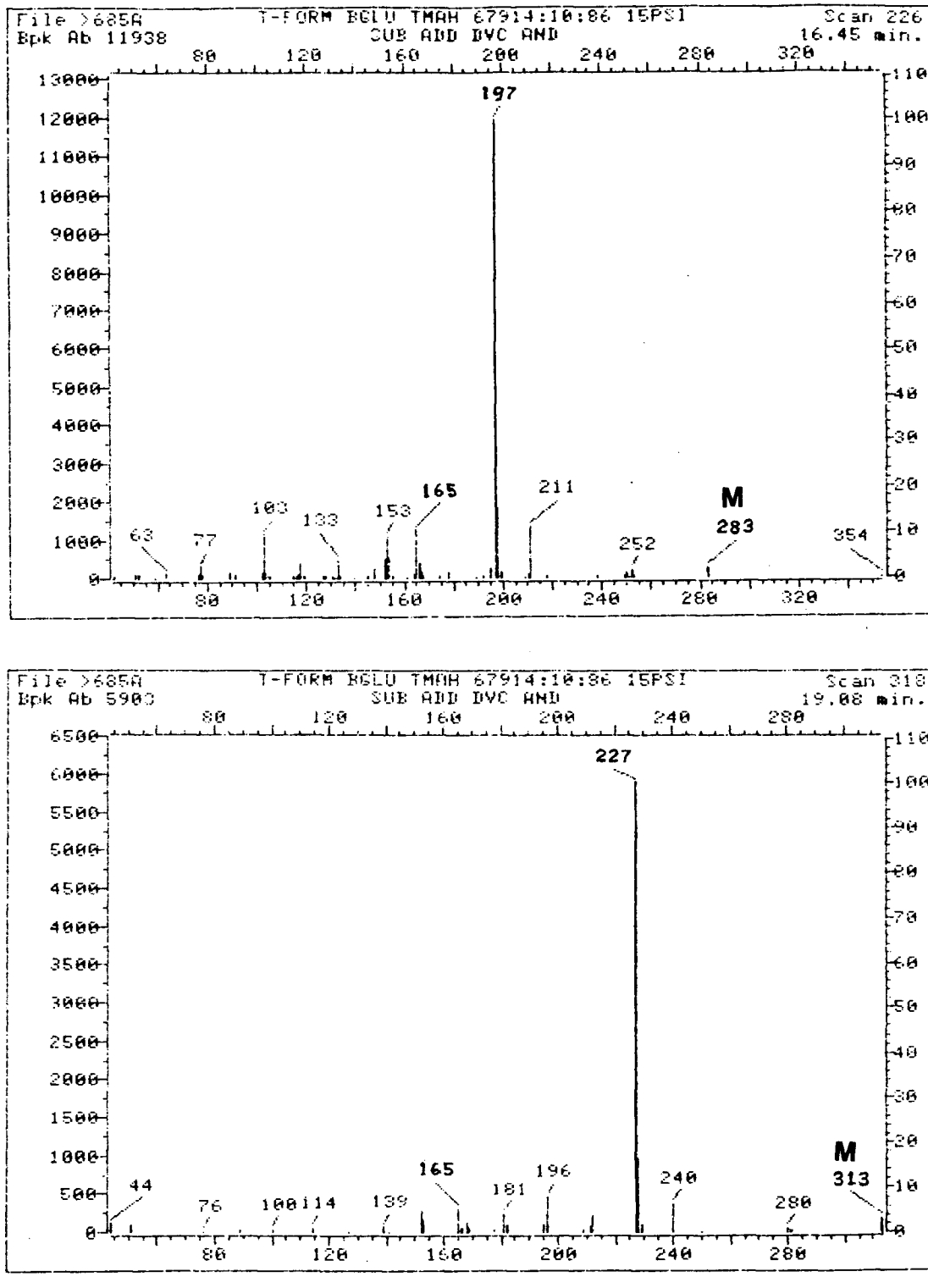


3.25. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) secondary formamide dimethylcatechol (109b). (bottom) tertiary formamide dimethylcatechol (108b).

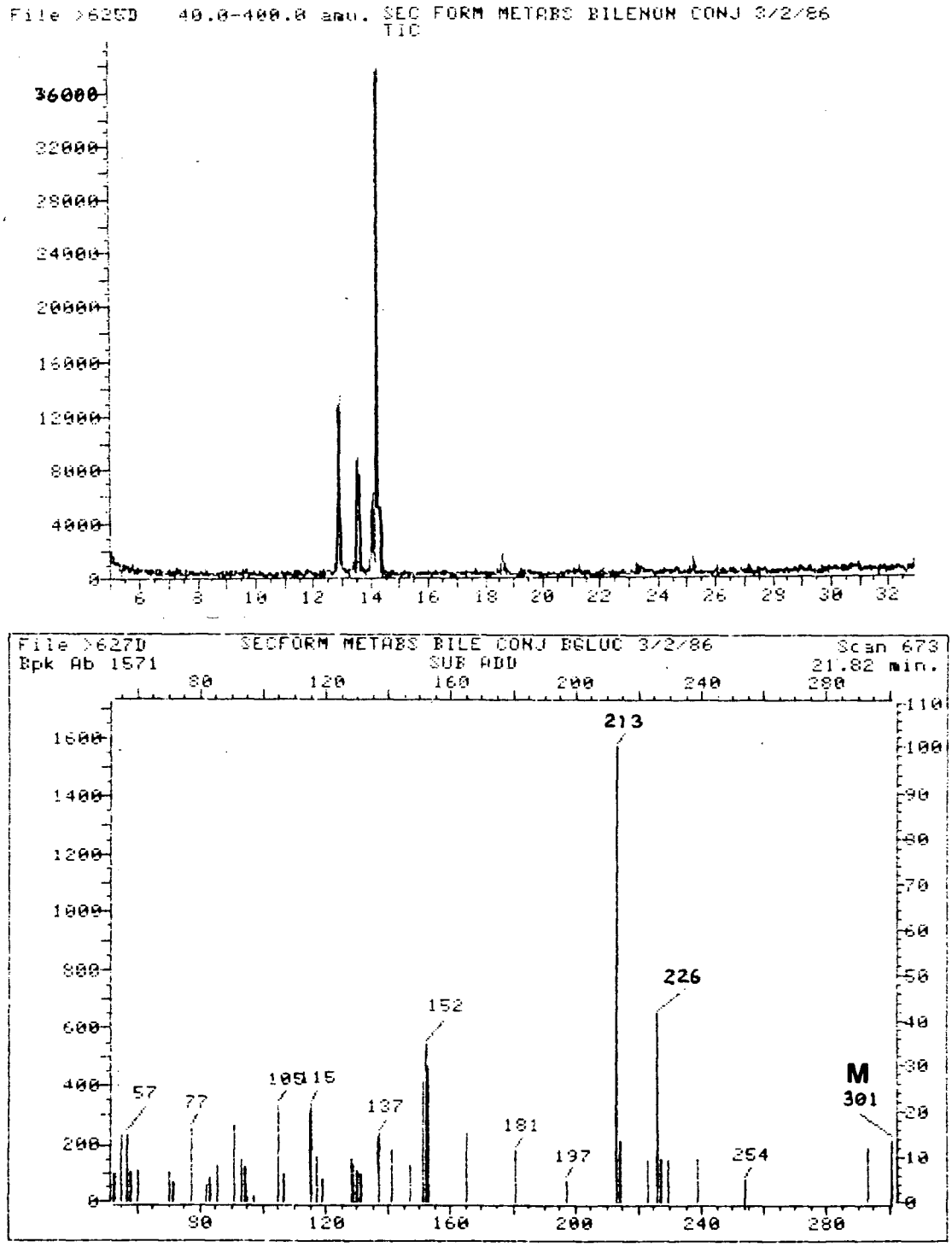




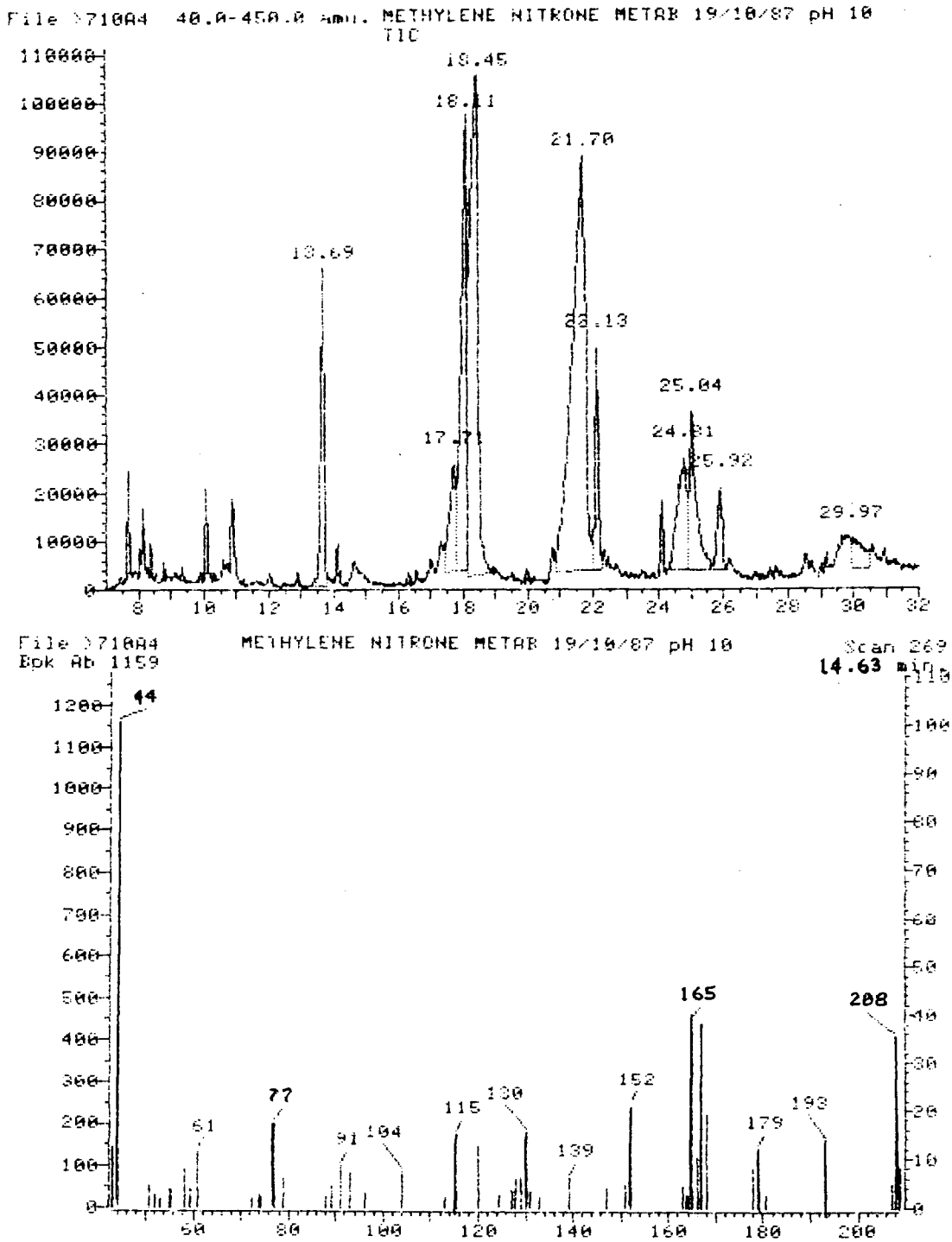
3.26. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) Diphenylbutanone dimethylcatechol (91b). (bottom) Diphenylbutanol dimethylcatechol (92b).



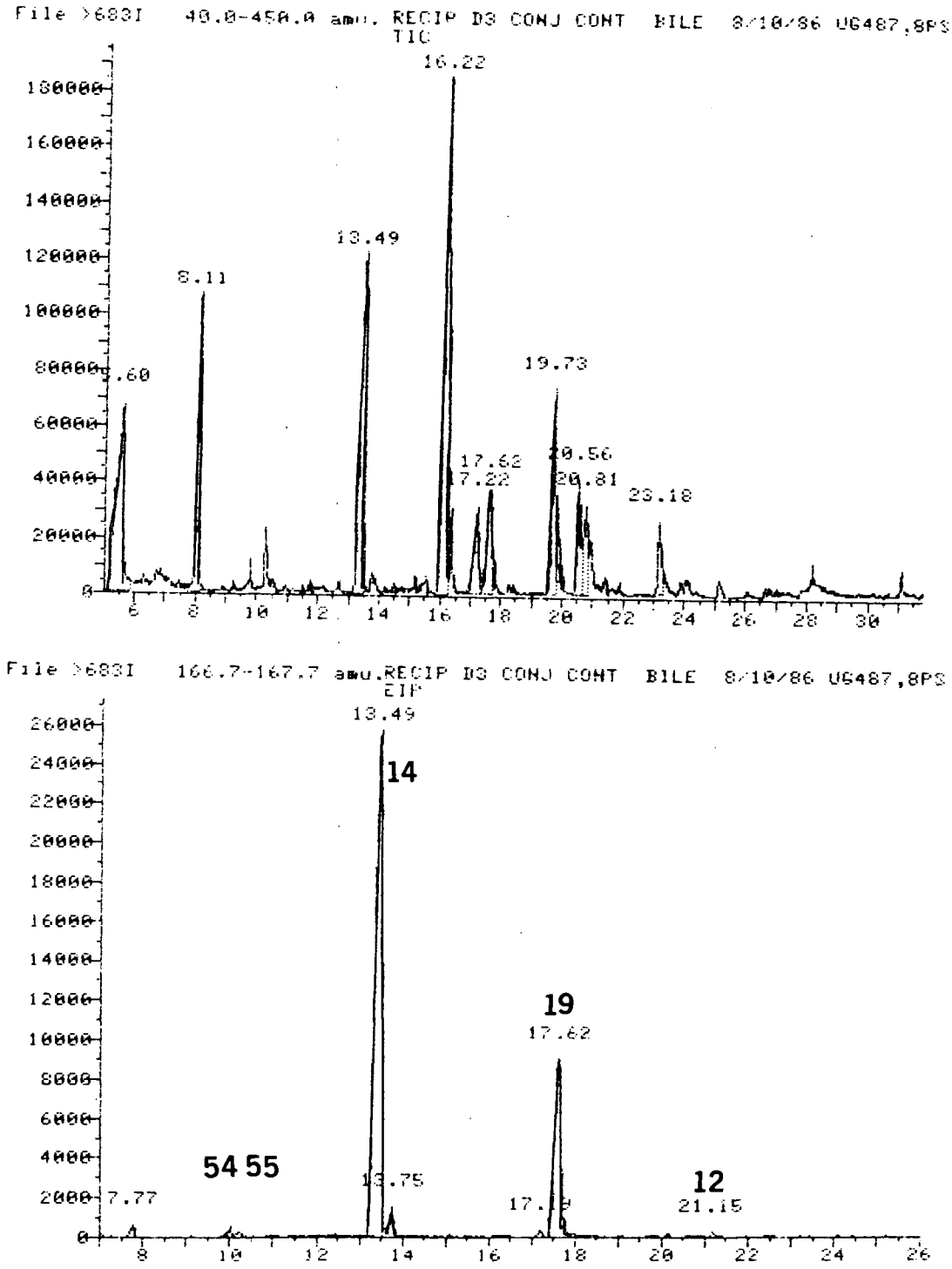
3.27. Mass spectra of TMAH derivatized tertiary formamide metabolites: (top) O-methyl oxime O-methyl phenol (89b). (bottom) O-methyl oxime dimethylcatechol (93b).



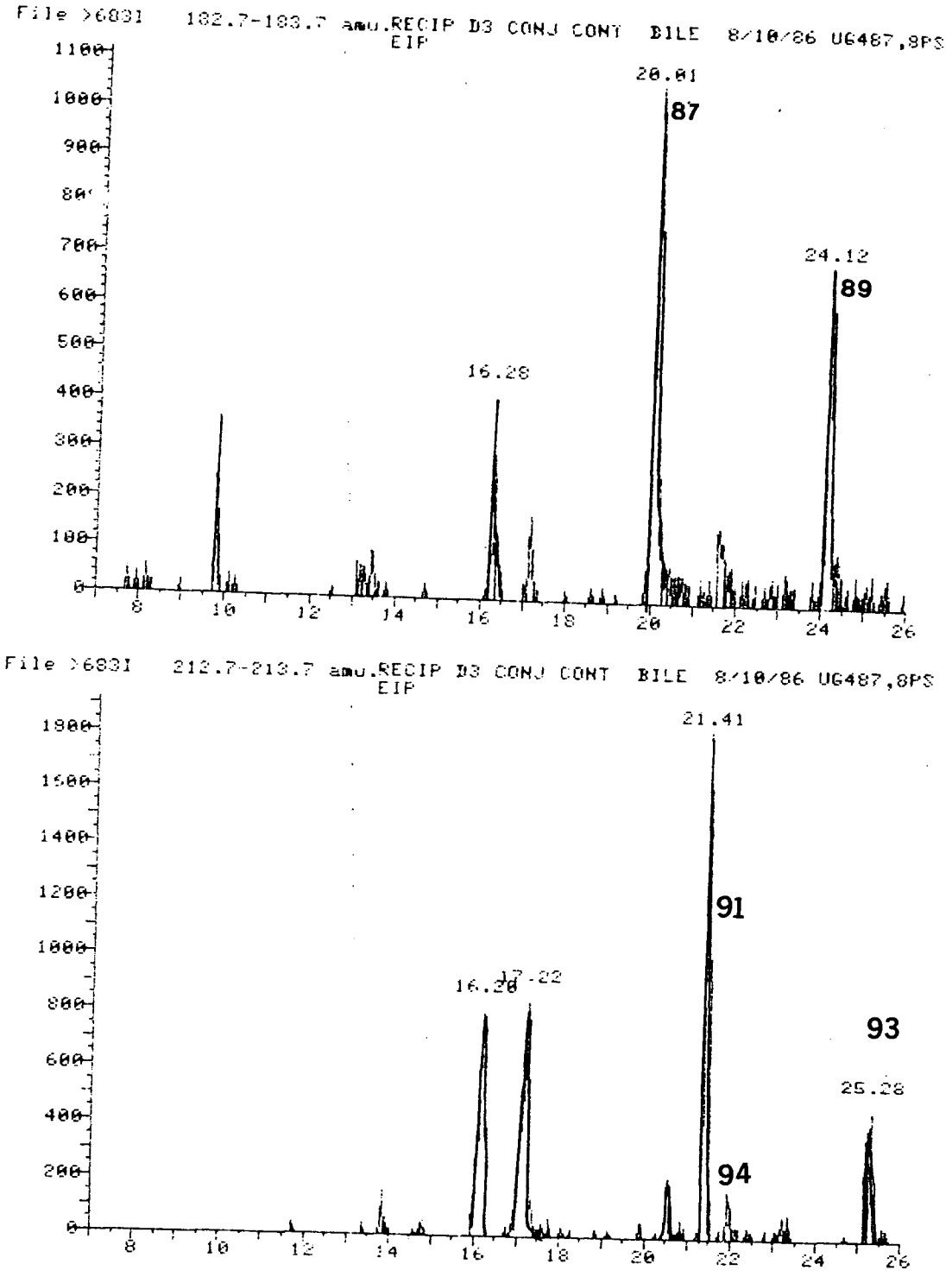
4.1 (top) Total ion current for the nonconjugated biliary metabolites of the secondary formamide (12). (bottom) Mass spectrum of the suspected nitro O-methyl catechol (98)



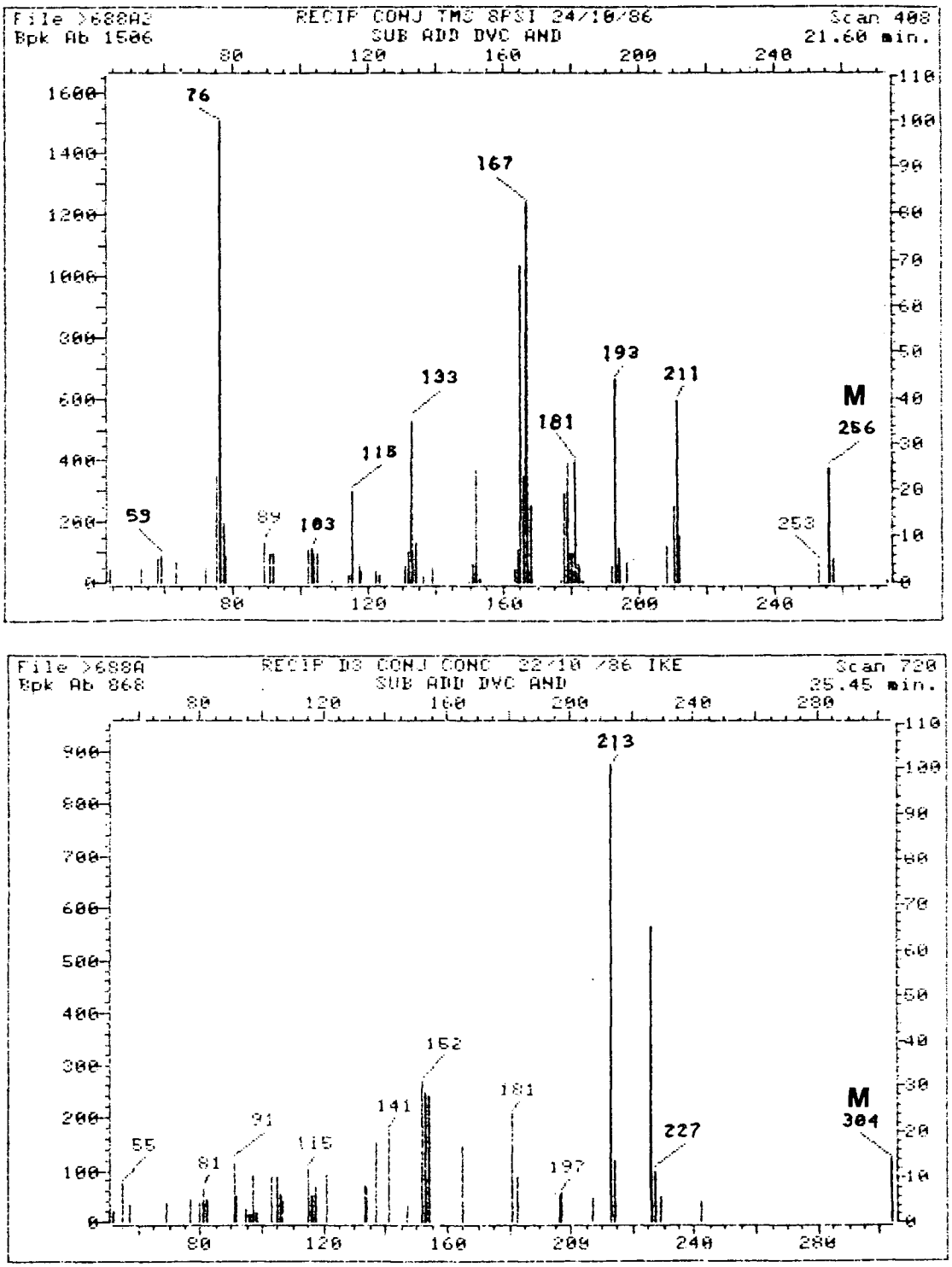
5.1 (top) Total ion current for the biliary metabolites of the methylene nitrone (24). Mass spectrum of the nitrone metabolite dinorrecipavrin (20).



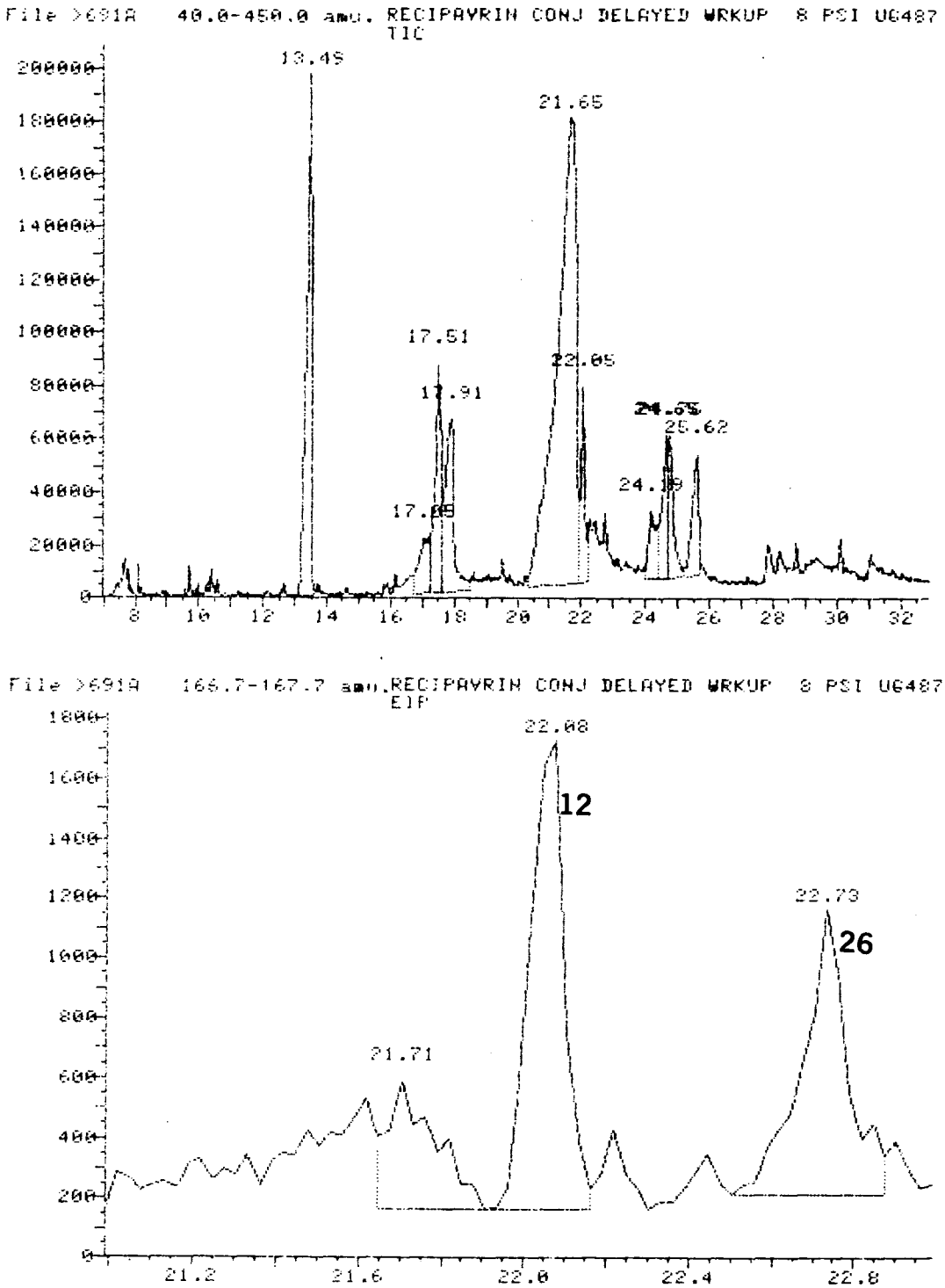
6.1. (top) Total ion current for an extract of β-glucuronidase hydrolyzed conjugated biliary metabolites of recipavrin D_3. (bottom) Mass chromatogram m/z 167 for the same sample (control experiment for the sulfatase hydrolysis experiment)



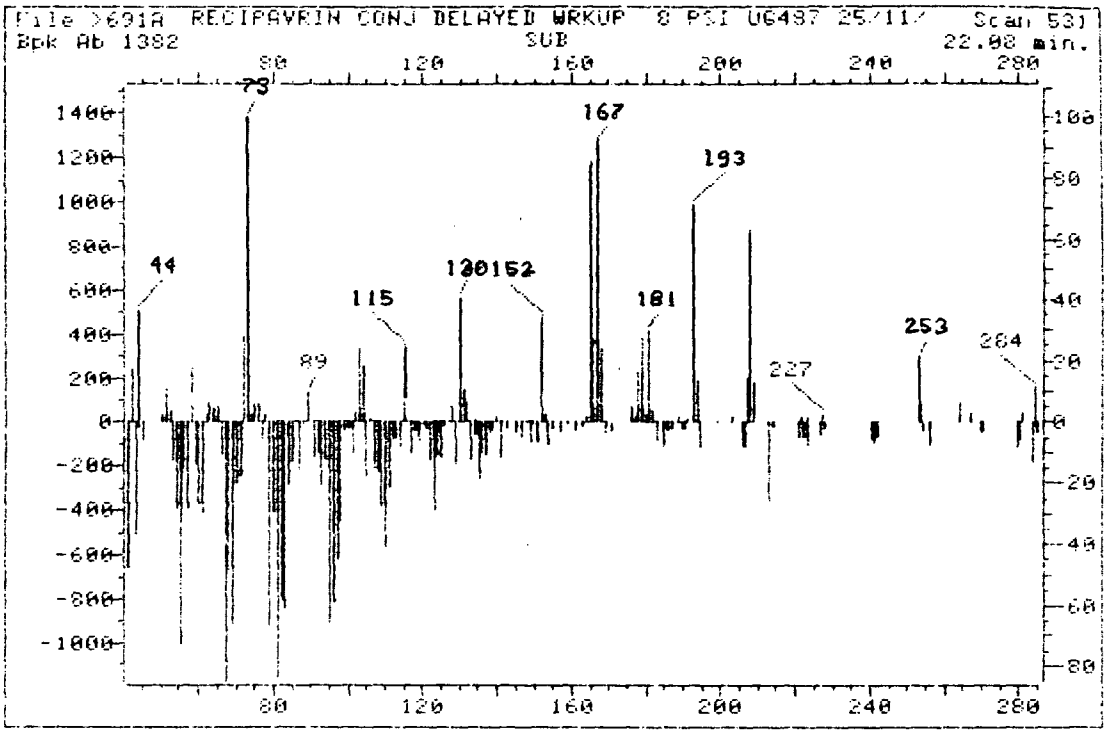
6.2 (top) Mass chromatogram m/z 187 for an extract of β-glucuronidase hydrolyzed conjugated biliary metabolites of recipavrin D_3. (bottom) Mass chromatogram m/z 213 for the same sample (control experiment for the sulfatase hydrolysis experiment)

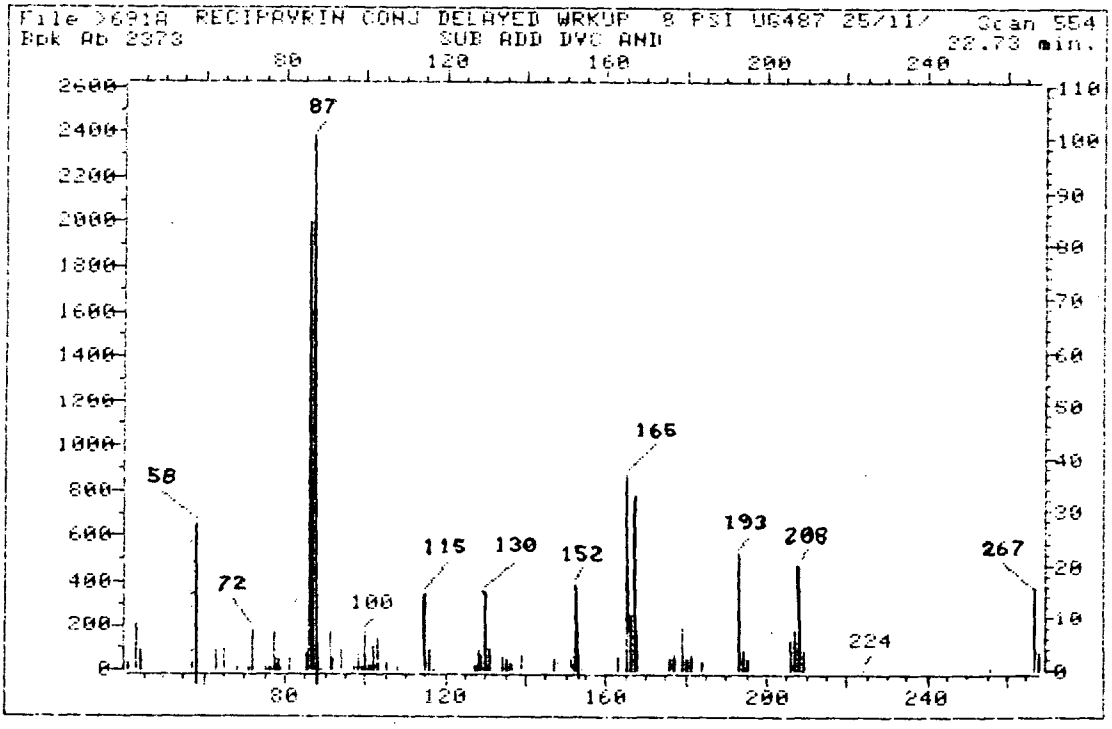


6.3. (top) Mass spectrum of the D_3 secondary formamide metabolite of recipavrin. (bottom) Mass spectrum of the D3 nitro O-methylcatechol.

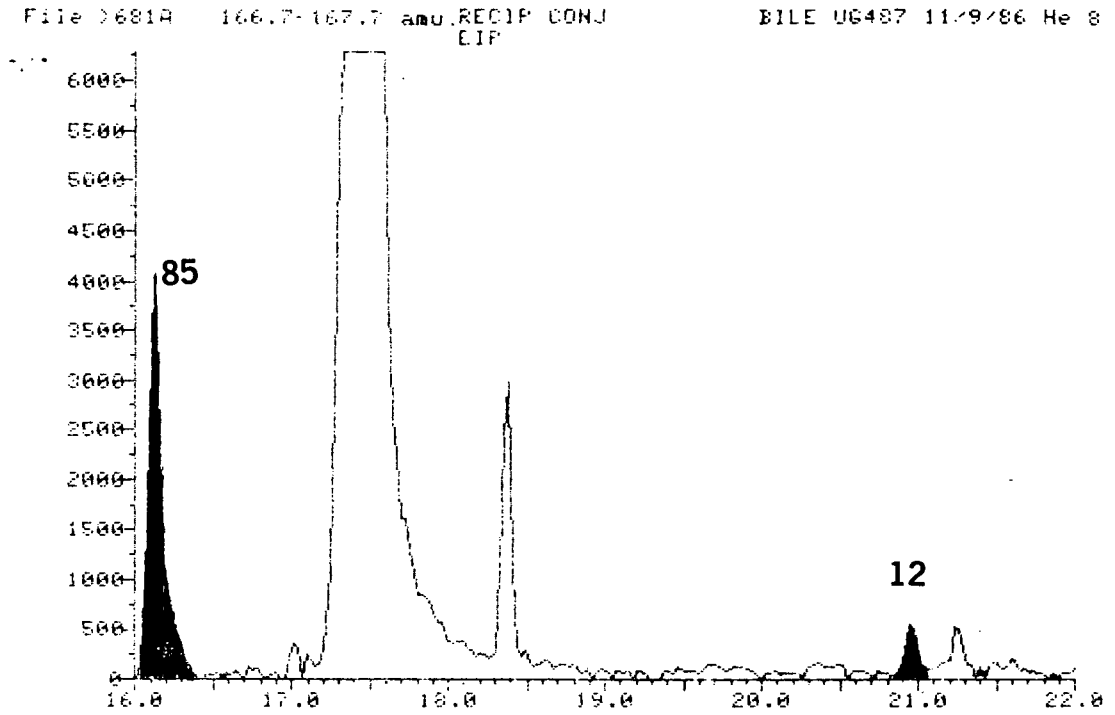


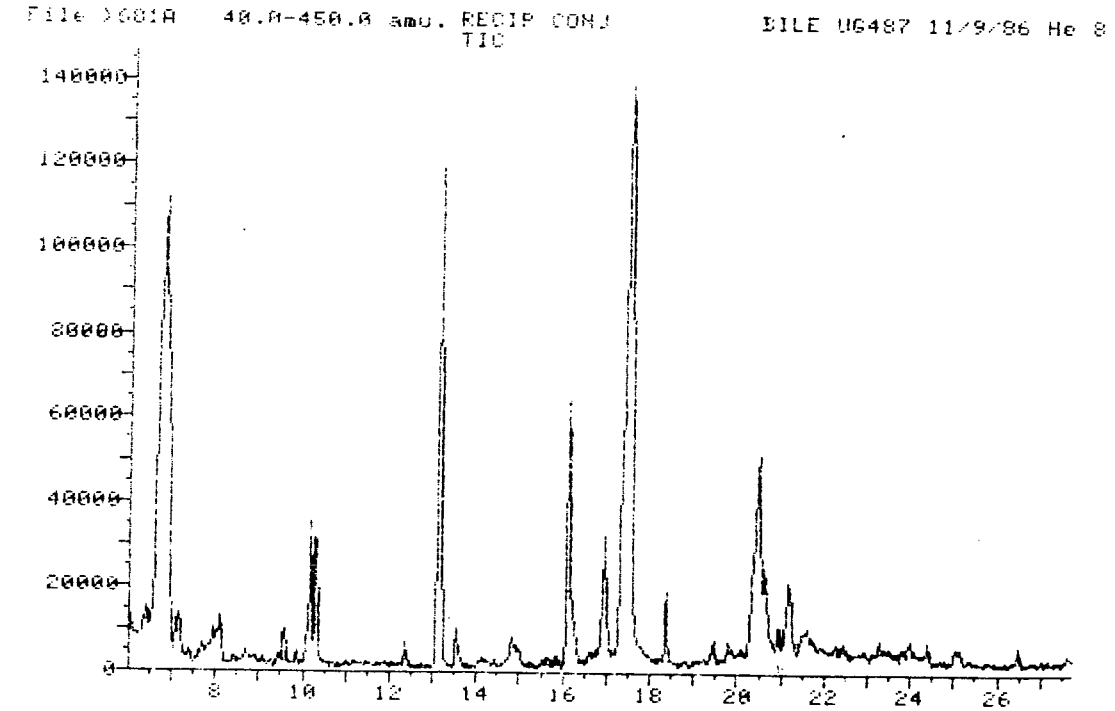
6.4. (top) Total ion current for an extract of nonconjugated biliary metabolites of recipavrin (worked up after prolonged storage at room temperature). (bottom) A close-up of mass chromatogram m/z 167 for the same sample showing both the secondary formamide (12) and tertiary formamide (26).



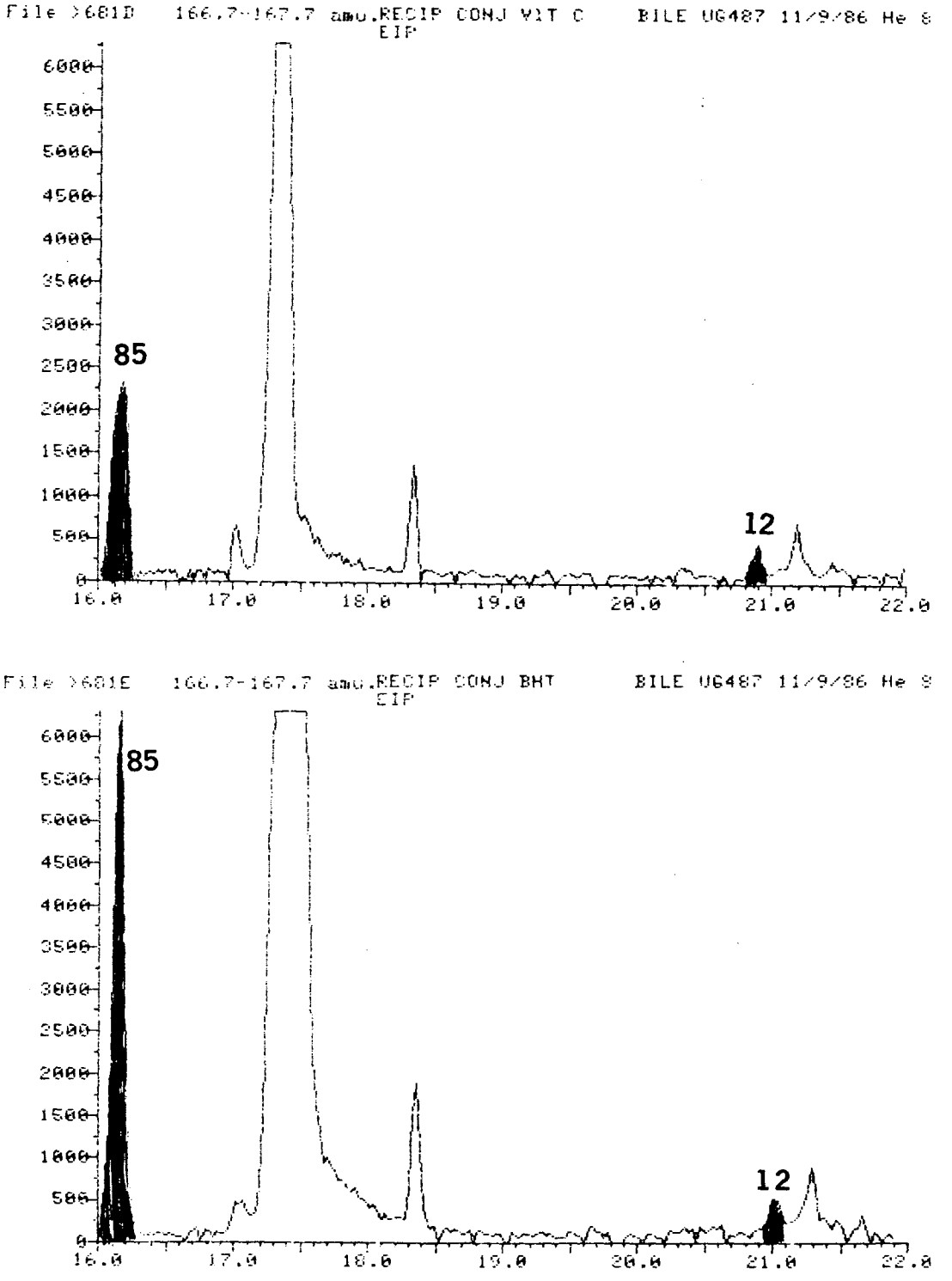


6.5. (top) Mass spectrum of the secondary formamide (12). (bottom) Mass spectrum of the tertiary formamide observed in the delayed workup sample.

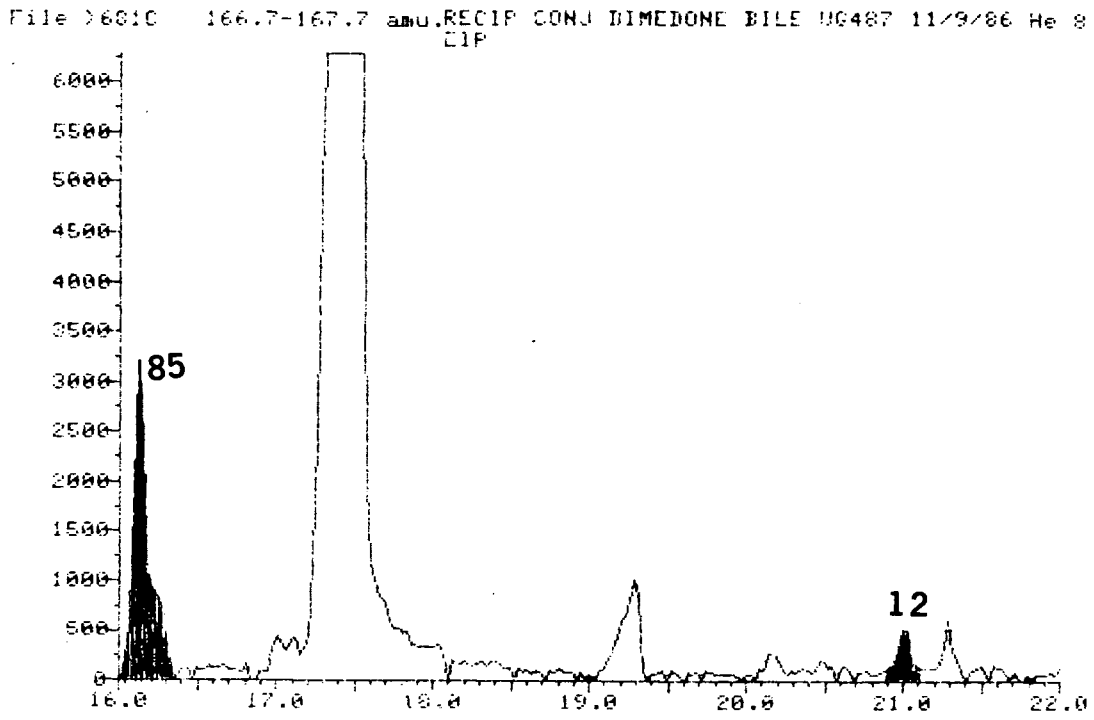


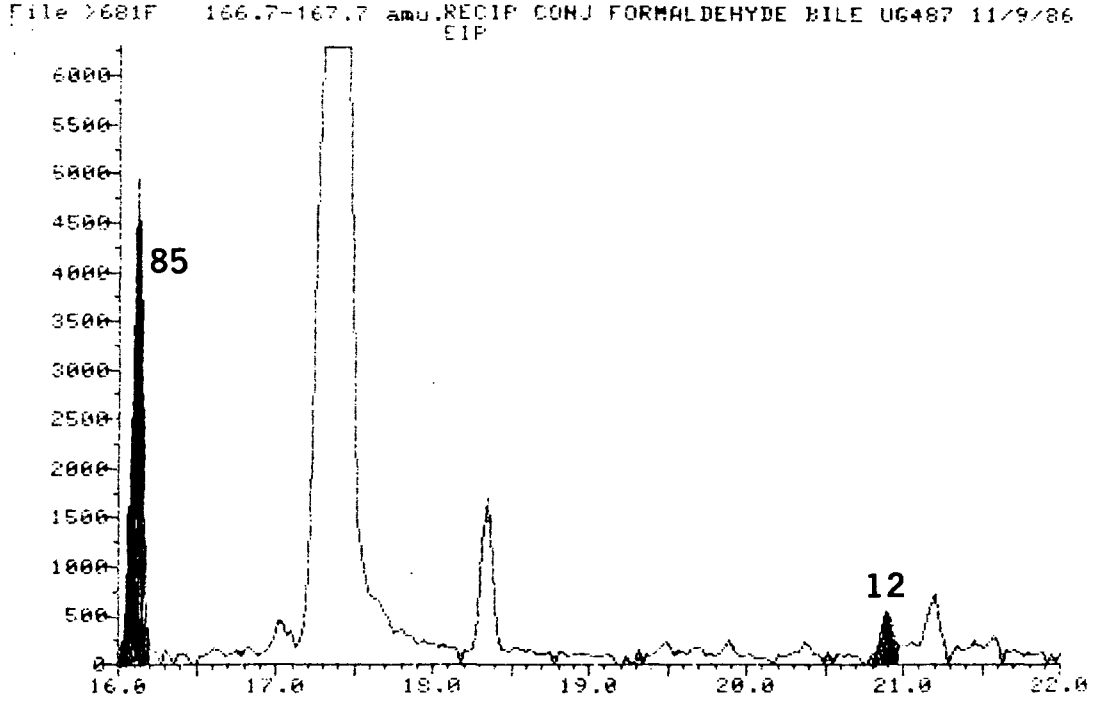


6.6. (top) Close-up of mass chromatogram m/z 167 for a control extract of β-glucuronidase hydrolyzed recipavrin metabolites. (bottom) Total ion current for the same sample.

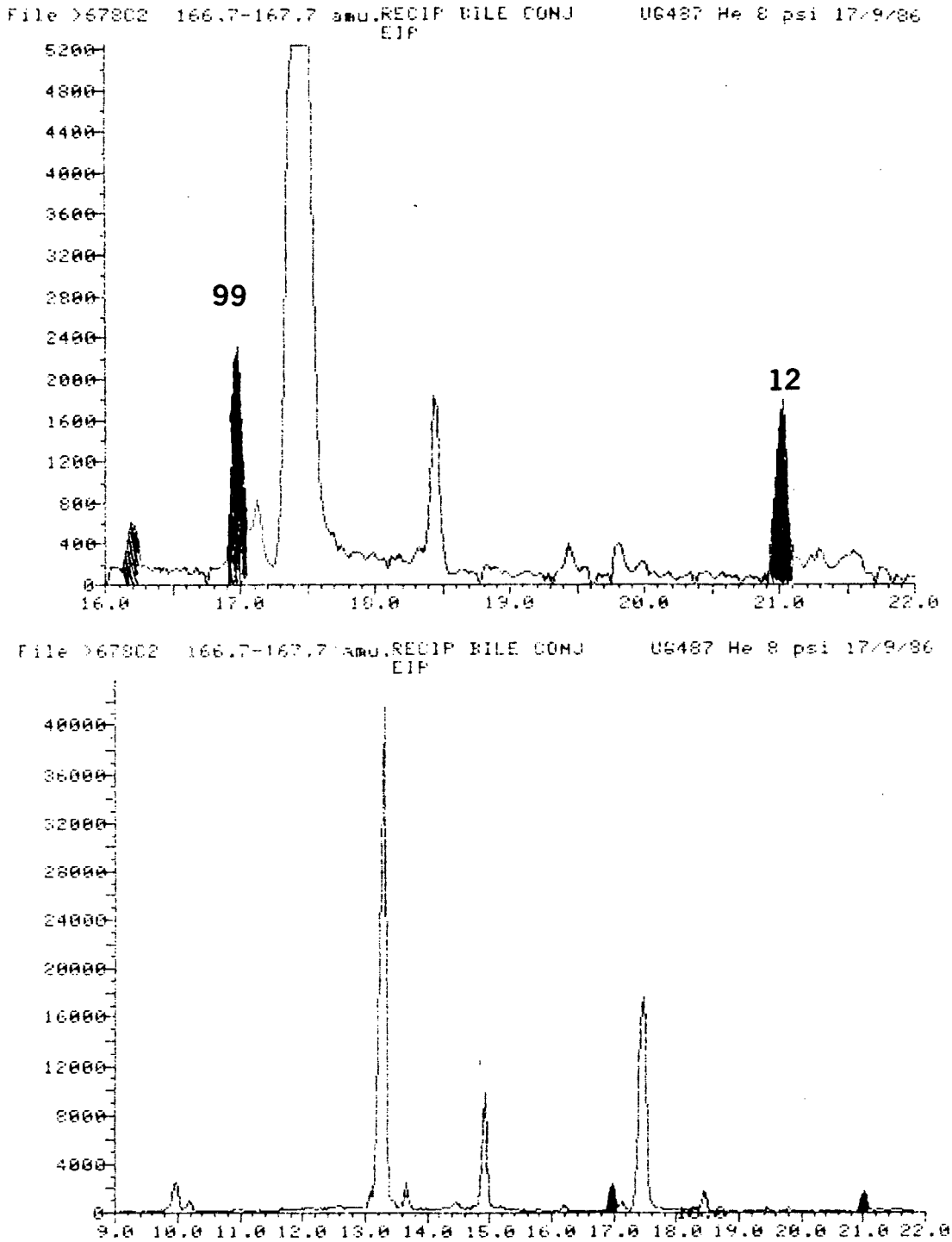


6.6.1. (top) Close-up of mass chromatogram m/z 167 for a vitamin C treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a BHT treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.

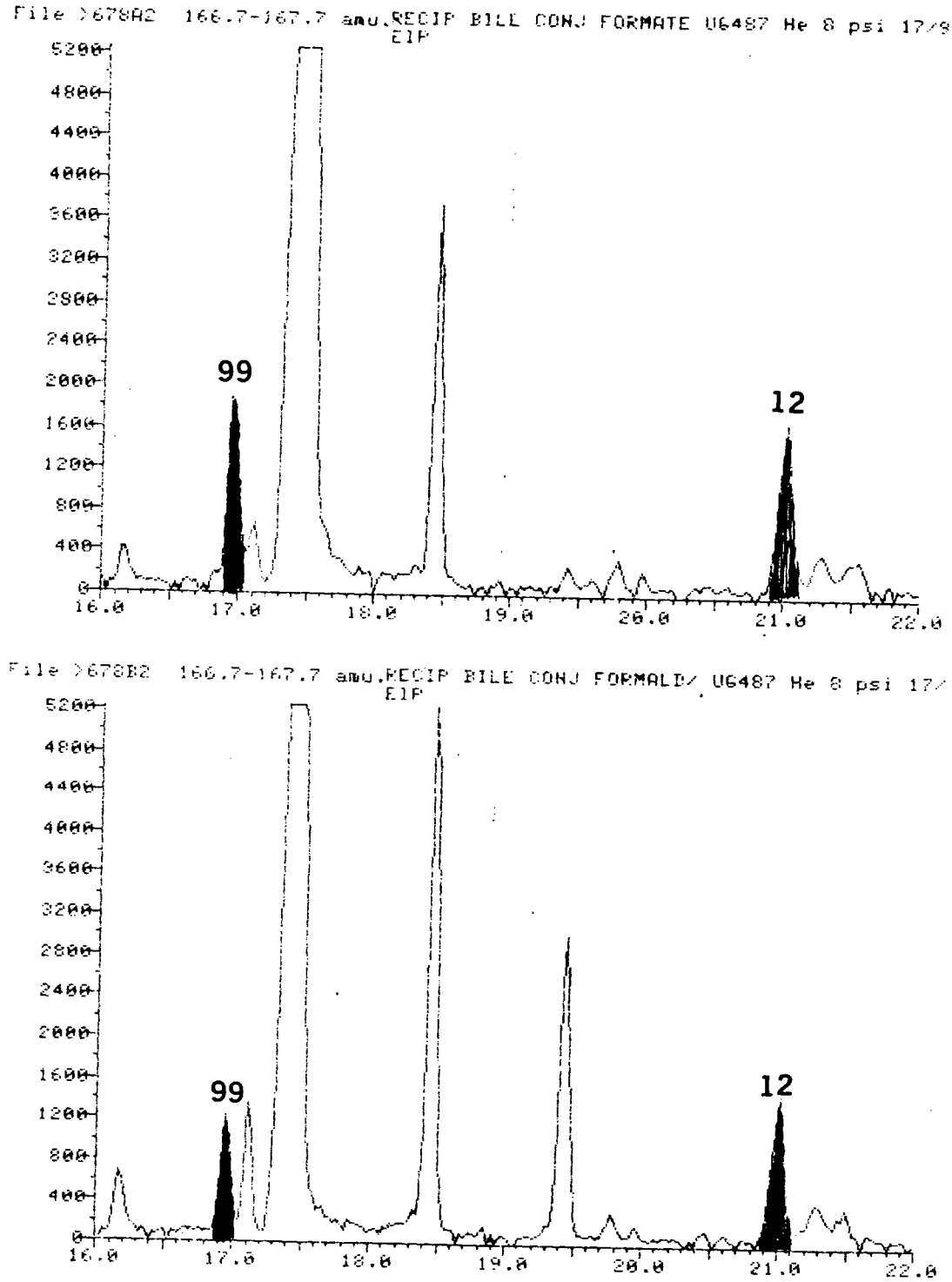




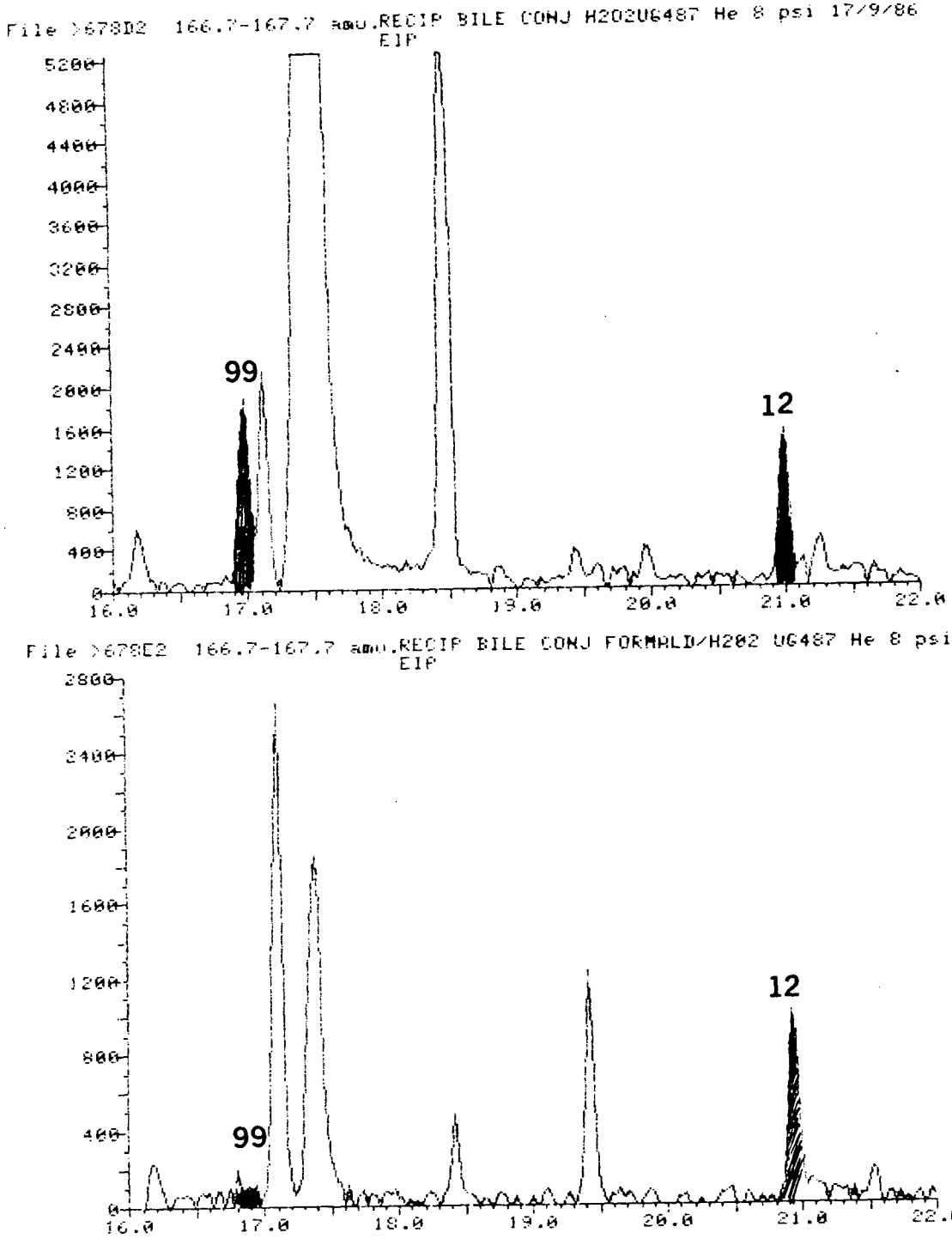
6.6.2. (top) Close-up of mass chromatogram m/z 167 for a dimedone treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a formaldehyde treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.



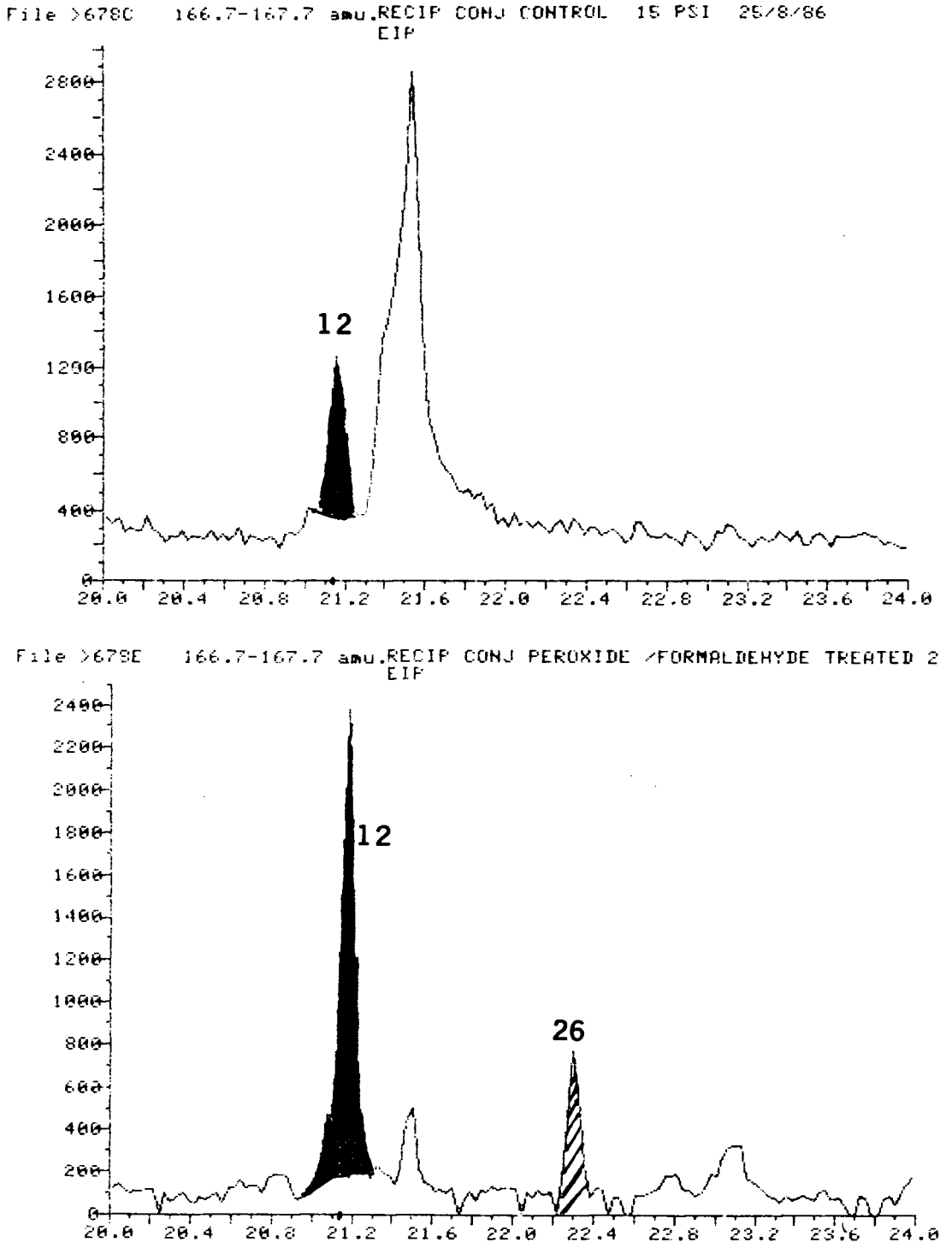
6.7. (top) Close-up of mass chromatogram m/z 167 for a control extract of β-glucuronidase hydrolyzed recipavrin metabolites. (bottom) Mass chromatogram m/z 167 for the same sample.



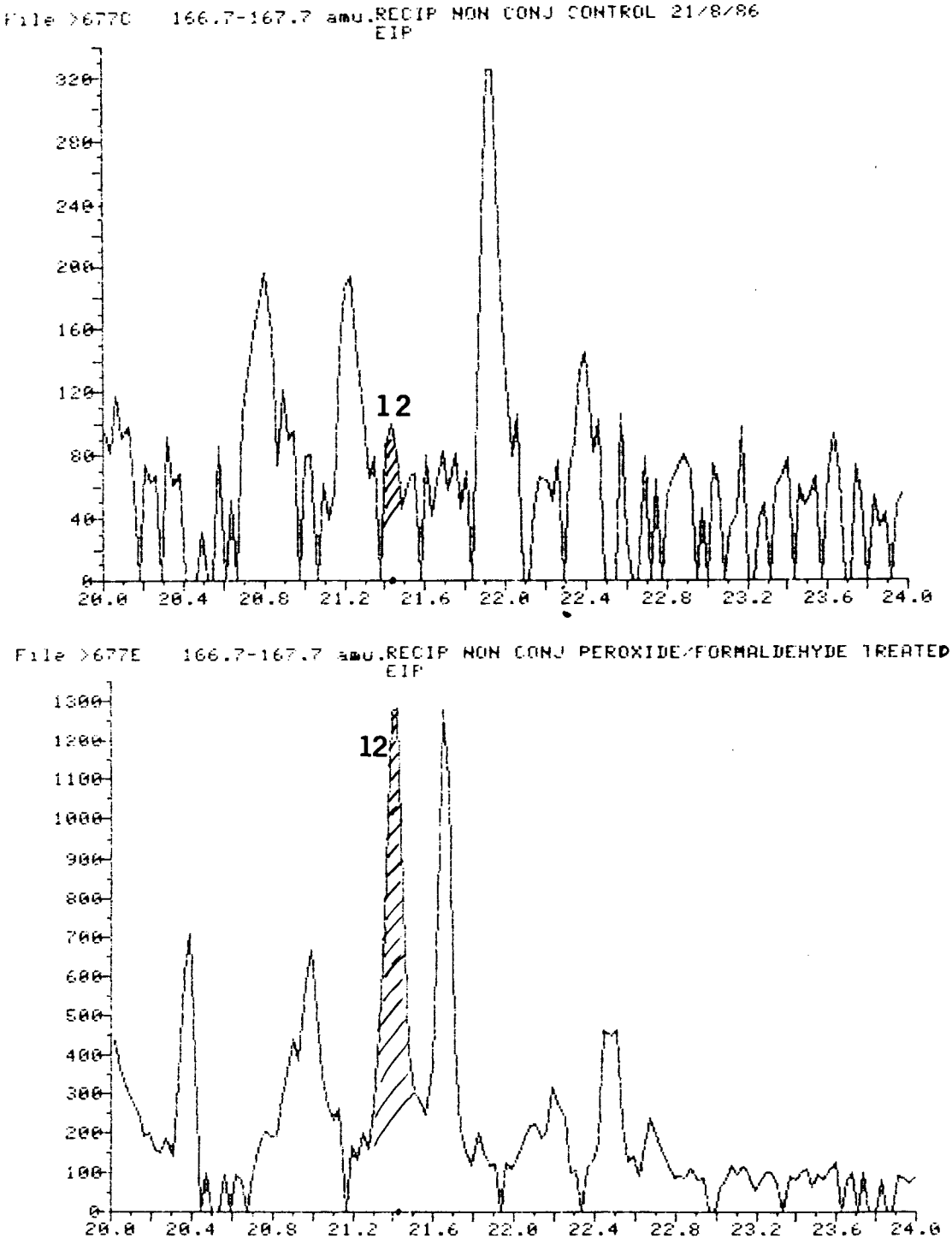
6.7.1. (top) Close-up of mass chromatogram m/z 167 for a formate treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a formaldehyde treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.



6.7.2. (top) Close-up of mass chromatogram m/z 167 for a hydrogen peroxide treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Close-up of mass chromatogram m/z 167 for a formaldehyde and hydrogen peroxide treated extract of β-glucuronidase hydrolyzed recipavrin metabolites.



6.8. (top) Mass chromatogram m/z 167 for a control extract of β-glucuronidase hydrolyzed recipavrin metabolites.(bottom) Mass chromatogram m/z 167 for a formaldehyde and hydrogen peroxide treated extract of β-glucuronidase hydrolyzed recipavrin metabolites, showing the secondary formamide (solid) and tertiary formamide (hatched).



6.9. (top) Mass chromatogram m/z 167 for a control extract of nonconjugated recipavrin metabolites.(bottom) Mass chromatogram m/z 167 for a formaldehyde and hydrogen peroxide treated extract of nonconjugated recipavrin metabolites showing the secondary formamide (hatched).

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