In Vitro and In Vivo Studies on the Biotransformation of β-Nicotyrine, a Minor Tobacco Alkaloid

by

Xin Liu

DISSERTATION

Submitted to the Faculty of the

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry

APPROVED

Dr. Neal Castagnoli, Jr., Chairman

Dr. David G. I. Kingston

Dr. Judy S. Riffe

Dr. Joseph S. Merola

Dr. Robert H. White

June, 1995

Blacksburg, Virginia
2D
5655
V858
1995
L583
2.2
IN VITRO AND IN VIVO STUDIES ON THE 
BIOTRANSFORMATION OF β-NICOTYRINE, A MINOR 
TOBACCO ALKALOID 

Xin Liu 

Dr. Neal Castagnoli, Jr., Chairman 

Chemistry 

ABSTRACT 

β-Nicotyrine is a minor tobacco alkaloid found in both tobacco plants and tobacco smoke. Preliminary studies have shown that β-nicotyrine is pneumotoxic and that the toxicity is mediated via its metabolites formed in reactions catalyzed by cytochrome P-450. The in vitro metabolic fate of β-nicotyrine has been examined in rat liver microsomal preparations and rabbit lung and liver microsomal preparations utilizing HPLC UV-diode array analysis. Four metabolites have been identified from the incubation mixtures of β-nicotyrine with rabbit lung and liver microsomal preparations. The primary in vitro metabolites are two unstable pyrrolinone species, 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one, which exist in equilibrium. The pyrrolinones undergo autoxidation to form a secondary metabolite, 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one, and hydrolysis to form 5'-hydroxycotinine. The autoxidation is likely to involve a free radical process. Evidence to support this proposal has been obtained by studies on 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole, a latent form of the pyrrolinones. Free radicals generated following hydrolysis of the acetoxypyrrole derivative have been trapped and detected by ESR analysis by spin trapping techniques. In contrast, only the starting substrate β-nicotyrine was found in the incubation mixture of β-nicotyrine with rat liver microsomes. 

The in vivo metabolic fate of β-nicotyrine has been examined in rabbits and mice by HPLC UV-diode array and GC-EIMS analysis of urine extracts. Three metabolites have been identified. Besides 5'-hydroxycotinine and 5-
hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one, observed in the in vitro studies, 3'-hydroxycotinine is also found and determined to be the principal urinary metabolite of β-nicotyrine in both species. The stereochemistry of this 3'-hydroxycotinine is cis. This is in contrast to the trans stereochemistry of the urinary metabolite of (S)-cotinine and (S)-nicotine. The mechanism of formation of cis-3'-hydroxycotinine from β-nicotyrine is proposed to involve a free radical process leading to the formation of the precursor 3-hydroxy-1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one. This intermediate then undergoes a carbon-carbon double bond reduction to yield the final product. This proposal is supported by in vivo studies on 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one which undergoes reduction to 5'-hydroxycotinine. Furthermore, 2-acetoxyl-1-methyl-5-(3-pyridyl)pyrrole goes to cis-3'-hydroxycotinine as the major urinary metabolite.

The results of these studies point to a potentially novel metabolic bioactivation pathway of (S)-nicotine that could be relevant to some of the toxic effects observed in chronic tobacco users.
To

My Mother and the Memory of My Father

for

Their Love, Nurturing and Moral Guidance

Especially in Those Bleak Years
Acknowledgements

I would like to express my gratitude from the bottom of my heart to my advisor, Dr. Neal Castagnoli, Jr., for his encouragement, support and guidance both in chemistry and in life, especially for his humor and the joyful discussions on Marco Polo.

My heartfelt appreciation goes to my committee member Dr. David G. i. Kingston, for his friendship and understanding, encouragement and help in many different ways.

My true appreciation also goes to my committee members, Dr. Joseph S. Merola, Dr. Judy S. Riffee, Dr. Robert H. White and Dr. James F. Wolfe, for their kind criticisms, advice and helpful suggestions.

Uncountable thanks go to my labmates, the members of Dr. Castagnoli’s research group, for their daily help, discussions, assistance and the friendly working atmosphere. Special thanks go to Dr. Kazuo Igarashi and Dr. You-Xiong Wang, for their friendship and sincere help, and to Mrs. Kay Castagnoli for her teaching me the highly energetic approach to science.

My thanks go to all of the Faculty and Staff in the Department of Chemistry, Virginia Tech, for the knowledge they taught me and the help they gave me to support the completion of my degree.

My sincere thanks also go to Mr. and Mrs. Robert McDuffie, for their family-like love and enthusiasm; to Mrs. Beulah Kline, my American mother, for her endless love, concern and help; to my best friends, Dr. James V. Petersen, Dr. Ipin Guo, Dr. Zhiyang Zhao, Dr. Lunyi Zang and Dr. Qin Liu, for their friendship and help in many ways.

Finally, I would like to acknowledge my sisters Xiuli Lü and Min Liu, for their endless love, understanding, encouragement and support.
List of Contents

List of Schemes xi
List of Tables xv
List of Figures xvi
Index of Compound Structures xxi
List of Compound Structures xxv
List of Abbreviations xxxiii

Chapter 1. Introduction and Literature review 1
1.1. Past and Present Day Uses of Tobacco 1
   1.1.1. Early Reports 2
   1.1.2. Medical Uses of Tobacco 3
   1.1.3. Agricultural Use of Tobacco 6
   1.1.4. Recreational Tobacco Use 8
   1.1.5. Health Related Problems 8
   1.1.6. Air Pollution 11
1.2. Tobacco Alkaloids 12
1.3. Biotransformation of the Minor Tobacco Alkaloids 14
   1.3.1. Metabolic Fate of Nornicotine (4) 14
   1.3.2. Metabolic Fate of (S)-Cotinine (3) 15
   1.3.3. Formation and Metabolism of Mycsmine (5) 19
   1.3.4. Metabolism of Anabasine (9) 20
1.4. β-Nicotyrine 20
   1.4.1. Physicochemical Properties and Absorption of β-Nicotyrine 20
   1.4.2. Sources of β-Nicotyrine 21
   1.4.3. Bioactivity of β-Nicotyrine 24
   1.4.4. Metabolic Characteristics of β-Nicotyrine 25
      1.4.4.1. The Pyridine Ring 25
      1.4.4.2. The Pyrrole Moiety 27
1.5. Research Proposal 34
References 36
Chapter 2. In Vitro Studies on the Metabolism of β-Nicotyline 54

2.1. Background 54

2.2. Results and Discussion 57

2.2.1. Chemistry of β-Nicotyline Metabolites 57

2.2.1.1. Synthesis of β-Nicotyline 61

2.2.1.2. Synthesis of 5’-Hydroxocotinine (20) and 5-Hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) 66

2.2.1.3. Synthesis of 1-Methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-Methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51) 84

2.2.1.4. Synthesis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) 96

2.2.1.5. Hydrolysis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) 111

2.2.1.6. ESR Studies on the Hydrolysis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) 137

2.2.2. In Vitro Metabolism 152

2.2.2.1. HPLC Analysis 153

2.2.2.2. GC-EIMS Analysis 154

2.2.2.3. Identification and Confirmation of the Metabolites of β-Nicotyline Found in the In Vitro Studies 157

2.2.2.4. In Vitro Studies on the Metabolism of β-Nicotyline with Rat Liver Microsomes 164

2.2.2.5. Summary of the In Vitro Studies 165

2.3. Conclusions from the In Vitro Studies 167

2.4. Materials and Methods 168

2.4.1. Chemicals 168

2.4.2. Analytical Instruments 168

2.4.3. Source of Tissues 170

2.4.4. Syntheses 170

2.4.4.1. 5-Methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (58) 170

2.4.4.2. 5’-Methoxocotinine (59) 171

2.4.4.3. 5’-Hydroxocotinine (20) 171
2.4.4.4. 5-Hydroxy-1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (52)  172
2.4.4.5. 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74)  172
2.4.4.6. 1-Methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrroline-2-one (76)  173
2.4.4.7. 1-Methyl-5-(3-pyridyl)-4-pyrroline-2-one (48) and 1-Methyl-5-(3-pyridyl)-3-pyrroline-2-one (51)  174
2.4.4.8. β-Nicotyrine Free Base and Its Tartrate Salt  175

2.4.5. Hydrolysis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74)  176
2.4.6. ESR Studies of the Hydrolysis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74)  177
2.4.6.1. Purification of DMPO  177
2.4.6.2. Spin Trapping with DMPO or PBN and ESR Measurement  177

2.4.7. Preparation of Tissues  179
2.4.7.1. Preparation of Rabbit Liver Microsomes  179
2.4.7.2. Preparation of Rat Liver Microsomes  180
2.4.7.3. Preparation of Rabbit Lung Microsomes  180

2.4.8. Incubations  181
2.3.8.1. Incubations with Rabbit Liver and Lung Microsomes  181
2.3.8.2. Incubations with Rat Liver Microsomes  181

References  182

Chapter 3. In Vivo Studies on the Metabolism of β-Nicotyrine  191
3.1. Background  191
3.2. Results and Discussion  192
3.2.1. Toxicity of β-Nicotyrine and Dosage Studies  192
3.2.2. Extraction of Urines  192
3.2.3. HPLC UV-diode Array Analysis of β-Nicotyrine Treated Rabbit and Mouse Urinary Samples  194
3.2.3.1. In Vitro Metabolites Not Observed In Rabbit Urine Samples  197
3.2.3.2. Characterization of the Metabolites Derived from β-Nicotyrine in Rabbit Urine  198
3.2.4. Synthesis of cis-3'-Hydroxycotinine (19b/c) and trans-3'-Hydroxycotinine (19a/d) 203
3.2.4.1. cis-3'-Hydroxycotinine (19b/c) 203
3.2.4.2. trans-3'-Hydroxycotinine (19a/d) 212
3.2.5. Characterization of the Structure of the Hydroxycotinine Metabolite of β-Nicotyrine 214
3.2.6. Stereochemistry of 3'-Hydroxycotinine 219
3.2.7. Mechanistic Pathway Leading to the Formation of cis-3'-Hydroxycotinine from β-Nicotyrine 224
3.2.8. Metabolic Relationship between (S)-Nicotine and β-Nicotyrine 236
3.3. Summary of In Vivo Studies on the Metabolism of β-Nicotyrine 239
3.4. Future Work 240
3.5. Materials and Methods 240
3.5.1. Chemicals 240
3.5.2. Analytical Instruments 241
3.5.3. Animals 242
3.5.4. Syntheses 242
3.5.4.1. 3-(3-Pyridyl)-N-methylnitrone (101) 242
3.5.4.2. 2-Methyl-3-(3-pyridyl)-5-methoxycarbonyl-isoxazolidine (102) 243
3.5.4.3. cis-3'-Hydroxycotinine (19b/c) 243
3.5.4.4. cis-3'-Hydroxycotinine Mesylate (107) 244
3.5.4.5. trans-3'-Hydroxycotinine Acetate (108) 244
3.5.4.6. trans-3'-Hydroxycotinine (19a/d) 245
3.5.5. Preparation of Urinary Samples 246
3.5.6. Preparation of the Silyl Ether Derivatives of 3'-Hydroxycotinine (109) 246
3.5.7. Preparation of 3'-Chloro derivatives of 3'-Hydroxycotinine Diastereomers (110) 247
3.5.8. In Vivo Transformation of trans-3'-Hydroxycotinine to Its cis-Isomer 248
3.5.9. In Vivo Studies of Metabolism of (S)-Nicotine in Rabbits 248
References 250
# List of Schemes

| I-1. | Metabolic pathway of nornicotine   | 15 |
| I-2. | Transformation of (S)-nicotine through Δ1′5′-iminium ion | 16 |
| I-3. | Metabolic pathway of (S)-cotinine | 18 |
| I-4. | Glucuronidation of (S)-cotinine and (3R,5S)-trans-3′-hydroxy-cotinine | 19 |
| I-5. | Formation of myosmine from (S)-nicotine | 19 |
| I-6. | Metabolism of anabasine | 20 |
| I-7. | Metabolism of N-methylanabasine | 20 |
| I-8. | Putative in vitro metabolic pathway of nicotine to β-nicotyrine | 22 |
| I-9. | Metabolism pathway of MTPP | 23 |
| I-10 | Proposed mechanism for the MAO-B catalyzed oxidation of (S)-nicotine Δ1′,5′-iminium ion to β-nicotyrine | 23 |
| I-11 | NIH shift | 29 |
| I-12 | Metabolism of pyrrolnitrine | 31 |
| I-13 | Nitrosation | 33 |
| I-14 | Pyrrole compound blocking nitrosation | 34 |
| I-15 | In vitro metabolism of β-nicotyrine | 35 |

| II-1. | Formation of the β-nicotyrine metabolites | 55 |
| II-2. | Proposed autoxidation mechanism of the β-nicotyrine metabolites | 48 and 51 |
| II-3. | Mass spectral fragmentation of 3-butylypyridine (56) | 56 |
| II-4. | Dehydrogenation of (S)-nicotine | 63 |
| II-5. | Reported synthesis of 5′-hydroxycotinine (20) and 5-hydroxy-1-methyl-5′-(3-pyridyl)-3-pyrroline-2-one (52) | 63 |
| II-6. | Mass spectral assignment of 5-methoxy-1-methyl-5′-(3-pyridyl)-3-pyrroline-2-one (58) | 66 |
| II-7. | Synthesis of 5-hydroxy-1-methyl-5′-(3-pyridyl)-3-pyrroline-2-one (52) and 5′-hydroxycotinine (20) | 68 |
| II-8. | Mass spectral fragmentation of 5-methoxy-1-methyl-5′-(3-pyridyl)pyrroolidine-2-one (59) | 71 |

xi
II-9. Decomposition of 5'-methoxycotinine (59) 75
II-10. Mechanism of ether cleavage with TMSI 77
II-11. Preparation of 5'-hydroxycotinine (20) and the 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) with TMSI 82
II-12. Pyrolysis of 5'-hydroxycotinine (20) and 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) to their ring opened isomers 82
II-13. Mass spectral fragmentation of 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) 83
II-14. Mass spectral fragmentation of 5'-hydroxycotinine (20) 84
II-15. Synthesis of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51) 85
II-16. Tautomer formations of synthetic pyrrolineones 87
II-17. The resonance of the pyrroline isomers 88
II-18. Attempts to form the pyrroline one 48 and 51 from 3',3'-dibromocotinine (57) 89
II-19. Elimination of two moles of HBr from (S)-3',3'-dibromocotinine (57) 90
II-20. Attempted elimination of HBr from 3',3'-dibromocotinine (57) with lithium carbonate 91
II-21. Elimination of 5'-methoxy cotinine (59) with TFA 92
II-22. The formation mechanism of 1-methyl-5-(3-pyridyl)-2-trifluoroacetoxypyrrole (75) 98
II-23. Syntheses of 2'-trifluoroacetoxypyrrole 75 and 2'-acetoxypyrrole 74 from 5'-hydroxycotinine (20) 98
II-24. Mass spectral assignment of 2-acetox-1-methyl-2-(3-pyridyl)-pyrrole (74) 103
II-25. Reactions of 5'-methoxycotinine (59) with TFAA and acetic anhydride 104
II-26. Mass spectral assignment of 1-methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrrolin-2-one (76) 110
II-27. The formation mechanism of 1-methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrrolin-2-one (76) 111
II-28. Hydrolysis of 2-acetox-1-methyl-2-(3-pyridyl)pyrrole (74) in HCl /H₂O 113
II-29. Proton-deuterium exchange of vinyl protons in 2-acetoxy-1-methyl-2-(3-pyridyl)pyrole (74) 118
II-30. The reduction of oxygen 138
II-31. DMPO radical adducts formed from ·OH and ·CH₃ 142
II-32. Spin splitting of DMPO free radical adducts 144
II-33. PBN trapped free radicals and its adducts 145
II-34. Spin splitting of PBN free radical adducts 147
II-35. Alkoxyl radical and possible spin trap adducts 147
II-36. Ethanol induced free radical adducts of DMPO and PBN 151
II-37. Spin splitting of ethanol induced free radical adducts of DMPO and PBN 151
II-38. Formation of nicotinamide 164

III-1. Synthesis of cis-3'-hydroxycotinine (19b/19c) 203
III-2. Formation of the four regio isomers of isoxazolidine 102 203
III-3. Fragment ions formed from 4'-methoxycarbonylisoxazolidine 203
III-4. Formation of ion at m/z 84 207
III-5. Formation of ion at m/z 191 207
III-6. Thermolysis of the isoxazolidines 102 208
III-7. Fragmentation of N-methyl-C-(3-pyridyl)enamine 103 208
III-8. Formation of 3'-hydroxycotinine from hydrogenation of the 3'-methoxycarbonylisoxazolidines 102a/b 211
III-9. Formation of cis-3'-hydroxycotinine (19b/c) from the cis-3'-methoxycarbonyl isoxazolidines 102a 212
III-10. Proposed hydrogenation of the 4'-methoxycarbonyl isoxazolidines 102c/d 212
III-11. Synthesis of trans-3'-hydroxycotinine (19a/d) 213
III-12. Synthesis of silyl ether of 3'-hydroxycotinine (109) 214
III-13. Fragmentation of dimethyl-tert-butylsilyl ether of 3'-hydroxycotinine (109) 218
III-14. Chloride derivatization of cis- and trans-3'-hydroxycotinine 219
III-15. Mass fragmentation of 3'-chlorocotinine 110 221
III-16. Proposed intramolecular substitution in the chlorination reaction 223
III-17. In vivo metabolism of the 2'-acetoxyprrole 74 228

III-18. Hydrolysis of the pyrrolinones 48 and 51 229

III-19. Proposed pathway for the formation of cis-3'-hydroxycotinine from β-nicotyrine 231

III-20. Examples of in vivo reduction of C-C double bonds 232

III-21. In vivo reduction of 5'-hydroxypyrroline 52 235

III-22. Proposed formation mechanism of cis-3'-hydroxycotinine from β-nicotyrine 235

III-23. Proposed biotransformation pathway of (S)-nicotine leading to 19a and 19b/c 238
List of Tables

I-1. Tobacco alkaloids found in tobacco and tobacco smoke 14

II-1. $^1$H NMR (CDCl$_3$) data assignment of the products from the dehydrogenation of (S)-nicotine 62
II-2. GC-EIMS data assignment of the products from the dehydrogenation of (S)-nicotine 62
II-3. $^1$H NMR (DMSO-d$_6$) data assignment of 5-methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (58) 67
II-4. $^1$H NMR (CDCl$_3$) data assignment of 5-methoxy-1-methyl-5-(3-pyridyl)pyrrolidin-2-one (59) 74
II-5. Mass spectra assignment of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51) 77
II-6. $^1$H NMR (CDCl$_3$) data assignment of the products from the sublimations of 5'-methoxyxocotinine (59) 93
II-7. $^1$H NMR (CDCl$_3$) data assignment of 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) 99
II-8. $^1$H NMR (CDCl$_3$) data assignment of 1-methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrrolin-2-one (76) 104
II-9. $^{13}$C NMR (CDCl$_3$) data assignment of 1-methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrrolin-2-one (76) 110
II-10. GC-EIMS analysis of the hydrolysis products of 74 176
II-11. ESR detection times and signals 179
II-12. Hyperfine splitting constants of the ESR signals 179

III-1 Mass spectra data of the products from the condensation of C-pyridyl-N-methylnitramine and methyl acrylate 206
III-2. Injection doses of (S)-nicotine for the rabbit studies 249
List of Figures

I-1. Proposed catalytic reaction cycle involving cytochrome P-450 in the oxidation of xenobiotics 30

II-1. $^1$H NMR spectrum of $\beta$-nicotyrine free base (2) in CDCl$_3$ 58
II-2. GC-EIMS data of $\beta$-nicotyrine (2) free base 59
II-3. $^1$H NMR spectrum of $\beta$-nicotyrine tartrate in CD$_3$OD 60
II-4. $^1$H NMR spectrum of 3-butylypyridine (56) in CDCl$_3$ 64
II-5. GC-EIMS spectrum of 3-butylypyridine (56) 65
II-6. $^1$H NMR spectrum of 5-methoxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (58) in CDCl$_3$ 69
II-7. GC-EIMS data of 5-methoxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (58) 70
II-8. $^1$H NMR spectrum of 5-methoxy-1-methyl-(3-pyridyl)pyrrolidin-2-one (59) in CDCl$_3$ 72
II-9a. GC-EIMS data of 5-methoxy-1-methyl-(3-pyridyl)pyrrolidin-2-one (59) 73
II-9b. GC-EIMS data of decomposed 5-methoxy-1-methyl-(3-pyridyl)-pyrrolidin-2-one (59) 76
II-10. $^1$H NMR spectrum of 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (52) in DMSO-d$_6$ 78
II-11. GC-EIMS data of 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (52) 79
II-12. $^1$H NMR spectrum of 5'-hydroxycoctinine (20) in DMSO-d$_6$ 80
II-13. GC-EIMS data of 5'-hydroxycoctinine (20) 81
II-14. $^1$H NMR spectrum of the sublimation products of 5'-methoxycoctinine (59) in CDCl$_3$ 94
II-15. GC-EIMS data of the pyrolysis products of 5'-hydroxycoctinine (20) 95
II-16. $^1$H NMR spectrum of 2-trifluoroacetxy-1-methyl-2-(3-pyridyl)-pyrrole (75) in CDCl$_3$ 96
II-17. $^1$H NMR spectrum of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in CDC$_3$ 100
II-18. GC-EIMS data of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) 101
II-19. UV spectrum of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in MeOH 102
II-20. $^1$H NMR spectrum of 1-methyl-2-(3-pyridyl)-4-trifluoroacetophenone-2-one (76) in CDC$_3$ 105
II-21. GC-EIMS data of 1-methyl-2-(3-pyridyl)-4-trifluoroacetophenone-2-one (76) 106
II-22. $^{13}$C NMR spectrum of 1-methyl-2-(3-pyridyl)-4-trifluoroacetophenone-2-one (76) in CDC$_3$ 107
II-23. High resolution mass spectrum of 1-methyl-2-(3-pyridyl)-4-trifluoroacetophenone-2-one (76), molecular ion at m/z 270.062 108
II-24. High resolution mass spectrum of 1-methyl-2-(3-pyridyl)-4-trifluoroacetophenone-2-one (76), base peak at m/z 201.066513 109
II-25a. $^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DCI/D$_2$O at time = 0 114
II-25b. $^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DCI/D$_2$O at 70 minutes 115
II-26. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in HCl/H$_2$O 116
II-27. Kinetic plots for the hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DCI/D$_2$O 117
II-28a. $^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DOAc/D$_2$O at time = 0 119
II-28b. $^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DOAc/D$_2$O after 8 days 120
II-29. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in HOAc/H$_2$O 121
II-30. Kinetic plots for the hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DOAc/D$_2$O 122
II-31. $^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer of pH 7.4 after 4 days (in D$_2$O) 124

xvii
II-32. Kinetic plots for the hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)-pyrrole (74) in phosphate buffer of pH 7.4

II-33. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer of pH 7.4 after 8 days

II-34. $^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% Na$_3$PO$_4$/D$_2$O

II-35. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% Na$_3$PO$_4$/D$_2$O, time = 0

II-36. GC-EIMS spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% Na$_3$PO$_4$/H$_2$O, time = 1 hour

II-37. Kinetic plots for the hydrolysis and subsequent reactions of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% of Na$_3$PO$_4$/D$_2$O

II-38. $^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in anaerobic 5% Na$_3$PO$_4$/D$_2$O

II-39. Kinetic plots for the hydrolysis and subsequent reactions of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in anaerobic 5% of Na$_3$PO$_4$/D$_2$O

II-40. HPLC-diode array chromatogram of the hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in anaerobic 5% of Na$_3$PO$_4$/H$_2$O

II-41. ESR signal of hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer, trapped with DMPO

II-42. ESR signal of hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer, trapped with PBN

II-43. ESR signal of hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer, added ethanol and trapped with DMPO

II-44. ESR signal of hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer, added ethanol and trapped with PBN

II-45. HPLC-diode array tracing of β-nicotyrine incubated with rabbit liver microsomes at time = 0 and 75 minutes

II-46. HPLC-diode array tracing of β-nicotyrine incubated with rabbit liver microsomes at time = 75 minutes, spiked with β-nicotyrine

II-47. UV spectra of 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51)

II-48. UV spectra of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48)
II-49. GC-EIMS data of β-nicotyrine incubated with rabbit liver microsomes at time = 75 minutes
II-50. HPLC-diode array tracing of β-nicotyrine incubated with rabbit liver microsomes at time = 75 minutes, spiked with 5'-hydroxycotinine (20)
II-51. HPLC-diode array tracing of β-nicotyrine incubated with rabbit liver microsomal at time = 75 minutes, spiked with 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrroline-2-one (52)
II-52. HPLC-diode array tracing of incubation mixture containing no β-nicotyrine at time = 75 minutes and the UV spectra of nicotinamide (98)

III-1. HPLC tracing of urine samples obtained from untreated rabbit and rabbit treated i.p. with β-nicotyrine
III-2. HPLC tracing of 24 hour urine sample obtained from mice treated i.p. with β-nicotyrine
III-3. HPLC tracing of rabbit urine samples spiked with 5'-hydroxycotinine (20), 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrroline-2-one (52), and β-nicotyrine (2)
III-4. UV spectra of synthetic and metabolic 5'-hydroxycotinine (20), and synthetic and metabolic 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrroline-2-one (52)
III-5. GC-EIMS TiC tracing and mass spectrum of unknown urinary metabolite derived from β-nicotyrine (2)
III-6. GC-EIMS data of the methoxycarboxylisoxazolidine (102) isomers
III-7. GC-EIMS data of hydrogenation products of methoxycarboxylisoxazolidines (102)
III-8. GC-EIMS data of synthetic 3'-hydroxycotinine and the unknown metabolite derived from β-nicotyrine
III-9. UV absorption of synthetic 3'-hydroxycotinine and unknown urinary metabolite derived from β-nicotyrine
III-10. GC-EIMS SIC (m/z 249) and mass spectra of silyl ether derived from 3'-hydroxycotinines (109)
III-11. GC-EIMS SIC (m/z 210) and mass spectra of 3'-chlorocotinines (110)  222

III-12. GC-EIMS SIC (m/z 210) of 3'-chloro derivatives (110) of trans-3'-hydroxycotinines derived from (S)-nicotine, (S)-cotinine and smoker's urine  225

III-13. GC-EIMS SIC (m/z 210) of 3'-chloro derivatives (110) of trans-3'-hydroxycotinines derived from standard and rabbit urine  226

III-14. HPLC tracing of urine samples obtained from untreated rabbit and rabbit treated i.p. with 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74)  227

III-15. GC-EIMS SIC (m/z 210) of 3'-chlorocotinines (110) derived from synthetic cis-3'-hydroxycotinine (19), β-nicotyrine (2) and 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74)  230

III-16. HPLC tracing of urine samples obtained from untreated rabbit and rabbit treated i.p. with 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (52)  233

III-17. GC-EIMS data of 5'-hydroxycotinine (20) in urine samples obtained from untreated rabbit and rabbit treated i.p. with 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (52)  234

III-18. GC-EIMS SIC (m/z 158) of β-nicotyrine derived from (S)-nicotine  237
## Index of Chemical Structures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-Nicotine</td>
<td>1</td>
</tr>
<tr>
<td>β-Nicotyrine</td>
<td>2</td>
</tr>
<tr>
<td>(S)-Cotinine</td>
<td>3</td>
</tr>
<tr>
<td>Nornicotine</td>
<td>4</td>
</tr>
<tr>
<td>Myosmine</td>
<td>5</td>
</tr>
<tr>
<td>(S)-Nicotine-N'-oxide</td>
<td>6</td>
</tr>
<tr>
<td>(S)-Nicotine-N,N'-dioxide</td>
<td>7</td>
</tr>
<tr>
<td>(S)-Nicotine-N-oxide</td>
<td>8</td>
</tr>
<tr>
<td>Anabasine</td>
<td>9</td>
</tr>
<tr>
<td>Anatabine</td>
<td>10</td>
</tr>
<tr>
<td>2,3-Bipyridyl</td>
<td>11</td>
</tr>
<tr>
<td>Nicotelline</td>
<td>12</td>
</tr>
<tr>
<td>Desmethylcotinine</td>
<td>13</td>
</tr>
<tr>
<td>5-Hydroxydesmethylcotinine</td>
<td>14</td>
</tr>
<tr>
<td>(S)-5-(3-pyridyl)-3,4,5-trihydripyrrole [(S)-Nicotine Δ¹,₅-iminium ion]</td>
<td>15</td>
</tr>
<tr>
<td>2-Hydroxy-(S)-nicotine</td>
<td>16</td>
</tr>
<tr>
<td>(S)-2-(3-Pyridyl)-2,3-dihydropyrrole</td>
<td>17</td>
</tr>
<tr>
<td>2-Cyano-(S)-nicotine</td>
<td>18</td>
</tr>
<tr>
<td>(3R,5S)-trans-3'-Hydroxycotinine</td>
<td>19a</td>
</tr>
<tr>
<td>(3R,5R,)-cis-3'-Hydroxycotinine</td>
<td>19b</td>
</tr>
<tr>
<td>5'-Hydroxycotinine</td>
<td>20</td>
</tr>
<tr>
<td>γ-(1-Methyl-3-pyridyl)-γ-oxobutryramide</td>
<td>21</td>
</tr>
<tr>
<td>2-(3-Pyridyl)acetic acid</td>
<td>22</td>
</tr>
<tr>
<td>2-(3-Pyridyl)ethanol</td>
<td>23</td>
</tr>
<tr>
<td>γ-(3-Pyridyl)-γ-oxobutryramide</td>
<td>24</td>
</tr>
<tr>
<td>(S)-Cotinine-N-oxide</td>
<td>25</td>
</tr>
<tr>
<td>trans-3'-Hydroxycotinine glucuronide</td>
<td>26</td>
</tr>
<tr>
<td>(S)-Cotinine N-glucuronide</td>
<td>27</td>
</tr>
<tr>
<td>N'-Methylmyosmine (Nicotine Δ¹,₂-iminium ion)</td>
<td>28</td>
</tr>
</tbody>
</table>
Anabasine-N'-hydroxide
Anabasine-N'-nitron
N'-Methylanabisine
\textit{cis}-Anabisine-N'-oxide
\textit{trans}-Anabasine-N'-oxide
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)
1-Methyl-4-phenylpyridinium ion (MPP+)  
N-Methyl-\( (S) \)-nicotine
N-Methyl-\( (S) \)-cotinine
Paraquat (4,4'-Bipyridyl-bismethiodide)
4-Ipomeanol
Methapyrine
Pyrolnitnine
4-Chloro-3-(3-chloro-2-nitrophenyl)maleimide
4-(2-Hydroxyethyl)mercapto-3-(3-chloro-2-nitrophenyl)maleimide
1,3,4-Trimethylpyrrole
1-Methyl-3,4-dihydroxymethylpyrrole
2-(N,N-diMethyl)methylenylpyrrole
2-Oximyl-3,4-pyrrolin-5-one
1-Methyl-5-(3-pyridyl)-4-pyrrolin-2-one
1-Methyl-5-(3-pyridyl)-2,3-epoxypyrrole
2-Hydroxy-1-methyl-5-(3-pyridyl)pyrrole
1-Methyl-5-(3-pyridyl)-3-pyrrolin-2-one
5-Hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one
5-Hydroperoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one
3-Hydroperoxy-1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one
3-Hydroxy-1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one
4-(3-Pyridyl)butane
3',3'-Dibromocotinine (DBC)
5-Methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one
5-Methoxy-1-methyl-5-(3-pyridyl)pyrrolidin-2-one (5'-Methoxycotinine)
1-Methyl-5-(3-pyridyl)-5-trimethylsiloxy-3-pyrrolin-2-one
1-Methyl-5-(3-pyridyl)-5-trimethylsiloxypropyrrolidin-2-one
N-Methyl-4-oxo-4-(3-pyridyl)but-2-enamide
3-Phenylselyl-(S)-cotinine 63
3-Phenylselyclox-(S)-cotinine 64
2-Hydroxyppyrrole 65
4-Pyrrolin-2-one 66
3-Pyrrolin-2-one 67
4-Methyl-3-pyrrolin-2-one 68
4-Ethoxycarbonyl-4-pyrrolin-2-one 69
4-Hydroxy-4-methylpyrrolidine-2-one 70
3-Hydroxy-4-ethoxycarbonylpyrrolidine-2-one 71
3-Bromo-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one 72
3-Bromo-1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one 73
2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole 74
1-Methyl-5-(3-pyridyl)-2-trifluoroacetoxypyrrole 75
1-Methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrrolin-2-one 76
4'-Hydroxycoctionine 77
bis[1-Methyl-2-(3-pyridyl)-3-pyrrolin-5-one-2-yl] peroxide 78
bis[1-Methyl-2-(3-pyridyl)-3-pyrrolin-5-one-2-yl] ether 79
5,5-Dimethyl-1-pyrrole-N-oxide (DMPO) 80
C-Phenyl-N-tert-butylNitron (PBN) 81
C-N'-Oxidepyridiy-N-tert-butylNitron 82
2-Methyl-2-nitosopropane 83
Nitrosobenzene 84
Nitrosodurene 85
Tris-tert-butylNitrosobenzene 86
5,5-Dimethyl-2-hydroxyppyrrolidine-N-oxide free radical 87
2-Alkyl-5,5-dimethylppyrrolidine-N-oxide free radical 88
5,5-Dimethyl-2-superxyppyrrolidine-N-oxide radical anion 89
5,5-Dimethyl-2-hydroperoxyppyrrolidine-N-oxide free radical 90
1-Hydroxy-1-phenyl-N-tert-butyl-N-oxide free radical 91
1-Alkyl-1-phenyl-N-tert-butyl-N-oxide free radical 92
2-Alkylxy-5,5-dimethylpyrrolidine-N-oxide free radical 93
1-Alkylxy-1-phenyl-N-tert-butyl-N-oxide free radical 94
2-(1-Hydroxyethyl)-5,5-dimethylpyrrolidine-N-oxide free radical 95
1-(1-Hydroxyethyl)-1-phenyl-N-tert-butyl-N-oxide free radical 96
Nicotinamide
Nicotinamide adenine dinucleotide phosphate (NADP+)
1,4-Dihydronicotinamide adenine dinucleotide phosphate (NADPH)
3-Pyridylcarboxyaldehyde
C-(3-pyridyl)-N-methylnitrene
3-Methoxy carbonyl-2-methyl-3-(3-pyridyl)isoxazolidine
N-Methyl-1-(3-pyridyl)methanamine
(1S,3R)-cis-(3-Hydroxyl)-3-methoxycarbononxyl-1-(N-methyl)-1-(3-pyridyl)propanamine
(1S,3S)-trans-(3-Hydroxyl)-3-methoxycarbononxyl-1-(N-methyl)-1-(3-pyridyl)propanamine
(1S,2R)-cis-(3-Hydroxyl)-2-methoxycarbononxyl-1-(N-methyl)-1-(3-pyridyl)propanamine
(1S,2S)-trans-(3-Hydroxyl)-2-methoxycarbononxyl-1-(N-methyl)-1-(3-pyridyl)propanamine
(1S,2S)-trans-2-Hydroxymethyl-1(3-pyridyl)-N-methyl-β-lactam
(1S,2R)-cis-2-Hydroxymethyl-1(3-pyridyl)-N-methyl-β-lactam
(1S)-3-Methoxycarboxyl-N-methyl-3-oxo-1-(3-pyridyl)propanamine
(3S,5S)-cis-3'-Hydroxycotinine mesylate
(3R,5S)-trans-3'-Hydroxycotinine acetate
3'-tert-Butylsiloxycotinine
(3R,5S)-trans-3'-Chlorocotinine
(3R,5S)-trans-3'-Chlorothionylcotinine
(3S,5S)-cis-3'-Chlorocotinine
(3S,5S)-cis-3'-Chlorothionylcotinine
Norethindrone
2,3-Dihydro-1-hydroxynorethindrone
1-[2-[bis[4-(trifluoromethyl)phenyl]methoxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (CI-966)
1-[2-[bis[4-(trifluoromethyl)phenyl]methoxy]ethyl]-3-piperidinecarboxylic acid (SKF 89976A)
List of Compound Structures

1. 
2. 
3. 
4. 
5. 
6. 
7. 
8. 
9. 
10. 
11. 
12. 
13. 
14. 
15. 
16.
POBN, 82

(CH₃)₃CN=O

NB, 84

PMNB, 85

TBNB, 86

87

88

89

90

91

92

93

94

95

96

97

98

99

100

101

102
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_N$</td>
<td>Alpha nitrogen hyperfine splitting constant</td>
</tr>
<tr>
<td>$a_B^H$</td>
<td>Beta hydrogen hyperfine splitting constant</td>
</tr>
<tr>
<td>$a_\gamma^H$</td>
<td>Gamma hydrogen hyperfine splitting constant</td>
</tr>
<tr>
<td>Br$_2$</td>
<td>Bromine</td>
</tr>
<tr>
<td>BuLi</td>
<td>n-Butyl lithium</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>CD$_3$OD</td>
<td>Deuterated methanol</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>Methylene chloride</td>
</tr>
<tr>
<td>CHCl$_3$</td>
<td>Chloroform</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>DCI</td>
<td>Deuterium chloride</td>
</tr>
<tr>
<td>DMAA</td>
<td>N,N-Dimethylacetamide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMPO</td>
<td>5,5-Dimethyl-1-pyrroline-N-oxide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DMSO-d$_6$</td>
<td>Deuterated dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOAc</td>
<td>Deuterated acetic acid</td>
</tr>
<tr>
<td>e$^-$</td>
<td>Electron</td>
</tr>
<tr>
<td>EGTA</td>
<td>[Ethylene-bis-(oxyethylenenitriolo)]tetraacetic acid</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron Spin Resonance</td>
</tr>
<tr>
<td>ETS</td>
<td>Environmental Tobacco Smoke</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Reduced Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>G</td>
<td>Gauss</td>
</tr>
<tr>
<td>g</td>
<td>Gram, graphic, g factor of electrons</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-EIIMS</td>
<td>Gas Chromatography with Electron Ionization Mass Spectrometry</td>
</tr>
</tbody>
</table>
GC-MS  Gas Chromatography with Mass Spectrometry
H     Hydrogen
H⁺    Proton
H₂     Hydrogen gas
H₂O₂   Hydrogen peroxide
HBr   Hydrobromic acid
HCl   Hydrochloric acid
HEPES N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HO    Hydroxyl group
HO⁻    Hydroxyl free radical
HOAc  Acetic acid
HOO⁻   Protonated superoxide
hrs   Hours
I     Spin constant
I.D.   Intradiameter
i.p.   Intraperitoneal
i.v.   Intravenous
KBr   Potassium bromide
KCl   Potassium chloride
KH₂PO₄ Potassium dihydrogenphosphate
K₂HPO₄ Potassium monohydrogenphosphate
kHz   Kiloohertz
KOH   Potassium hydroxide
LD₅₀   Lethal dose-50%
Li₂CO₃ Lithium carbonate
M     Molar
m/z   Mass/charge
MAO-B Monoamine oxidase B
Me    Methyl group
MeOH  Methanol
μg    Microgram
mg    Milligram
MgCl₂ Magnesium chloride
MHz   Megahertz
m  Meter
min  Minute
μL  Microliter
mL  Milliliter
mM  Millimolar
mm  Millimeter
mmHg  Millimeter of mercury column
μmol  Micromole
mmol  Millimole
MNP  2-Methyl-2-nitrosopropane
MPP+  1-Methyl-4-phenylpyridinium ion
MPTP  1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine
mW  Milliwatt
N  Nitrogen
Na₂CO₃  Sodium carbonate
Na₂SO₄  Sodium sulfate
Na₃PO₄  Trisodium phosphate
NADP+  Nicotinamide adenine dinucleotide phosphate
NADPH  1,4-Dihydronicotinamide adenine dinucleotide phosphate
NB  Nitrosobenzene
ND  Nitrosodurene
nm  Nanometer
NMR  Nuclear magnetic resonance
O₂⁻  Superoxide
°C  Degree centigrade
ODS  Octadecyl silane
PBN  C-Phenyl-N-tert-butyl nitrone
Pd  Palladium
pH  Minus log of the hydrogen ion concentration
PhSeCl  Phenylselenyl chloride
POBN  C-N'-Oxidepyridyl-N-tert-butyl nitro
PTLC  Preparative Thin Layer Chromatography
Py  Pyridine
Rf  Retention factor

XXXV
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SIC</td>
<td>Selected Ion Chromatography</td>
</tr>
<tr>
<td>SOCl₂</td>
<td>Thionyl chloride</td>
</tr>
<tr>
<td>SCX</td>
<td>Strong cation exchange</td>
</tr>
<tr>
<td>TBNB</td>
<td>Tris-&lt;i&gt;tert&lt;/i&gt;-butylnitrosobenzene</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TFAA</td>
<td>Trifluoroacetic anhydride</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatography</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>TMSI</td>
<td>Trimethylsilyl iodide</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(Hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSNA</td>
<td>Nicotine-derived nitrosamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>v</td>
<td>Volume</td>
</tr>
<tr>
<td>w</td>
<td>Weight</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction and Literature Review

β-Nicotyrine (2) is one of the important minor tobacco alkaloids and a member of the family of cyclopentadienoid heterocyclic compounds. These electron rich compounds have been implicated in numerous organ specific toxicities.1 β-Nicotyrine itself has been reported to cause various biological effects which we will discuss in detail later. This thesis will discuss the biotransformation of β-nicotyrine and the potential bioactivity of its metabolites. In order to help the reader to understand better this presentation, we will provide background information regarding this interesting compound. The introduction will include a discussion of the historical use of tobacco and the physicochemical, structural, pharmaceutical and metabolic properties of β-nicotyrine.

In this thesis, compounds will be identified both by IUPAC nomenclature and by common names. For generally known compounds like (S)-nicotine and β-nicotyrine, the common names will be used. In general, in order to distinguish positions on the pyridine ring from positions on the additional ring present in these alkaloids, the pyrrole, piperidine or pyrroline ring substituents will be identified by primed numbers, e.g. 3'.

* The reader is advised that the structures of all the compounds discussed in this thesis are presented in numerical order on pages xxv-xxxi. Reference to these pages may assist the reader in following certain sections of the thesis where compounds, which have been presented in earlier schemes, are discussed.

1.1. Past and Present Day Uses of Tobacco

Tobacco has been in use for over 8,000 years2 and remains one of the most popular drugs in the world. This history, the known health hazards and the
Chapter 1 Introduction and Literature Review

potential therapeutic use of tobacco components continue to encourage scientific investigation on the biological, chemical, medical, psychological and environmental properties of the tobacco plant. The following is a brief review of the history of and studies on tobacco that will relate to the research pursued in this thesis which focuses on the metabolic fate of the minor, but important tobacco alkaloid β-nicotyrine.

1.1.1. Early Reports

The use of tobacco for about the last 500 years has been well documented. When Europeans first explored the new world, they observed the use of tobacco by the native population who smoked a product prepared from the plant. Columbus and his men were offered tobacco by the native Indians but were puzzled about what to do with the shriveled leaves. They wondered why such withered matter should be considered an appropriate gift for what the Europeans considered a momentous occasion. The use of tobacco was entirely new to the Spanish explorers who found that South American Indians not only smoked but also chewed the plant. However, the American Indians, as more recent investigations revealed, had been using tobacco in one form or other for at least 8,000 years. About 11,000 years ago, originating in Asia, the ancestors of American Indians after traversing North and Central America had reached the southern subcontinent and occupied the open lowland of South America where tobacco grew wild. They discovered and started cultivating the wild tobacco plant some 8,000 years ago. On the other hand, some writers assert that tobacco came to America from Asia and that it was first grown in China where it had been used long before the narcotic properties of opium were known. Conclusions regarding the first cultivation of tobacco are still open to debate.

The name tobacco traces its origins to either Tabasco, a province of Mexico, or the island Tabasco in the gulf of Florida, or even perhaps to another island called Tobago which is located in the Caribbean. The name tobacco also might be derived from the Indian word, tobaccos, which belongs to the ancient language of Haydi, and was the name given by the Caribbeans to the pipe in which they smoked the plant.
Chapter 1 Introduction and Literature Review

The formal “discovery of tobacco” is attributed to Fernando Cortez who reported its extensive use in Yucatan in the Gulf of Mexico. He sent plants to the King of Spain in 1519 as part of the spoils and treasures of his new-found world. Sir John Nicot obtained tobacco seed in Portugal in 1559 from a Dutch merchant, who obtained it in Florida, and eventually took it to France in 1561. Sir Walter Raleigh carried tobacco from Virginia to England in 1586. As soon as tobacco was introduced into Europe by the Spaniards, its use became a general custom which has increased over the years.³

Besides the early cultivation by the native Indians, the cultivation of tobacco began in the islands of the West Indies and in South America early in the Sixteenth Century by the Spanish, English and Dutch and afterward in Cuba, Virginia and Florida by the French colonists. As soon as the cultivation of tobacco had spread to the West Indies and South America, large quantities of this plant were shipped to Europe. At this period the entire product was sent to Spain, Portugal, France, Great Britain and other countries of Europe. Tobacco was first planted in Portugal in 1559, Holland in 1615, France in 1626, and Switzerland in 1686.³

In the Seventeenth Century, with the increase in the cultivation of tobacco in America, tobacco became very popular, but precious. It was even used as money. Public, county, or parish taxes were payable in tobacco.³ Although tobacco is no longer so precious, it remains today the most popularly used drug in the world that does not require a prescription.

1.1.2. Medical Uses of Tobacco

Tobacco, in addition to its very early uses, has been in use also for at least 500 years in the form of pipe tobacco, cigars, cigarettes, snuff, chewing tobacco and tobacco extracts. Tobacco itself has a much longer medicinal history than does smoking of the leaf. In fact, the initial introduction of tobacco into various Old World countries almost always has been for medicinal purposes. Silvette et al. have reviewed the medical uses of tobacco.⁴⁵
Chapter 1 Introduction and Literature Review

The first recorded use of tobacco as a medicinal agent was by the American Indian. From prehistoric times until roughly 1,700 A.D., South American Indians took tobacco primarily for magico-religious and related medicinal purposes. In both North and South America, many Indian traditions involved tobacco use. They held the plant in veneration and considered it a gift that the Great Spirit had provided for their comfort and enjoyment. The use of tobacco in sufficient quantities to produce intoxication seemed to be a favorite remedy for many diseases. The Spaniards concluded that this was a method of choice in the attempt to cure many diseases. When introduced to the Old World, its claims as a remedy for diseases helped to establish its popularity and served to increase its use.

The first use of tobacco as a medicine in Europe was credited to Sir John Nicot when he was the French ambassador to Portugal from 1559 to 1561. He planted tobacco seeds obtained from Florida in his garden in Lisbon. It was claimed that a man had cured a facial ulcer by applying tobacco juice and pulverized herb from the resulting plants. Some time later, one of Nicot's cooks, who had almost all of his thumb cut off with a knife, was treated by dressing the wound with the leaves of "Nicotiana". As the wound healed, the medicinal reputation of tobacco increased. The plant began to be famous throughout all of Portugal for its ability to cure cutaneous ulcers, ringworm and scrofula. It was also sent to France to treat ulcers, ringworm, asthma and "the king's evil".

About this time, physicians claimed that it was "the most sovereign and precious weed that ever the earth tendered to the use of man." Dr. Edmund Gardiner issued in 1610 a volume entitled "The Trial of Tobacco". He described tobacco as a noble medicine and claimed that "Tobacco is not violent and therefore may in my judgement be safely put in practise." Dr. William Barclay in 1614 and Dr. Verner Bath in 1637 also declared their theories concerning the taking of tobacco and said "that it worketh wondrous cures".

When tobacco began to creep into use in France it was known as the Queen Herb or Medicine Herb. Since John Nicot was the first person to plant tobacco in Europe, it is in his honor that the principal alkaloid of tobacco, nicotine, was named. Even the name of this plant, Nicotiana, is named after
Chapter 1 Introduction and Literature Review

him. Many of the early synonyms for Nicotiana bear witness to the supposed therapeutic virtues of the plant: Herba Panacea, Herba propre a tous maux, Heilkraut, etc. The pharmacopeias, official or unofficial, invariably included Tabacum, as late as the United States Pharmacopeia of 1890.5

Some records from the middle of the Nineteenth Century have reported the therapeutic benefits of tobacco. Haskell claimed to successfully treat a case of strychnine poisoning with tobacco smoke in 1859.6 Johnson used tobacco both externally and, when combined with alcoholic stimulation, internally as antidotes to bites of poisonous reptiles and insects in 1860.7 O'Neill reported a case to stop bleeding with chopped, wet tobacco for a woman with a wounded leg in 1879.5 More commonly, tobacco was used to treat nervous system disorders such as apoplexy and epilepsy (Sigmond, 1838),8 and hysteric (Thompson, 1842).9 It also has been used for the relief of pain (Somervail, 1838).10 It was even mentioned to be helpful for stammering by putting a pipe between the stammering lips to improve the respiratory power.5 Parkinsonism was reported to be improved after treatment with i.v. nicotine.11 In the Nineteenth Century, the tobacco enema was a popular therapeutic method used to treat muscle contraction (Ranney, 1848),12 strangulated hernia and constipation (O'Donovan, 1858),13 hemorrhoidal bleeding (Sigmond, 1838),14 and for deworming.5 Unfortunately, many fatal poisonings following tobacco enemas were also reported.5

Tobacco smoking was also reported to be therapeutic. It was reported in 1931 that one or two cigarettes induced both the amplitude and frequency of the involuntary movements of Hemiballism.15 Hooper reported in 1885 that tobacco smoking could abort heart disorders.16 After smoking two weeks, it was claimed the arrhythmias ceased. It was observed that tobacco smoking shortened the gastrointestinal passage time and improved the bowel movement and intestinal habits of some patients.17 Tobacco smoking was used to treat incarcerated hernias (Haskell, 1859),6 and problems with the ileum (Copland, 1860).18 It was prescribed for diabetes insipidus.5 It was also considered of merit in oral hygiene and as prophylaxis for paradentosis.5 Relaxation of laryngeal spasms in cynanche trachealis19 and the prevention of some forms of
Chapter 1  Introduction and Literature Review

nasal catarrhs\textsuperscript{5} were reported. Even tobacco fumes were used as a respiratory stimulant\textsuperscript{5} and for treating gout.\textsuperscript{20,21}

Tobacco snuff has been recommended for its analgesia properties\textsuperscript{10} and to treat tracheal irritation and harsh crouping cough,\textsuperscript{22} hiccough,\textsuperscript{23,24} and receding polyps in the nostrils.\textsuperscript{25} Other diseases treated with tobacco include dropsy, bladder paralysis, constipation, fecal obstruction of the intestines, paralytic ileus, colic, gout, muscle spasm including strangulated hernia, skin disorders as mentioned above and pustulua dermatoses of the extremities, canker sores, itching, scabies and pediculosis.\textsuperscript{5}

Use of tobacco as a prophylactic in the treatment of contagious diseases also has a long history, going back as far as the Great Plague in London in 1665. It was reported to be used as a deodorizer for individuals in charge of "dead-carts" in 1783.\textsuperscript{5} It was thought to help individuals trying to escape from cholera and other epidemics.\textsuperscript{5}

In the Nineteenth and early Twentieth Centuries, tobacco was almost a universal panacea applied to all human health problems. It was used in various forms including tobacco extract preparations in ointments, tobacco powder and chopped wet leaf, smoke, fumes, sniff and pipe juice. The administration methods included intravenous injection, instillation into the lungs, enema, oral, topical and by smoke fumes.

However, from the beginning it was clear that tobacco was used more for amusement than for its medicinal virtues. Before too long the potential dangers of tobacco were noted. As early as 1762, a report in Acta Helvetica\textsuperscript{5} pointed out the dangers of tobacco. Sigmond\textsuperscript{14} and Copland\textsuperscript{18} urged great caution in the employment of such dangerous remedies. Weaks reported a case in 1852-1853\textsuperscript{26} involving a seven-day-old infant who was fatally poisoned when given two tablespoons of water impregnated with tobacco smoke. Some fatal results caused by tobacco enemas also were reported at this time.\textsuperscript{14,27} Doctors had started suggesting that their patients stop smoking. Today, tobacco products are recognized as a major source of health related problems.
Chapter 1  Introduction and Literature Review

1.1.3. Agricultural Use of Tobacco

Tobacco plants originally were cultivated by Europeans in Europe and America for horticultural interest. However, even in the early years the commercial value of tobacco as an insecticide was well recognized. As early as in 1919, Horn reported the results of his investigation on the manufacture of a tobacco insecticide in Canada. He described the extraction of nicotine from tobacco waste, such as leaves, stalks, roots and suckers. In 1926 Huczek reported the toxicity of tobacco dust to aphids under laboratory conditions and found a direct correlation between nicotine content and toxicity. In the same year, Das Bhasin found that tobacco was a fairly good repellant against insect infestations that did not affect germination of the desired crop. Gansser reported in 1928 that tobacco juice was useful to treat and eradicate warble-fly larvae. In 1937 Richter and Calef reported that nicotine, when dissolved in refined petroleum oil, would not injure tender plants but was highly toxic to many insects. Its toxicity to insects was equal to a dose of 0.1% of pyrethrum in oil. Volck obtained a patent on finely ground tobacco powder as a component of an insecticide in 1939.

In all of the above reports, the characterized toxicities and activities of the insecticides containing tobacco plant materials are due to the toxicity of nicotine itself. In 1940, Hansberry and Norton reported the insecticidal actions of d-nornicotine and l-nornicotine, and the unnatural d-nicotine for the first time. They prepared the unnatural d-nicotine by epimerizing l-nicotine. Although d-nicotine was definitely less toxic than l-nicotine, no difference in activity was found between the two nornicotine isomers. This was the first description of the insecticidal activities of the tobacco alkaloids besides that of natural nicotine.

In the same year Yothers and Griffin reported that another tobacco alkaloid, anabasine, showed insecticidal activities against the woolly apple aphid. They found that anabasine was also toxic to the woolly aphid: it killed about 90% of the aphid in a 1:4800 ratio in petroleum. Since then until the early 1960's numerous literature citations from the United States, Cuba, Greece, Japan, Spain, and India refer to the insecticidal use of tobacco and nicotine. It has been claimed that in the USA alone, the use of
Chapter 1 Introduction and Literature Review

nicotine for insecticidal purposes amounted to over 500 tons per year in the 1960s. However, during the past 30 years, as research has demonstrated the toxicity of nicotine to humans, the interest in tobacco insecticides has decreased. One reason for this is the development of safer and more potent insecticides, such as pyrethroids.

1.1.4. Recreational Tobacco Use

Although tobacco has been used in medicine and agriculture, the most important and extensive use of tobacco, usually through smoking (pipe, cigars and cigarettes), is for enjoyment. Today, tobacco is still widely used around the world for enjoyment. The number of tobacco users has increased since the middle of this century. In 1915, fewer than 20 billion cigarettes were sold whereas in 1987 some 575 billion cigarettes were sold. A 1985 report stated that more than 30% of the adult US population smoked. According to a 1982 European Economic Community report, 80 million Europeans smoked. In the USA, the proportion of heavy smokers (those who smoke more than 25 cigarettes a day) has increased in the past 20 years from 24% to 33% in the male and from 13% to 20% in the female population. Smokeless tobacco users are comparatively fewer. In 1986, 2.2% of men and 0.5% of women aged 21 and older used snuff and 3.1% of men and 0.1% of women used chewing tobacco. In the European Economic Community between 1960 and 1979 the adult population (15 years and older) who used tobacco increased by 16%. A report from the World Health Organization Expert Committee on Smoking Control Strategies in 1983 states that in all the developing countries surveyed about 50% or more of adult men use some form of tobacco. The people in Latin America have proportionally more smokers than in Asia and Africa. In Latin America, 45% of men and 18% of women were cigarette smokers. In 1985, smokeless tobacco was used by at least 1.2 million people in the USA with half of those being regular users. The use of smokeless tobacco has increased among male adolescents and young male adults. In 1991, 19% of male high school students used smokeless tobacco.
Chapter 1  Introduction and Literature Review

1.1.5. Health Related Problems

Even in the Seventeenth Century, the golden age of tobacco, when it was very popular, the toxicity of tobacco had been noticed and reported. The human health related problems associated with tobacco use have been documented increasingly during this century. The data have been presented in many scientific articles and in the summaries provided by reports of the Surgeon General since 1964. The statistics in 1989 showed cigarette smoking to be the most devastating preventable cause of disease and premature death in the USA. Each year it causes the death nearly 400,000 Americans, 115,000 from coronary heart disease, 27,000 from stroke, 136,000 from cancer, 60,000 from chronic obstructive pulmonary disease and an estimated 50,000 from other diseases and other causes. Comparing smokers and non-smokers, 57% of smokers are dead by age 75, but only 30% of non-smokers are dead by the same age. Approximately 2.5 million people die worldwide each year as a result of smoking.

Smoking, hypertension and elevated cholesterol are major risk factors for heart disease. Smoking alone doubles the risk. When it is combined with either high blood pressure or elevated cholesterol, the risk increases four fold. When all three risk factors are present, the total risk is eight times greater. Other diseases that may be linked to tobacco smoking are arteriosclerotic peripheral vascular disease, atherosclerosis of the aorta and coronary arteries, angina pectoris, cerebrovascular disease and peptic ulcer disease. The toxicological implications of smoke constituents on cardiovascular diseases are not clearly understood. Carbon monoxide in smoke may act to precipitate cardiac symptomatology or ischemic episodes in individuals already compromised by coronary disease. In addition, carbon monoxide binds to hemoproteins, potentially inhibiting their functions.

Cancer is second to heart disease as a cause of death, and cigarette smoking is the most important preventable cause of cancer. Some 30% of all cancer deaths and 87% of lung cancer deaths are due to smoking. Other cancers that may be caused by smoking are cervix, larynx, oral cavity, esophagus, bladder, pancreas and kidney cancers. The known carcinogens
Chapter 1 Introduction and Literature Review

identified in the gas phase of tobacco smoke are nitrosamines, such as N-nitrosodiethylamine, hydrazine and vinyl chloride. In the particulate phase of tobacco smoke are carcinogenic polycyclic aromatic hydrocarbons, such as benzo-[a]-pyrene. In addition, both phases contain tumor promoters such as formaldehyde, volatile phenols, e.g. cresol and other unknown weak acidic and neutral compounds. Co-carcinogens are also present. The same carcinogens that have been identified in the condensed phase of tobacco smoke are present in the tar of cigars, pipes and cigarettes. The carcinogenic metals, like As and Ni, are found in tobacco plants and smoke. Also present are radioelements, such as $^{226}$Ra, $^{210}$Pb, $^{210}$Po and $^{40}$K.

In 1965 the percentage of female smokers (31.9%) was lower than that of male smokers (50.2%). However, since 1965 the number of male smokers has declined by 20% while the number of female smokers has declined only 5%. About 30% of pregnant women are cigarette smokers. Smoking is associated with spontaneous abortion (1.7 times higher risk with a smoking mother), pre-term births (1.36 times greater), low birth-weight full-term babies (1.98 times greater) and fetal infant deaths (1.25 times greater). The greater the exposure, e.g. two smoking parents, the greater the risks. Contributing to these problems are:

1) Toxic components of tobacco smoke,
2) Secondary effects due to impaired nutrition of the mother. Smoking mothers tend to eat less than non-smoking mothers so the fetus gets less nourishment,
3) Reduction of blood supply to the placenta,
4) Release of oxytocin which affects the uterus,
5) Disturbance of vitamin B$_{12}$ and vitamin C metabolism since serum levels of both these vitamins are lower in smokers.

In addition, lung cancer in women was expected to top breast cancer as the leading cause of cancer deaths among women after 1985.

The decline in smoking has been coupled to an increase in the use of smokeless tobacco. In the United States, less than 4% of people used snuff and chewing tobacco in 1975. That number increased to 10% by 1985.
Chapter 1 Introduction and Literature Review

Many of those who now use snuff or chewing tobacco are either current or former smokers. The use of smokeless tobacco can lead to nicotine dependence and may contribute to health problems. Many studies on tobacco chewing have been done in Asia where the prevalence of both oral cancer and tobacco chewing is high.\textsuperscript{54,55} The positive relationship between the use of smokeless tobacco and oral cancer, nasal cancer and oral leukoplakia, has been studied and confirmed. Chemical analyses of smokeless tobacco indicate that three types of known carcinogenic agents are present: N-nitrosamines, polycyclic aromatic hydrocarbons, and $^{210}$Po, a radioactive $\alpha$-emitter.\textsuperscript{56}

1.1.6. Air Pollution

Since it has been known that tobacco use may be a human health hazard, air pollution caused by tobacco smoke has been taken more seriously, particularly since the 1980s. Environmental tobacco smoke (ETS) is a potential source of indoor air pollution. Recent epidemiologic research indicates that ETS may be a health hazard for nonsmokers. The sources for ETS are sidestream smoke, which is emitted from the burning tobacco, and the exhaled mainstream smoke. The sidestream contains higher concentrations of some toxic compounds, including ammonia, volatile amines, volatile nitrosamines, nicotine decomposition products, aromatic amines like quinoline and polycyclic aromatic hydrocarbons.\textsuperscript{57} The composition of the total ETS includes carbon monoxide and carbon dioxide, nicotine, nitrogen oxides, acrolein, N-nitrosamines, polycyclic aromatic hydrocarbons, pyrenes, volatile phenols, and the radioelements $^{226}$Ra, $^{210}$Pb, $^{210}$Bi, and $^{210}$Po.\textsuperscript{57}

About 66\% of men and 60\% of women have been exposed to ETS in childhood; 32\% of men and 62\% of women reported ETS exposure in the home in adulthood; and 60\% of men and 62\% of women who worked outside the home reported ETS exposure at work.\textsuperscript{58} ETS exposure is thought to lead to a higher risk of lung cancer morbidity. Non-smokers married to smokers have a 30-40\% higher risk of lung cancer than that of non-smokers married to non-smokers.\textsuperscript{59} Some findings imply that ETS exposure may cause respiratory symptoms, usually coughing and wheezing, in some children whose parents are smokers. Bronchitis, pneumonia and other lower-respiratory-tract illnesses
Chapter 1  Introduction and Literature Review

occur up to twice as often during the first year of life in children who have smoking parents than in children whose parents are non-smokers. A few studies have reported that children of smokers have reduced growth and development and an increased risk of persistent middle-ear effusions.\textsuperscript{57} The American Cancer Society reported that ETS causes an estimated 53,000 deaths annually in the USA, about two thirds from heart disease and about 4,000 from lung cancer.\textsuperscript{54} The studies on the health effects associated with ETS exposure continue.

1.2. Tobacco Alkaloids

More than 4000 compounds have been identified in tobacco smoke. About 2200 components have been separated from tobacco plant extracts.\textsuperscript{60} The biological activities of some of these compounds, including carcinogenic polycyclic aromatic hydrocarbons, pyrenes and nitrosamines and the cardiotoxic carbon monoxide are well-known. Some of the components are common toxins, e.g. the radioactive species and heavy metals mentioned above. Over 600 (27\%) of the compounds isolated from the plant are alkaloids.\textsuperscript{60} (S)-Nicotine (1) is the major tobacco alkaloid. Its content in tobacco leaves ranges from 2-6\% depending on the species of tobacco.\textsuperscript{61,62,63} It represents 85-90\% of the total tobacco alkaloids.\textsuperscript{60} The biological activity of (S)-nicotine has been widely studied. Shigenaga summarized (S)-nicotine's pharmacokinetics and absorption characteristics, pharmacological activities, toxicities, cytogenetic effects and metabolism.\textsuperscript{64} Gorrod and Jenner\textsuperscript{65} as well as Kyerematen and Vesell\textsuperscript{66} have reviewed the metabolism of (S)-nicotine in considerable detail.
Chapter 1 Introduction and Literature Review

Besides (S)-nicotine, at least twenty other alkaloids have been identified in tobacco. β-Nicotyrine (2), (S)-cotinine (3), nornicotine* (4), myosmine (5), nicotine-N-oxides including 6, 7 and 8, anabasine* (9), anatabine** (10), 2',3'-bipyridyl (11) and nicotelline (12) are the most important minor alkaloids found in both tobacco plants and tobacco smoke (Table I-1).60,61,67,68,69,70,71 Their contents in tobacco plants and tobacco smoke normally are very low, only in the tens to hundreds of ppm. Even though they may exist in small amounts, tobacco users are exposed to these minor tobacco alkaloids and therefore some appreciation of their biological properties may be useful in characterizing the pharmacological and toxicological properties of tobacco.

** Nornicotine (4), anabasine (9) and anatabine (10) are chiral. However, up to now, none of the literature discusses the stereochemistry of these three compounds.
Table I-1. Tobacco alkaloids found in tobacco and tobacco smoke

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tobacco Dry Leaves (µg/g)</th>
<th>Tobacco Smoke (µg/cigarette)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabasine (9)</td>
<td>36-103</td>
<td>3-12</td>
</tr>
<tr>
<td>Anatabine (10)</td>
<td>36-120</td>
<td>3.7-14</td>
</tr>
<tr>
<td>2',3-Bipyridyl (11)</td>
<td>12-13</td>
<td>+ ***</td>
</tr>
<tr>
<td>(S)-Cotinine (3)</td>
<td>45-96</td>
<td>9-57</td>
</tr>
<tr>
<td>Myosmine (5)</td>
<td>16-30</td>
<td>9</td>
</tr>
<tr>
<td>Nicotelline (12)</td>
<td>+ ***</td>
<td>+ ***</td>
</tr>
<tr>
<td>(S)-Nicotine (1)</td>
<td>20,000-60,000</td>
<td>800-3000</td>
</tr>
<tr>
<td>Nicotine-N-oxides</td>
<td>+ ***</td>
<td>+ ***</td>
</tr>
<tr>
<td>β-Nicotyrine (2)</td>
<td>54-305</td>
<td>+ ***</td>
</tr>
<tr>
<td>Nornicotine (4)</td>
<td>640</td>
<td>27-88</td>
</tr>
</tbody>
</table>

*** + Indicates the compounds are detectable but quantitative data are not available.

1.3. Biotransformation of the Minor Tobacco Alkaloids

Information on the bioactivities and biotransformation pathways of the minor tobacco alkaloids is very limited. It has been reported that β-nicotyrine (2), nornicotine (4), and anabasine (9) possess insecticidal activities as was mentioned above. β-Nicotyrine shows some hypotensive properties. Nornicotine and (S)-cotininem, reported metabolites of (S)-nicotine (1), are present in tobacco plants and tobacco smoke and have been studied the most extensively. The discussion that follows will review the literature related to the metabolism of the tobacco alkaloids.

1.3.1. Metabolic Fate of Nornicotine (4)

Rabbit liver preparations are reported to convert (S)-nicotine to nornicotine. Nornicotine has been identified as a urinary metabolite of (S)-nicotine in rats and cigarette smokers. Nornicotine may be oxidized to norcotinine (13), which has been identified in the urine of both smokers and
Chapter 1 Introduction and Literature Review

subjects given (S)-nicotine intravenously but not in the urine of volunteers after they receive (S)-cotinine.\textsuperscript{76,77} Norcotinine may be further oxidized to 5'-hydroxynorcotinine (14, Scheme I-1).\textsuperscript{66} Myosmine (5), which is derived metabolically from nornicotine in tobacco plants,\textsuperscript{78} apparently has not been detected as a human metabolite of nornicotine.

\begin{center}
\begin{tikzpicture}
  \node (4) at (0,0) {4};
  \node (13) at (1,0) {13};
  \node (14) at (2,0) {14};
  \draw[->] (4) -- (13);
  \draw[->] (13) -- (14);
\end{tikzpicture}
\end{center}

**Scheme I-1** Metabolic pathway of nornicotine

1.3.2. Metabolic Fate of (S)-Cotinine (3)

After (S)-nicotine itself, the metabolic fate of (S)-cotinine has been examined more extensively than any other tobacco alkaloid. It is one of the most abundant minor tobacco alkaloids and is present in both the tobacco leaves and in tobacco smoke (57 mg per smoked cigarette\textsuperscript{79}). (S)-Cotinine may be formed from (S)-nicotine during curing of the tobacco leaf.\textsuperscript{80}

Interest in the metabolic fate of (S)-cotinine (3) is a direct consequence of its pivotal role in (S)-nicotine metabolism. (S)-Cotinine was first reported as a urinary metabolite of (S)-nicotine in the dog and rabbit.\textsuperscript{81,82,83} Studies in humans indicate that as much as 70% of the metabolites formed from (S)-nicotine involves the initial formation of (S)-cotinine.\textsuperscript{84} (S)-Cotinine is formed in a 2-step process involving initial cytochrome P-450 catalyzed oxidation to the iminium species 15\textsuperscript{85,86,87} followed by a second 2-electron oxidation catalyzed by aldehyde oxidase to yield the lactam 3.\textsuperscript{88,89,90} The initial step of this oxidation is thought to be a one electron transfer from the nitrogen lone pair to an electron deficient perflyl oxygen complex, Fe^{V}=O or (FeO)\textsuperscript{III} and results in the generation of an aminium radical i and Fe^{IV}=O. Loss of a proton at C-5' may lead to the formation of a transient carbon centered radical ii and an Fe^{IV}-OH complex. Radical ii can then recombine with the activated oxygen species, in this case the equivalent of a neutral hydroxyl radical, to yield the corresponding carbinolamine 16. On the other hand, the carbon centered
radical ii could give up a second electron to the Fe$^{IV}$-OH complex to yield the Fe$^{III}$-OH complex and corresponding two electron oxidized species (S)-nicotine $\Delta^{1.5''}$-iminium ion 15, respectively. The iminium species 15 is present in equilibrium with its conjugate base, 5-(3-pyridyl)-4,5-dihydropyrrole (17).\textsuperscript{86,91} Recombination of the iminium ion with the equivalent of a hydroxide anion would then yield the carbinolamine species 16. Alternatively, the iminium ion 15 might escape from the P-450 pocket and later recombine with hydroxide anion present in the cytosol also to give 16. The same oxidation process could occur on the N-methyl carbon to form the corresponding N-carbinolamine species. This, however, is a minor pathway at best.\textsuperscript{87} Oxidation on the 2'-carbon has not been observed, perhaps due to the steric constraints that limit the accessibility of cytochrome P-450 to this position. Evidence for the formation of the $\Delta^{1.5''}$-iminium intermediate 15 comes from trapping studies with cyanide to form the 5'-cyano-(S)-nicotine species 18.\textsuperscript{86,91}

Scheme I-2 Transformation of (S)-nicotine through $\Delta^{1.5''}$-iminium ion 15
Chapter 1 Introduction and Literature Review

(S)-Cotinine undergoes extensive further metabolism in vivo (Scheme 1-3). The metabolic fate of (S)-cotinine was first examined in mice in 1964.92 The C-oxidation of (S)-cotinine leads to 3'-hydroxycotinine (19), which is the major metabolite isolated from the urine of humans administered large doses of (S)-cotinine.93 The configuration about the newly introduced chiral center at the C3' position initially was not documented. Subsequent studies established that 3'-hydroxycotinine isolated from the urine of monkeys treated with (S)-cotinine has the (3R,5S)-trans stereochemistry as shown in 19a.94 This metabolite is also the principal metabolite excreted in the urine of humans and experimental animals administered (S)-nicotine.66,95,96,97,98 The metabolic pathway of the (S)-cotinine oxidation to 19a has not been totally explored. However, carbon-hydrogen bond cleavage at the 3'-position is likely to be involved.99 The deuterium isotope effect for the 3'-hydroxylation of (S)-cotinine was calculated to be 6-7 by in vivo studies in the monkey, indicating that carbon-hydrogen bond cleavage is involved in the rate determining step in this metabolic conversion. (S)-Cotinine also is metabolized to another carbinolamide, namely 5'-hydroxycotinine 20.100 Compound 20 is in equilibrium with the corresponding ring opened isomer, the ketoamide 21.65 Compound 21 undergoes degradation to smaller molecules such as 3-pyridylacetic acid (22) and 3-pyridylcarbinol (23).101,102 The enzymatic pathway leading to the two carbinolamides 19 and 20 remains poorly defined. Clearly more detailed mechanistic studies are required to provide a better understanding of these important biotransformations.

Desmethylcotinine (13) also has been reported to be present in the urine of dogs administered (S)-cotinine.103,104 Like (S)-cotinine, desmethylcotinine apparently is converted to 5'-hydroxydesmethylcotinine (14), which is in equilibrium with the corresponding ring opened isomer, the ketoamide (24).105 Finally, (S)-cotinine undergoes N-oxidation on the pyridine ring to give the corresponding N-oxide (25). This conversion has been observed in vivo in human,74 guinea pig,106 and monkey,107 and in vitro in hamster and guinea pig (Scheme 1-3).108
Recent studies indicate that (3R,5S)-trans-3'-hydroxycotinine (19a) may be converted to the corresponding O-glucuronide 26\textsuperscript{109} and (S)-cotinine to the N-glucuronide 27 (Scheme I-4).\textsuperscript{110} The O-glucuronide 26 has been identified in smokers' urine. Compound 26 was also found to be a longer lived metabolite than (S)-cotinine.\textsuperscript{109} The N-glucuronidation of (S)-cotinine, however, involves quaternization of the pyridyl nitrogen atom to generate the zwitterionic species 27 which has been recently synthesized and converted to cotinine upon treatment with β-glucuronidase (Scheme I-4).\textsuperscript{110} The identical material was isolated from smokers' urine and therefore 27 is considered to be a major urinary metabolite of (S)-nicotine.
1.3.3. Formation and Metabolism of Myosmine (5)

Myosmine (5), which is present in aged tobacco, is reported to be a pyrolysis product of (S)-nicotine (Scheme I-5). Myosmine may be derived by demethylation of N-methylmyosmine (28) which was postulated as the first intermediate of (S)-nicotine degradation in the tobacco leaf fermentation process. N-Methylmyosmine has also been obtained as a product of bacterial degradation of (S)-nicotine. Even under extremely mild conditions, (S)-nicotine is oxidized chemically to nornicotine (4) which may also give rise to myosmine.
1.3.4. Metabolism of Anabasine (9)

In vitro studies in rodents on the metabolism of anabasine (9) have established its conversion to the corresponding N'-hydroxy derivative 29, which subsequently is oxidized to the nitrone 30 (Scheme I-6).\(^{115}\) N-methylanabasine (31) is reported to undergo N-oxidation to form the diastereomeric N'-oxide 32 and 33 but apparently does not undergo \(\alpha\)-carbon oxidation as is the case with (S)-nicotine.\(^{116}\) (Scheme I-7) Gorrod has reviewed the metabolism of these compounds.\(^{65}\)

![Scheme I-6 Metabolism of anabasine]

\[
\begin{align*}
\text{Py} & \quad \rightarrow \\
\text{Py} & \quad \rightarrow \\
\text{Py} & \quad \\
\text{9} & \quad \rightarrow \\
\text{29} & \quad \rightarrow \\
\text{30} & \quad \\
\end{align*}
\]

Scheme I-6 Metabolism of anabasine

![Scheme I-7 Metabolism of N-methylanabasine]

\[
\begin{align*}
\text{Py} & \quad \rightarrow \\
\text{Py} & \quad \rightarrow \\
\text{Py} & \quad \\
\text{N} & \quad \rightarrow \\
\text{N} & \quad \rightarrow \\
\text{32} & \quad R = \text{CH}_3, \ R' = \text{O}\text{\textsuperscript-} \\
\text{33} & \quad R = \text{O}\text{\textsuperscript-}, \ R' = \text{CH}_3
\end{align*}
\]

Scheme I-7 Metabolism of N-methylanabasine

1.4. \(\beta\)-Nicotyrine

1.4.1. Physicochemical Properties and Absorption of \(\beta\)-Nicotyrine

\(\beta\)-Nicotyrine is a yellow oily liquid with a characteristic pungent odor. It darkens on standing in air. Its boiling point is 280-281 °C / 744 mm Hg and 117 °C / 1.2 mm Hg. Its density at 20 °C is 1.241 mg/mL and its index of refraction at 20 °C is 1.6057. \(\beta\)-Nicotyrine is a weak base and soluble in dilute acid. It is
Chapter 1 Introduction and Literature Review
also soluble in alcohols and is sparingly soluble in water. Its tartrate salt is yellow needles which melt at 105-106 °C.\textsuperscript{117} Absorption of β-nicotyrine in the oral cavity (buccal absorption) is estimated to be approximately 23% at pH 5.0 and 27% at pH 7.4.\textsuperscript{118} The percent of β-nicotyrine protonated at these pH values has been reported to be 99.8 and 66.6%, respectively.\textsuperscript{119}

1.4.2. Sources of β-Nicotyrine

β-Nicotyrine is a minor tobacco alkaloid and is present in both tobacco plants and tobacco smoke (Table I-1). In the dry green tobacco leaves, its content is about 60-300 ppm.\textsuperscript{61,65} β-Nicotyrine forms from the autoxidation of (S)-nicotine.\textsuperscript{112,113} Consequently commercial (S)-nicotine often contains trace amounts of β-nicotyrine. It is likely that β-nicotyrine in tobacco plants results from the photochemical oxidation or autoxidation of (S)-nicotine. The TiO$_2$ catalyzed photooxidation of (S)-nicotine to β-nicotyrine has been reported.\textsuperscript{120,121}

β-Nicotyrine has been reported as a urinary metabolite of (S)-nicotine in dogs and rats\textsuperscript{122} and was described as an intermediate product in (S)-nicotine metabolism as early as the 1950s.\textsuperscript{123,124,125} Recent studies have been concerned with the possible metabolic pathway of the conversion of (S)-nicotine to β-nicotyrine.

As we discussed earlier, (S)-nicotine can be first oxidized to the iminium intermediate, 5-(3-pyridyl)-3,4,5-trihydropyrroline (15) catalyzed by cytochrome P-450. The iminium species 15 can be oxidized further to (S)-cotinine (3) by a reaction catalyzed by aldehyde oxidase.\textsuperscript{64,91} However, since aldehyde oxidase is only present in liver,\textsuperscript{126,127} the above metabolic pathway is not likely to occur in extrahepatic tissues. In most mammalian species, (S)-nicotine is also susceptible to cytochrome P-450 catalyzed oxidation in the lung and kidney.\textsuperscript{128} The fate of the iminium metabolite formed in the lung is not clear. Of particular interest is the possible metabolic formation of β-nicotyrine in tissues such as the lung, kidney and possibly brain which are known to contain cytochrome P-450 oxidase activity but which lack aldehyde oxidase activity. Under these circumstances it is possible that the iminium ion intermediate 15,
Chapter 1  Introduction and Literature Review

formed in the initial 2-electron oxidation of (S)-nicotine, may undergo further oxidation to β-nicotyrine via the corresponding enamine 17 in a reaction catalyzed by an amine oxidase such as monoamine oxidase (MAO, Scheme I-8).

![Chemical structures](image)

Scheme I-8  Putative in vitro metabolic pathway of nicotine to β-nicotyrine

Since the iminium species 15 is present in equilibrium with its conjugate base 17, which is a tertiary enamine, it could be oxidized by MAO. An example of a related cyclic amine undergoing MAO and cytochrome P-450 catalyzed oxidation in tissues containing no aldehyde oxidase is the oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 35) to the Parkinsonian neurotoxic species 1-methyl-4-phenylpyridinium species (MPP+, 36).129,130 The metabolism of MPTP proceeds via an initial two electron oxidation which yields the corresponding dihydropyridinium species MPDP+ (i, Scheme I-9). After a further two electron oxidation via an unknown mechanism, MPDP+ is converted to the 1-methyl-4-phenylpyridinium species MPP+. The nicotine iminium species 15 belongs to the same tertiary cyclic amine class, therefore the proposed metabolic pathway may be reasonable for the formation of β-nicotyrine from the oxidation of 15 catalyzed by MAO.
Shigenaga has performed in vitro incubations of the perchlorate salt of 15 with monoamine oxidase B (MAO-B) and has detected β-nicotyrine as a product. A proposed metabolic pathway of the conversion of 15 to β-nicotyrine is shown in Scheme I-10.64 The oxidized flavin of MAO-B abstracts one electron from the pyrroline nitrogen atom to yield the iminium cation radical intermediate i which spontaneously loses a proton to afford the carbon centered radical species ii. Abstraction of the second electron by the one electron reduced flavin of MAO-B yields a fully reduced flavin and the cationic intermediate iii. Spontaneous proton loss from iii yields the pyrrole containing end product β-nicotyrine.
1.4.3. Bioactivity of β-Nicotyrine

β-Nicotyrine has been reported to have insecticidal properties as mentioned above.\textsuperscript{72} This pyrrolic analog of (S)-nicotine possesses some weak nicotine-like pharmacological activity. Its cholinergic stimulating properties are only about 1% that of (S)-nicotine.\textsuperscript{131,132} The effects induced by β-nicotyrine include the contraction of guinea pig ileum and the elicitation of the presser response which, as a consequence of sympathetic ganglion stimulation and adrenal medulla release of catecholamines, results in vasoconstriction. The early literature also reported that β-nicotyrine shows some hypertensive properties.\textsuperscript{73} Gassell \textit{et al.} described the muscle relaxing effects of β-nicotyrine.\textsuperscript{133} Based on these skeletal muscle relaxing effects of β-nicotyrine, it was under consideration as a potentially useful therapeutic agent. However, at minimally toxic doses β-nicotyrine caused a general depression in respiratory and central nervous system activities.\textsuperscript{64}

Stålhandske \textit{et al.} reported that β-nicotyrine inhibits the metabolism of (S)-nicotine without increasing its toxicity.\textsuperscript{134} In their studies, mice were pretreated with β-nicotyrine and then injected with radiolabeled (S)-nicotine. Results showed a significant dose-dependent increase of (S)-nicotine in the liver, blood and brain. Concomitantly there was a significant decrease in the (S)-cotinine concentrations suggesting an inhibition of (S)-nicotine liver metabolism. Despite the higher (S)-nicotine concentrations in the brain of the pretreated mice, the LD\textsubscript{50} after an intraperitoneal injection of (S)-nicotine was not different between the two groups of mice. This protection against the lethal effects of increased (S)-nicotine levels suggested a direct protective action of β-nicotyrine on (S)-nicotine mediated effects in the central nervous system. More recently, Capland has observed that β-nicotyrine blocks (S)-nicotine induced constriction of vascular smooth muscle.\textsuperscript{134}

Another recent finding related to the potential pneumotoxicity of β-nicotyrine.\textsuperscript{135} Since the lung is exposed to β-nicotyrine, its biological activity relating to this organ is of interest. In an attempt to explore its pneumocytotoxic potential, β-nicotyrine was incubated with Clara cells isolated from rabbits lungs which are rich in cytochrome P-450. Alveolar macrophages, which contain
Chapter 1 Introduction and Literature Review

much less cytochrome P-450 were used as controls.\textsuperscript{136} Trypan blue exclusion and release of the cytoplasmic enzyme lactate dehydrogenase were utilized to assess toxicity. It was found that β-nicotyrine was more toxic to Clara cells than to alveolar macrophages. Consistent with a metabolic bioactivation pathway the cytochrome P-450 suicide substrate 1-aminobenzotriazole protected the Clara cells against the toxic effects of β-nicotyrine. These results indicate that the oxidative metabolites of β-nicotyrine catalyzed by cytochrome P-450, but not β-nicotyrine itself, contributed to the cytotoxicity of β-nicotyrine. Since β-nicotyrine is present in tobacco smoke and lung is the major organ directly exposed to tobacco smoke, this finding may be of toxicological relevance.

1.4.4. Metabolic Characteristics of β-Nicotyrine

1.4.4.1. The Pyridine Moiety

Based on the findings of the bioactivities of β-nicotyrine and its unidentified metabolites, it is necessary to explore the metabolic fate of this pyrrolic derivative. From the point of view of its chemical structure, β-nicotyrine should be metabolically reactive. Structurally β-nicotyrine possesses both a pyridine ring and a pyrrole ring. At present, we know nothing about the fate of the pyridyl moiety of β-nicotyrine. However, from an examination of the metabolism of (S)-nicotine, (S)-cotinine and nornicotine, which also possess a pyridine ring, it may be possible to obtain some insight into possible reaction pathways involving the pyridine ring of β-nicotyrine. While the metabolic degradation of the pyrroloidine of (S)-nicotine is extensive,\textsuperscript{137,138,139} the pyridine ring tends to be metabolically stable since 3-pyridylacetic acid (22) and 3-pyridylcarbinol 23 have been detected as urinary metabolites of (S)-nicotine.\textsuperscript{100,101} On the other hand, the pyridine ring is known to undergo N-oxidation to form (S)-nicotine-N-oxide (8), (S)-nicotine-N,N'-dioxide (7) and (S)-cotinine-N-oxide (25).\textsuperscript{140} (S)-Nicotine-N,N'-dioxide accounts for approximately 5% of the total N-oxidation pathway of (S)-nicotine.\textsuperscript{141} Therefore, the pyridine ring in β-nicotyrine might be oxidized to corresponding β-nicotyrine N-oxide.
Pyridine N-Oxide Derivatives of Tobacco Alkaloids

The N-methyl pyridinium species 36 and 37, which could be formed from nicotine and cotinine have been reported as urinary metabolites.\textsuperscript{142} However, in vitro, the N-methylation pathway was shown to be stereospecific, involving only the unnatural (R)-(+) enantiomer of nicotine.\textsuperscript{143,144} N-Methylation of (R)-nicotine has been demonstrated in human liver cytosolic preparations.\textsuperscript{145} In vitro studies characterizing guinea pig lung aromatic azaheterocycle-N-methyl transferase\textsuperscript{146} and studies using cytosolic azaheterocycle-N-methyl transferase purified from rabbit liver\textsuperscript{66} report that this enzyme is responsible for catalyzing the stereospecific formation of both N- and N'-methylated metabolites of nicotine.

The N-methylpyridinium moiety is of considerable toxicologic interest because of compounds such as MPP+ (35), the ultimate toxin derived from the Parkinsonian inducing agent MPTP (34).\textsuperscript{147} Recent evidence shows that N-methylpyridinium derivatives 36 and 37 may be substrates for the dopamine transporter as is the case with MPP+. Such interactions are thought to be associated with the nigrostriatal toxicity of MPP+\textsuperscript{148} A second toxic N-methylpyridinium system is the herbicide paraquat (38). This compound causes gastrointestinal irritation (hematemesis and bloody stool) and respiratory distress with the development of congestive hemorrhagic pulmonary edema that is accompanied by widespread cellular proliferation.\textsuperscript{149} Consequently, the N-methylation product derived from β-nicotyrine could contribute to the reported lung disorders caused by tobacco smoke.
Chapter 1  Introduction and Literature Review

MPTP (34) and N-Methylpyridinium Ions

1.4.4.2. The Pyrrole Moiety

The pyrrole moiety of β-nicotyrine should be much more metabolically reactive since the pyrrole moiety is a π-electron excessive cyclopentadienoid heteroaromatic system which would be expected to undergo oxidative transformations. As a class, the cyclopentadienoid heteroaromatic systems include some known toxins which may be converted to toxic metabolites. These reactive metabolic species may covalently bind to biological macromolecules and cause tissue injuries.⁹¹⁸ For example, the furan containing toxin 4-ipomeanol (39) has been reported to undergo cytochrome P-450 mediated bioactivation.⁹⁵⁰ A second example, the thiophene containing hepatocarcinogen methapyrilene (40), was also reported to undergo cytochrome P-450 mediated bioactivation and covalent binding to DNA.⁹⁵¹ The bioactivities of both of these compounds are based in part on their cyclopentadienoid heteroaromatic moieties.⁹⁵¹,⁹⁵² The metabolic processes and results of these cytochrome P-450 mediated bioactivations have been reviewed by Shigenaga in detail.⁶³

Toxic Heterocyclic Analogs
Chapter 1 Introduction and Literature Review

With the exception of acute receptor mediated toxic responses, the majority of toxic and carcinogenic chemicals do not produce their detrimental biological effects by themselves. In most cases the xenobiotics are oxidized to electrophilic intermediates capable of reacting irreversibly with macromolecular nucleophiles. The bioactivations can be catalyzed by alcohol dehydrogenase, monoamine oxidase, microsomal flavin-containing monooxygenases, myeloperoxidase and peroxidases. However, the majority of oxidative bioactivation reactions can be attributed to cytochrome P-450.

The cytochrome P-450 family of enzymes are hemoproteins. They are located in the endoplasmic reticulum of many cell types. Cytochrome P-450 activity is found in high concentrations in the liver, the major organ involved in the metabolism of xenobiotics. In addition, it is present in lung, kidney, brain, intestine, skin, placenta and adrenal cortex. An important feature of the cytochrome P-450 enzymes is their ability to metabolize almost an unlimited number of diverse substrates by a variety of oxidative transformations. Because of its membrane-bound nature, the cytochrome P-450 monooxygenase system is in a lipoidal environment. This is why this system shows catalytic activity towards the oxidation of lipophilic xenobiotics.

Cytochrome P-450 is responsible for transferring an oxygen atom to the substrate molecule R-H in a reaction coupled to the oxidation of the cofactor NADPH. The process involves an NADPH-dependent cytochrome P-450 reductase and/or an NADH-linked cytochrome b5. The latter two components, along with the cofactors NADPH and NADH, supply the reducing equivalents needed in the overall metabolic oxidation of foreign compounds. The catalytic role that the cytochrome P-450 monooxygenase system plays in the oxidation of xenobiotics is summarized in the cycle shown in Figure I-1. It starts with the bonding of substrate to the oxidized ferric (Fe$^{3+}$) resting state of cytochrome P-450 to form an enzyme substrate complex. This complex then undergoes transfer of one electron from NADPH in a reaction catalyzed by cytochrome P-450 reductase. This one electron transfer leads to the ferrous (Fe$^{2+}$) form of the
Chapter 1  Introduction and Literature Review

enzyme. This reduced P-450-substrate complex is capable of binding dioxygen (O₂). The dioxygen-P-450-substrate complex then undergoes a second NADPH-dependent one-electron reduction (catalyzed by cytochrome P-450 reductase-NADPH and/or cytochrome b5 reductase-NADH) to yield a peroxide dianion-P-450 (Fe³⁺)-substrate complex. Water (containing one of the oxygen atoms from the original dioxygen molecule) is released from the later intermediate to form an activated oxygen-P-450-substrate complex. The oxygen in this complex is highly electron-deficient and is a potent oxidizing agent. The activated oxygen is transferred to the substrate (RH) and the oxidized substrate product (ROH) is released from the enzyme complex to regenerate the oxidized form of cytochrome P-450 (Figure I-1).¹⁶⁵

Cytochrome P-450 is able to metabolize an almost unlimited number of diverse substrates by a variety of oxidative transformations including alkene epoxidation, alcohol and halide oxidation to carbonyl compounds, aldehyde oxidations to acids, carbon-nitrogen system oxidations and aromatic hydroxylations. These aromatic oxidations, for example, the oxidation of benzene to phenol, proceed by a mechanism involving an NIH shift, which is illustrated by the oxidation of p-deuterated anisole (Scheme I-11).¹⁶⁶

Scheme I-11  NIH shift
Fig. 1.1. Proposed catalytic reaction cycle involving cytochrome P-450 in the oxidation of xenobiotics.

\[ \text{Cytochrome P-450 Reductase} \]

\[ \text{Oxidized Product} \]

\[ \text{R-OH} \]

\[ \text{R-H} \]

\[ \text{Substrate} \]

\[ \text{Catalytic Cycle} \]

\[ \text{Activated Oxygen Species} \]

\[ \text{H}_2\text{O} \]

\[ \text{2H}^+ \]

\[ \text{O}_2 \]

\[ \text{NADPH or NADH} \]

\[ \text{CO} \]

\[ \text{Absorbs at 450 nm} \]

\[ \text{CO} \]

\[ \text{450 nm} \]

\[ \text{Cytochrome P-450 Reductase} \]

\[ \text{Cytochrome b} \]

\[ \text{e}^- \text{NADPH} \]
Chapter 1 Introduction and Literature Review

The aromatic ring i is first oxidized by cytochrome P-450 to the epoxide ii, followed by ring opening in the direction that generates the most resonance-stabilized carbocation iii, (positive charge on C-3 is resonance-stabilized by OCH₃ group). The zwitterionic species iii then undergoes a spontaneous rearrangement, by a 1,2-deuteride shift (NIH shift) to form the dienone iv. Final transformation of the dienone to 3-deuterio-4-hydroxyanisol occurs with the preferential loss of a proton because of the weaker bond energy of the C-H bond compared with the C-D bond. Thus the deuterium is retained in the molecule by undergoing this intramolecular NIH shift. The results of this experiment are taken as indirect evidence for the involvement of an arene oxide.

Arene oxidation of pyrroles also may be plausible. As an example, the pyrrole containing analog pyrrolnitrine (41) is a broad-spectrum antifungal agent which is degraded metabolically in vivo and in vitro.¹⁶⁷ The pyrrole ring of 41 is metabolized rapidly in an NADPH dependent reaction by microsomal enzymes to yield 4-chloro-3-(3-chloro-2-nitrophenyl)-maleimide (42), the keto form of an analog of phenol. Since in general, maleimide derivatives are chemically reactive, it was anticipated that covalent binding of 42 to protein may occur. This hypothesis has been examined with synthetic 42. The synthetic 42 was incubated with β-mercaptoethanol and found to form the thiol adduct 43, evidence which illustrates the bioalkylating potential of the maleimide (Scheme I-12).¹⁶⁷

![Scheme I-12 Metabolism of pyrrolnitrine](image)

31
Chapter 1  Introduction and Literature Review

In addition, Guengerich et al. studied the covalent binding of tritium labeled 1,3,4-trimethylpyrrole (44) and 1-methyl-3,4-bis(hydroxymethyl)pyrrole (45) and found that both pyrrole derivatives formed covalent bonds to protein, DNA and RNA via reactive metabolites. The proposal that the covalent binding of these pyrrole derivatives is due to cytochrome P-450 dependent bioactivation is supported by in vitro studies which showed that the covalent binding of 44 to liver microsomal fractions was inhibited by SKF 525-A, carbon monoxide, anaerobiosis and glutathione. Furthermore, reconstituted vesicles containing cytochrome P-450 and cytochrome P-450 reductase also catalyzed the bioactivation of the trimethyl compound 44. These examples indicate a common potential for the oxidative metabolism of 5-membered aromatic heterocyclic compounds and the possibility that their reactive metabolites combine with biological macromolecules.

Since β-nicotyrine possesses an electron rich pyrrolic moiety, it is reasonable to propose that this system may undergo enzyme catalyzed oxidation that could lead to reactive species. These reactive intermediates may form covalent bonds to biological macromolecules and cause tissue damage. The cytotoxic effects of β-nicotyrine evaluated using isolated Clara cells, which are rich in cytochrome P-450 provided some support for this hypothesis. The metabolic pathway that might be responsible for the generation of reactive metabolites, however, remains to be identified.

The other research interest is the nitrosation of tobacco alkaloids or the formation and the bioactivities of nicotine-derived nitrosamines, or namely tobacco-specific N-nitrosamines (TSNA). The nitrosamines constitute a family of powerful animal carcinogens. They may be carcinogenic to humans as well. Nitrosoamines may form naturally in humans who excrete small quantities of N-nitrosopropylene in their urine daily.
Chapter 1 Introduction and Literature Review

Nitrosamines form easily from ubiquitous precursors. Amines or other nitrogen containing compounds react readily with nitrosating agents (Y-NO) to give nitrosamines. This can be described in a general equation shown in Scheme I-13.\(^\text{172}\)

\[
\ce{N-H + Y-NO <=> N-NO + HY}
\]

Scheme I-13 Nitrosation

Y-NO can be considered an NO\(^+\) carrier. Neither nitrous acid (HO-NO) nor nitrite ion (NO\(_2^+\)) acts as an effective nitrosating agent, except during photolysis or radiolysis, when nitrogen oxides are generated. Under mild acidic conditions, the nitrosating agent N\(_2\)O\(_3\) is formed from two molecules of nitrous acid, and reacts with unprotonated amines to form nitrosamines.

The tobacco alkaloids including secondary amines, such as nornicotine, anatabine and anabasine, and tertiary amines, such as (S)-nicotine, are not carcinogens, but they may be transformed to the carcinogenic nitrosamines. In the study on carcinogenic agents in snuff, a high concentration of carcinogenic N-nitrosamines was found, especially nicotine-derived nitrosamines (TSNA) formed during tobacco processing\(^\text{173}\) and storage, with the greatest concentration occurring under condition of high moisture and high temperature.\(^\text{174,175}\) The content of tobacco alkaloids and TSNA is different in fresh green tobacco leaves, dried tobacco leaves and cured tobacco leaves. For example, TSNA levels increased to 0.9-17.8 mg/g during curing of green mature tobacco leaves, which contain a smaller amount of TSNA, 0.6-1.5 mg/g.\(^\text{176}\) Sunning tobacco leaves also increases the content of TSNA.\(^\text{177}\)

A current study has reported that compounds containing a pyrrole moiety may block N-nitrosation.\(^\text{178}\) Pyrrole itself reacts with nitrosating agents, like nitrous acid, to produce a C-nitrosated product instead of the nitrosamine. Although the initial reaction may occur on the pyrrole nitrogen, the resulting product is unstable and undergoes rapid rearrangement to give a ring carbon nitrosated product, e.g. Compound 46 when treated with nitrous acid gives 47 (Scheme I-14).\(^\text{178}\) In some cases nitrosation with a large excess of nitrous acid

33
Chapter 1  Introduction and Literature Review

leads to diazo formation.\textsuperscript{179} The details of the reaction mechanism still need more evidence to be established. However, unfortunately, Wilcox et al. found that β-nicotyrine is an exception because the electron-withdrawing group, the pyridyl group, stabilizes the pyrrole ring and reduces the blocking efficiency. Whether the nitrosation of β-nicotyrine itself occurs has not been reported.

\begin{center}
\textbf{Scheme I-14 Pyrrole compound blocking N-nitrosation}
\end{center}

1.5. Research Proposal

Tobacco users will be exposed to β-nicotyrine since it is present in both the tobacco plants and tobacco smoke and is a potential metabolite of (S)-nicotine. Because of the readily oxidizable pyrrole moiety in β-nicotyrine and the preliminary evidence of its cytotoxicity, this compound may contribute to human health disorders which are associated with tobacco use. Consequently, the metabolic fate of β-nicotyrine becomes an important research topic. At the present, relatively little is known about the metabolic fate of β-nicotyrine, both in vitro and in vivo. Therefore, this program was initiated in an effort to characterize more fully the metabolic fate of this tobacco alkaloid.

Limited in vivo metabolic studies have shown that β-nicotyrine is more extensively metabolized than (S)-nicotine.\textsuperscript{116} Beckett et al. reported GC analysis of urine samples obtained from human treated with β-nicotyrine which established that β-nicotyrine is extensively metabolized in the human.\textsuperscript{118} However, in these studies no attempts were made to characterize the metabolites of β-nicotyrine. An in vitro study reported by Shigenaga on the metabolism of β-nicotyrine has led to characterization of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48), a metabolite which accounted for only about 10\% of the total metabolites formed (Scheme I-15).\textsuperscript{180} In this thesis both the in vitro and in vivo metabolic fate of this minor tobacco alkaloid is examined in detail.
Scheme I-15 In vitro metabolism of β-nicotyrine
Chapter 1  Introduction and Literature Review

References


Chapter 1 Introduction and Literature Review


16 Hooper, W. D. (1885) A case of irritation of the ganglion of Remark, or Inhibitory nerve of the heart, cured by smoking tobacco. Virginia Med. Month. 12, 473-475.


24 Slevin, P. (1895) A case of obstinate hiccoughing which continued for twelve days without intermission. Lancet (London) 2, 92.
Chapter 1  Introduction and Literature Review


38


Martin, E. P. (1951) Insecticides. Span Patent 194,089


Chapter 1 Introduction and Literature Review


57 Committee on Passive Smoking, Board on Environmental Studies and Toxicology, National Research Council (1986) Environmental tobacco smoke. Measuring
Chapter 1 Introduction and Literature Review

exposures and assessing health effects. p 337, National Academy Press, Washington, D.C.


Chapter 1 Introduction and Literature Review


Chapter 1 Introduction and Literature Review


Chapter 1 Introduction and Literature Review


Chapter 1 Introduction and Literature Review


45
Chapter 1  Introduction and Literature Review


Chapter 1 Introduction and Literature Review


Chapter 1 Introduction and Literature Review


Chapter 1 Introduction and Literature Review


Chapter 1 Introduction and Literature Review


Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2.1. Background

As discussed in Chapter 1, β-nicotyrine (2) is an important minor tobacco alkaloid existing in both tobacco plants and smoke. Preliminary evidence indicates that this compound may be a pneumotoxin\textsuperscript{1} and it is a potential extrahepatic metabolite of (S)-nicotine. As an electron rich aromatic system, it is reasonable to postulate that it should undergo extensive oxidative metabolism to products of potential toxicological significance. However, up to now, only limited information on the in vitro and in vivo metabolism of β-nicotyrine has been available. This thesis has been pursued to expand the body of knowledge describing the metabolic fate of β-nicotyrine.

Jenner and Gorrod reported the results of their in vitro studies on the oxidative metabolism of (S)-nicotine (1), (S)-N'-methylanabasine (31) and β-nicotyrine (2) in 1973.\textsuperscript{2} Compound 1 and 31 underwent N'-oxidation to form the corresponding N'-oxide metabolites nicotine-N'-oxide (6) and N'-methylanabasine-N'-oxide (32). However, as expected the pyrrolic β-nicotyrine did not undergo N'-oxidation. Of these three compounds, β-nicotyrine appeared to be the most extensively metabolized in almost all species studied, mouse, guinea pig, rabbit and hamster. The rat was an exception. No unchanged β-nicotyrine was recovered from the hamster or guinea pig. Rat liver preparations had the lowest metabolic activity toward β-nicotyrine. Jenner and Gorrod postulated that the greater overall metabolism of β-nicotyrine relative to (S)-nicotine and N'-methylanabasine could be explained in terms of its lower pKa\textsubscript{1} value (4.73) compared to the pKa\textsubscript{1} values of (S)-nicotine (7.9) and N'-methylanabasine.\textsuperscript{3} With a lower pKa\textsubscript{1} value, β-nicotyrine should display greater lipid solubility at physiological pH and have greater access to the membrane bound cytochrome P-450 monooxygenase system.\textsuperscript{4} The metabolic products of β-nicotyrine were not determined in these studies.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Shigenaga has examined the in vitro metabolic fate of β-nicotyrine using rabbit liver and lung microsomal preparations. He reported that β-nicotyrine is metabolized by the NADPH dependent cytochrome P-450 system. In the absence of NADPH or in the presence of a cytochrome P-450 inhibitor, such as octylamine or amphetamine, the rate of β-nicotyrine metabolism was decreased. Low temperature (4 °C) also inhibited the metabolism of β-nicotyrine. Shigenaga identified one of the metabolites of β-nicotyrine as 1-methyl-5-(3-pyridyl)-4,5-pyrroline-2-one (48) by HPLC, GC, UV and mass spectroscopic methods. He also proposed a mechanism to account for the formation of 48. A cytochrome P-450 catalyzed oxidation of the pyrrole ring proceeds via a pathway that leads to the unstable arene oxide intermediate 49. This intermediate rearranges to 2-hydroxy-1-methyl-5-(3-pyridyl)pyrrole (50) via the protonated species i. Subsequently tautomerization leads to the final product 48 which is in equilibrium with its tautomer 51 (Scheme II-1).

![Scheme II-1 Formation of the β-nicotyrine metabolites 48 and 51](image)

This mixture of pyrrolinone metabolites was found to be unstable. At room temperature overnight, the mixture of 48 and 51 underwent autoxidation to form 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (52, Scheme II-2). It was proposed that this autoxidation reaction might involve a free radical process. The pyrrolinones may undergo tautomerization to form the corresponding enol, 2-hydroxy-1-methyl-5-(3-pyridyl)pyrrole (50). This unstable enol may lose a proton to form the corresponding anion i, a highly
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine
electron rich species. When i is exposed to air, it may lose an electron to
dioxygen to generate superoxide radical anion and the resonance stabilized
radical ii <-> iii <-> iv <->. This radical could undergo various reactions
including addition of protonated superoxide to form the hydroperoxypyrrrolinone
species 53 and 54. After subsequent hydrolysis, 53 and 54 could form the
hydroxypyrrrolinone isomers 52 and 55 (Scheme II-2).

\[
\begin{align*}
&\text{51} & &\text{50} & &\text{52} \\
&\text{53} & &\text{54} & &\text{55}
\end{align*}
\]

**Scheme II-2** Proposed autoxidation mechanism of the β-nicotyrine
metabolites 48 and 51

This was the first reported metabolic pathway for β-nicotyrine. However,
the pyrrrolinones accounted for only about 10% of the original substrate. Since
β-nicotyrine was extensively metabolized and no recovered substrate was
detected, the fate and identity of the other 90% remained unknown. One
reasonable hypothesis is that the unknown metabolites were more hydrophilic
species that are not extracted into the methylene chloride which Shigenaga
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

used in his purification procedures. Owing to the instabilities of the pyrrolinone metabolite and the documented formation of the hydroxypyrrrolinone 52, additional secondary polar metabolites, such as 55, might be formed from the unstable primary metabolites by oxidation or hydrolysis.

In an attempt to determine and identify the unknown in vitro metabolites of β-nicotyrine and to be able to assess better the biological contribution which these metabolic intermediates, metabolites and products may make to β-nicotyrine's putative pneumotoxic properties, the following studies have been undertaken:

1. An HPLC based assay that will provide quantitative estimations of both polar and non-polar metabolites was developed.

2. The in vitro metabolic profile of β-nicotyrine was investigated in a variety of settings.

3. The putative metabolites were synthesized.

4. A more complete characterization of the chemical behavior of the pyrrolinone metabolites derived from β-nicotyrine was pursued with particular attention to the potential involvement of antioxidative radical chemistry.

2.2. Results and Discussion

2.2.1. Chemistry of β-Nicotyrine Metabolites

The in vitro studies on the metabolism of β-nicotyrine were initiated with an attempt to repeat the studies with rabbit liver microsomal preparations as previously performed by Shigenaga. In this case, however, we wanted to employ an analysis that would lead to the detection of polar metabolites. Synthetic standards of the substrate molecule and known metabolites had to be prepared. In addition to the pyrrolinone species 48 and 51, other candidate compounds, such as 5'-hydroxycotinine (20) and the 5'-hydroxypyrrrolinone 52 had to be prepared.
Fig. II-1. $^1$H NMR spectrum of $\beta$-nicotyrine free base (2) in CDC$_3$. 
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Fig. II-2. GC-EIMS data of β-nicotyrine free base (2).

Abundance

5.0E-4
1.0E-4
5.0E-5
1.0E-5
0.0E+0
51
77
89
105
117
130
143
158
140
120
100
80
60

Mass/Charge

Scan 4.181 min. of DATA: L590E.D

Abundance

5.0E-4
1.0E-4
5.0E-5
1.0E-5
0.0E+0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0

Time (min.)

TIC of DATA: L590E.D

2
N

N

CH₃
Fig. II.3. $^1$H NMR spectrum of $\beta$-nicotyrine tartrate in CD$_3$OD.
2.2.1.1. Synthesis of β-Nicotyrine

The first compound required for our in vitro studies was the target compound β-nicotyrine. Several methods have been reported for the preparation of β-nicotyrine. Shigenaga reported the dehydrogenation of (S)-nicotine catalyzed by Pd/C. Mitano reported a palladium-phosphine complex catalyzed cross-coupling reaction to link the pyridine and pyrrole ring. Mullen reported a method using phenyl vinyl sulfoxide as a masked equivalent of an acetylene dipolarophile. Yamada reported a photo-induced electron transfer oxidation of (S)-nicotine to prepare β-nicotyrine. Mitano's method involves three steps and requires carefully controlled reactions. Mullen's method involves problems with purification of the final product β-nicotyrine because of the complexity of the reaction mixture. Shigenaga's method is a classic catalytic dehydrogenation reaction, a one-step conversion starting with the commercially available (S)-nicotine. However, the reported yield of the pure tartrate salt of β-nicotyrine was only 3.6%. Shibagaki has compared the catalytic dehydrogenation of (S)-nicotine with different catalysts and under different reaction temperatures. The dehydrogenation products of (S)-nicotine were found to be a mixture of myosmine (5) and β-nicotyrine. At 500 °C, hydrous zirconium oxide is reported to give a 100% conversion of (S)-nicotine to give principally β-nicotyrine. However, at this high temperature, β-nicotyrine undergoes polymerization and therefore decreases the final yield. Lowering the reaction temperature, sharply decreases the conversion of (S)-nicotine and decreases the selectivity for the formation of β-nicotyrine. A mixture of palladium and hydrous zirconium oxide at a lower reaction temperature (300 °C) was found to be the best catalyst and conditions for this reaction. Unfortunately, a reference for the preparation of the catalyst, hydrous zirconium oxide, could not be found. A consideration of these factors led to the selection of Pd/C as catalyst, using a lower reaction temperature (240 °C) and a longer reaction time (6 hours) compared to Shigenaga's conditions. A high vacuum distillation of the crude product led to the separation of two major fractions with boiling ranges of 38-40 °C / 0.17 mmHg and 92-94 °C / 0.35 mmHg. The higher temperature fraction provided a 22% yield of β-nicotyrine. The ^1H NMR (Fig. II-1) and GC-EIMS (Fig. II-2) spectra were consistent with the
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

desired product β-nicotyrine (Table II-1 and II-2). The tartrate salt could be obtained in pure form in 89% yield giving an overall yield of 20%. The melting point and ¹H NMR spectra (Fig. II-3) of β-nicotyrine tartrate were consistent with the published data.⁶

**Table II-1.** ¹H NMR (CDCl₃) data assignment of the products from the dehydrogenation of (S)-nicotine

<table>
<thead>
<tr>
<th>Compound</th>
<th>¹H NMR Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Butylpyridine</td>
<td>8.40 (2H, m, C2- and C6-H), 7.45 (1H, m, C4-H), 7.16 (1H, m, C5-H), 2.58 (2H, q, CH₂), 1.60 (2H, m, CH₂), 1.38 (2H, m, CH₂), 0.90 (3H, t, CH₃)</td>
</tr>
<tr>
<td>β-Nicotyrine</td>
<td>8.67 (1H, q, C2-H), 8.50 (1H, q, C6-H), 7.65 (1H, q, C4-H), 7.28 (1H, m, C5-H), 6.74 (1H, d, C3'-H), 6.28 (1H, m, C4'-H), 6.21 (1H, m, C2'-H), 3.84 (3H, s, N'-Me)</td>
</tr>
</tbody>
</table>

**Table II-2.** GC-EIMS data assignment of the products from the dehydrogenation of (S)-nicotine

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>M⁺⁺ (%)</th>
<th>Mass Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Butylpyridine (C₉H₁₃N)</td>
<td>2.35</td>
<td>135 (75)</td>
<td>120 (5%, [M-Me]+), 106 (18%, [M-Et]+), 92 (100%, [PyCH₂]+), 93 (97%, [PyHCH₂]+), 79 (8%, PyH⁺), 78 (8%, Py⁺)</td>
</tr>
<tr>
<td>β-Nicotyrine (C₁₀H₁₀N₂)</td>
<td>4.18</td>
<td>158 (100)</td>
<td>143 (5%, [M-Me]+), 130 (21%, [PyC₃H₂N]+), 116 (9%, [PyC₃H₂]+), 105 (4%, [PyC₂H]+), 104 (5%, [PyC₂]+), 90 (6%, [PyC]+), 79 (6%, PyH⁺), 78 (5%, Py⁺)</td>
</tr>
</tbody>
</table>

The lower temperature fraction was also examined by both ¹H NMR (Fig. II-4) and GC-EIMS (Fig. II-5). The GC retention time of this fraction was 2.35 minutes. The mass spectrum gave a molecular ion at m/z 135, which is assigned to a C₉H₁₃N species and could be a butylpyridine. The base peak was observed at m/z 92 which is due to C₆H₆N and could be the conjugation stabilized [PyCH₂]+ species generated by a loss of C₃H₇. Other fragments are summarized in Table II-2 and the major fragments are showed in Scheme II-3.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

The molecular radical cation at m/z 135 (i) could fragment in a variety of ways: (1) to yield the ion at m/z 120 (ii) by loss of a methyl radical; (2) to ion at m/z 106 (iii) by loss of an ethyl radical; (3) to ion at m/z 92 (iv) by loss of an propyl radical and (4) to ion at m/z 78, the pyridyl ion (v), by loss of a butyl radical. The ions at m/z 107 (vi), 93 (vii) and 79 (viii) could be the hydrogenated radical ions of iii, iv and v, probably formed via McLafferty rearrangement.

Scheme II-3  Mass spectral fragmentation of butylpyridine

The \(^1\)H NMR spectra showed in addition to the aromatic proton signals three groups of methylene proton signals at δ 2.58 (quartet), 1.60 (multiple) and 1.38 (multiple), and one methyl group signal at δ 0.90 (triplet). These data suggested that this low boiling fraction was 3-butylpyridine (56) which may be formed from (S)-nicotine by hydrogenolysis of the 1,2 and 1,5 carbon-nitrogen bonds of the pyrrolidine ring with loss of methylamine. The boiling point (38-40 °C / 0.17 mm) is consistent with the published data (86-88 °C / 8 mm) for 3-butylpyridine.\(^{11}\) This is the first report that this compound is a by-product formed from the catalytic dehydrogenation of (S)-nicotine (Scheme II-4).

Scheme II-4  Dehydrogenation of (S)-nicotine
Fig. II-4. $^1$H NMR spectrum of 3-butylypyridine (56) in CDC$_6$.
FIG. II-5. GC-EI-MS spectrum of 3-butyrylpyridine (56).
2.2.1.2. Synthesis of 5'-hydroxycytosine (20) and 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolidin-2-one (52)

5'-Hydroxycytosine (20) has been detected as a minor metabolite of (S)-nicotine in smoker's urine.\textsuperscript{12} It also is formed from (S)-cotinine both in vitro and in vivo in the human and animals by hydroxylation of the pyrrolidinone ring of (S)-cotinine.\textsuperscript{13,14} Since 90% of the β-nicotyrine substrate was unaccounted for, 20 was considered to be a possible β-nicotyrine metabolite since other pyrrole derivatives are known to undergo oxidative conversion to pyrrolidinone and succinimides.\textsuperscript{15}

The synthesis of 20 has been reported by Daghe\textsuperscript{16} and was repeated by Nguyen.\textsuperscript{17} The synthetic sequence started with commercially available (S)-nicotine (1). By oxidation with bromine in aqueous acetic acid, 1 was converted to 3',3'-dibromocytosine (57), which is the intermediate product for the preparation of (S)-cotinine from (S)-nicotine.\textsuperscript{18,19} Treatment of 57 with potassium methoxide in methanol leads to 5'-methoxypyrrrolinone 58. Cleavage of the ether bond with aqueous hydrobromic acid gave the 5'-hydroxypyrrrolinone 52 which was hydrogenated in the present of Pd/C to form 5'-hydroxycytosine (20) (Scheme II-5).

![Scheme II-5](image)

**Scheme II-5** Reported synthesis of 5'-hydroxycytosine (20) and 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52)
Chapter 2 In Vitro Studies on the Metabolism of $\beta$-Nicotyrine

In this synthetic pathway the 5'-hydroxypyrrolinone 52 was also prepared as an intermediate product. This is another target compound because it has been identified as an autooxidation product of the pyrrolinones 48 and 51. An unanswered question concerns the possible metabolic vs spontaneous oxidation of 48/51 to 52. Therefore, a synthetic sample of 52 also was needed as a standard to compare with the incubation products.

In order to prepare 20 and 52, the 5-methoxypyrrolinone 58 was required as starting material. In the above published synthetic methods, however, the free base of 58 was not characterized. We were able to isolate 58 as a free base by vacuum distillation and to characterize the resulting yellow oil. The data from the 270 MHz $^1$H NMR spectrum of 58 (Fig II-6) are summarized in Table II-3.

Table II-3. $^1$H NMR (DMSO-d$_6$) data assignments for 5-methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (58)

<table>
<thead>
<tr>
<th>$\delta$ (ppm)</th>
<th>$J$ (Hz)</th>
<th>Coupling</th>
<th>Integration</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.66</td>
<td>4.7, 1.6</td>
<td>dd</td>
<td>1H</td>
<td>C6-H</td>
</tr>
<tr>
<td>8.60</td>
<td>1.0, 2.2</td>
<td>dd</td>
<td>1H</td>
<td>C2-H</td>
</tr>
<tr>
<td>7.71</td>
<td>8.0, 1.6, 2.2</td>
<td>m</td>
<td>1H</td>
<td>C4-H</td>
</tr>
<tr>
<td>7.48</td>
<td>8.0, 4.7, 1.0</td>
<td>m</td>
<td>1H</td>
<td>C5-H</td>
</tr>
<tr>
<td>7.25</td>
<td>1.3, 5.9</td>
<td>dd</td>
<td>1H</td>
<td>C4'-H</td>
</tr>
<tr>
<td>6.50</td>
<td>1.3, 5.9</td>
<td>dd</td>
<td>1H</td>
<td>C3'-H</td>
</tr>
<tr>
<td>3.20</td>
<td></td>
<td>s</td>
<td>3H</td>
<td>O-CH$_3$</td>
</tr>
<tr>
<td>2.59</td>
<td></td>
<td>s</td>
<td>3H</td>
<td>N'-CH$_3$</td>
</tr>
</tbody>
</table>

The pyridyl ring protons at C2 and C6 are adjacent to nitrogen and therefore the corresponding signals appear the most downfield with the more upfield signal at $\delta$ 8.60 being assigned to the C-2 proton since only long range coupling is observed with $J_{2,4} = 2.2$ and $J_{2,5} = 1.0$ Hz. The quartet observed for the C6-proton is due to the coupling with the C5-proton ($J = 4.7$ Hz) and long range coupling with the C4-proton ($J = 1.6$ Hz). For the same reason, the C4-proton signal should be more downfield than the C5-proton signal. The C4-
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

and C5-proton coupling constant is 8.0 Hz. The protons with chemical shifts δ 6.89 and 6.40 are due to the two vinyl protons, the C3'- and the C4'-protons. Because they only have one adjacent proton, both appear as doublets with J = 5.9 Hz. The coupling constant of C3' and C4' protons of J=1.3 Hz probably is due to the long range coupling between the vinyl protons with the aromatic protons. Since C3' is attached to a carbonyl group, the C3'-proton should be more downfield. The two singlet peaks are due to the methyl groups. Since oxygen is more electronegative than nitrogen, the O-methyl protons should be more downfield than the N-methyl protons (Fig II-6).

Scheme II-6  Mass spectral assignment of 5-methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (58)

The mass spectrum (Fig II-7, Scheme II-6) of this product is fully consistent with its assigned structure. The molecular ion (i) of 58 appears at m/z 204 and corresponds to the molecular weight C_{11}H_{12}N_{2}O_{2}. The base peak appears at m/z 173 (C_{10}H_{9}N_{2}O) and corresponds to the loss of a methoxyl group to form the resonance stabilized iminium species ii. The other major ion was found at m/z 126 (iii, relative abundance 86%) and can be rationalized by loss of a pyridyl group from M+. The ion at m/z 145 could be due to loss of carbon monoxide from the ion iii and rationalized as iv. We have also assigned the other major fragments at m/z 119 to v and the ion at m/z 104 to vi. The peak at m/z 78 is consistent with a pyridyl cation.
Fig. II-6. \(^1^H\) NMR spectrum of 5-methoxy-1-methyl-(3-pyridyl)-3-pyrrolyl-2-one (58) in DMSO-d6.
Fig. II-7. GC-EIMS data of 5-methoxy-1-methyl-(3-pyridyl)-3-pyrroline-2-one (58).
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

In the published synthetic route (Scheme II-5), 5'-methoxypyrrolinone 58 was converted to 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) by cleavage of the ether bond with hydrobromic acid followed by a palladium on carbon catalyzed hydrogenation to form 5'-hydroxycotinine (20). Many by-products were encountered due to the vigorous reaction conditions and the reactivity of the pyrrolinone system. An alternative approach was pursued in which 58 was first reduced to 5-methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolidin-2-one (59) by a palladium on carbon catalyzed hydrogenation in methanol. Then the ether bond of 59 could be cleaved by trimethylsilyl iodide (TMSI) to form a silyl ether 60, which is hydrolyzed to gain 20. We also examined the conversion of 58 to 52 through 61 with TMSI (Scheme II-7).

![Chemical Structures](image)

**Scheme II-7** Synthesis of 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolidin-2-one (52) and 5'-hydroxycotinine (20)

The reduction of 58 gave an essentially quantitative yield of 59 which appeared to be pure according to the $^1$H NMR (Fig. II-8) and GC-EIMS spectra (Fig. II-9). The sample for CHN analysis of this unknown compound was obtained following recrystallization from ether/ethyl acetate.

The $^1$H NMR (CDCl$_3$) spectrum of 59 confirmed the reduction of the double bond since the two signals at 6.89 (3'-H) and 6.40 (4'-H) of the starting material were no longer present. Three multiplets at δ 2.65 (C3' methylene protons), 2.49 and 2.21 (C'4 methylene protons) were present. These and the remaining assignments are summarized in Table II-4.
Fig. 11.8. 1H NMR spectrum of 5-methoxy-1-methyl-3-(3-pyridyl)pyrrolidin-2-one (59) in DMSO-d6.
FIG. II-9A. GC-EIMS data of 5-methoxy-1-methyl-3-pyridylpyrrolidin-2-one (59).

[Diagram showing mass/charge, time (min.), and abundance plots.]

Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

73
Table II-4. $^1$H NMR (CDCl$_3$) data assignment of 5-methoxy-1-methyl-5-(3-pyridyl)-pyrrolidin-2-one (59)

<table>
<thead>
<tr>
<th>$\delta$ (ppm)</th>
<th>$J$ (Hz)</th>
<th>Coupling</th>
<th>Integrate</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.67</td>
<td>2.1</td>
<td>d</td>
<td>1H</td>
<td>C2-H</td>
</tr>
<tr>
<td>8.60</td>
<td>1.6, 4.7</td>
<td>dd</td>
<td>1H</td>
<td>C6-H</td>
</tr>
<tr>
<td>7.82</td>
<td>2.1, 8.0, 1.6</td>
<td>m</td>
<td>1H</td>
<td>C4-H</td>
</tr>
<tr>
<td>7.49</td>
<td>8.0, 4.7</td>
<td>dd</td>
<td>1H</td>
<td>C5-H</td>
</tr>
<tr>
<td>3.23</td>
<td></td>
<td>s</td>
<td>3H</td>
<td>O-CH$_3$</td>
</tr>
<tr>
<td>2.63-2.58</td>
<td></td>
<td>m</td>
<td>2H</td>
<td>C3'-H</td>
</tr>
<tr>
<td>2.53-2.47</td>
<td></td>
<td>m</td>
<td>1H</td>
<td>C4'-H</td>
</tr>
<tr>
<td>2.44</td>
<td></td>
<td>s</td>
<td>3H</td>
<td>N-Me</td>
</tr>
<tr>
<td>2.28-2.20</td>
<td></td>
<td>m</td>
<td>1H</td>
<td>C4'-H</td>
</tr>
</tbody>
</table>

Scheme II-8 Mass spectral fragmentation of 5-methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolidin-2-one (59)

The GC-EIMS total ion chromatogram (TIC) of 59 gave a peak at retention time 5.59 minutes (Fig. II-9a). The mass spectrum showed a very weak parent ion i at m/z 206 which is due to C$_{11}$H$_{14}$N$_2$O$_2$ (the nominal mass of 59) and two major fragment ions at m/z 175 (ii) due to loss of CH$_3$O- and at m/z 128 (iii) due to loss of a pyridyl moiety. Similar to the fragmentation of 58, ii loses a cyclopropanone moiety to form iv at m/z 119. By loss of carbon monoxide, iii is converted to v (m/z 100). The parent ion also may lose the
amid group to form vi (m/z 149). Fragment vi then loses a methyl and a vinyl group to form vii (m/z 106). As seen with 58, iv also loses a methyl radical to form vii (m/z 104, Scheme II-9).

Sometimes the GC-EIMS TIC of 59 gave three peaks with retention times of 4.00, 4.18 and 4.57 minutes (Fig. II-9b). The mass spectra of the major peak at 4.57 minutes showed that it was 59. Both of the other two minor chromatographic peaks displayed a molecular ion at m/z 174. This chromatographic behavior and the mass fragmentation pattern indicate the thermal instability of 59 which decomposed in the GC system with loss of methanol to form the two pyrrolinone species 48 and 51, both of which have the molecular weight 174 (Scheme II-8).

![Scheme II-9](image)

**Scheme II-9** Decomposition of 5'-methoxycotinine (59)

As mentioned above, the two minor peaks gave the same parent ion peak at m/z 174. The peak with the shorter retention time can be assigned to the Δ³-pyrrolinone species 51 and the one with longer retention time to the Δ⁴-pyrrolinone species 48 on the basis of published mass spectral data. The mass spectra of the two pyrrolinone isomers were similar. Both showed a parent ion (i) at m/z 174 as the base peak suggesting that the pyrrolinones 48 and 51 are reasonably thermally stable. The major fragment ions include one at m/z 145 (ii) due to the loss of carbon monoxide, an ion at m/z 119 (iii) due to the loss of a vinyl group similar to what happened with 58 and 59 and the pyridyl ion (iv) at m/z 78. A notable exception is the peak at m/z 96 (v), which was observed in the mass spectrum of 51 only. This fragment ion is due to the loss of a pyridyl group from the parent ion and formation of the conjugation stabilized pyrrolinone iminium species v. A summary of the spectral data is presented in Table II-5.
Fig. II-9b. GC-EIMS data of decomposed 5-methoxy-1-methyl-(3-pyridyl)-pyrrolidin-2-one (59).
Chapter 2  in Vitro Studies on the Metabolism of β-Nicotyrine

Table II-5. Mass spectral ion assignments from 1-methyl-5-(3-pyridyl)-4-pyrroline-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (51)

<table>
<thead>
<tr>
<th>m/z</th>
<th>174</th>
<th>145</th>
<th>119</th>
<th>96</th>
<th>78</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Structure of 48" /></td>
<td>M⁺</td>
<td>[M - HCO]⁺</td>
<td>Py - C - N - Me</td>
<td>Py⁺</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Structure of 51" /></td>
<td>M⁺</td>
<td>[M - HCO]⁺</td>
<td>Py - C - N - Me</td>
<td></td>
<td>Py⁺</td>
</tr>
</tbody>
</table>

TMSI, which is used for the cleavage of ether bonds was developed in the 1970s, primarily to cleave protecting groups.²⁰ Normally, these protecting groups are tert-butyl, benzyl, and triphenylmethyl, all of which can be removed under relatively mild conditions. TMSI was found to react with alkyl methyl ethers very well.²⁰ The ether oxygen atom first attacks the silyl group to generate the oxonium intermediate i which undergoes subsequent attack by iodide anion to generate methyl iodide and the trimethylsilyl ether ii which undergoes hydrolysis to yield the final hydroxyl product iii (Scheme II-10).

\[
R-O-\text{CH}_3 + \text{Me}_3\text{SiI} \rightarrow R-O-\text{SiMe}_3 + \text{MeI}
\]

\[
R-O-\text{SiMe}_3 + \text{H}_2\text{O} \rightarrow R-\text{OH} \quad \text{(iii)}
\]

Scheme II-10  Mechanism of ether cleavage with TMSI
Figure II-10. 1H NMR spectrum of 5-hydroxy-1-methyl-(3-pyridyl)-3-pyroline-2-one (52) in DMSO-d6.
Fig. II-11. GC-EIMS data of 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (52).
Fig. II-12. 1H NMR spectrum of 5-hydroxycoimnine (20) in DMSO-d6.
Fig. II-13. GC-EIMS data of 5'-hydroxycotinine (20).
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Scheme II-11 Preparation of 5'-hydroxycotinine (20) and the 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolidin-2-one (52) with TMSI

When TMSI was applied to ethers 58 and 59, the expected products 52 and 20 were obtained in excellent yield (Scheme II-11). The melting points of the two hydroxy compounds matched the published data by Dagne and Nguyen.\textsuperscript{16,17} The \textsuperscript{1}H NMR spectra of 52 and 20 obtained from these reactions also were in agreement with the published spectra (Fig. II-10 and II-12).\textsuperscript{16,17}

Scheme II-12 Pyrolysis of 5'-hydroxycotinine (20) and 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolidin-2-one (52) to their ring opened isomers

The GC-EIMS analysis of 52 showed two peaks (Fig. II-11). The mass spectra of both peaks gave a molecular ion at m/z 190, indicating isomeric structures. However, the \textsuperscript{1}H NMR spectrum was consistent with a single compound. A possible explanation could be that the pyrrolidyl ring of 52 opens
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine
to generate the isomeric ketoamide 62. Compound 20 also undergoes an
analogous reaction during gas chromatography to generate 21 (Scheme II-12).

The mass spectrum of the main peaks (tr = 4.1 minutes) in this analysis
gave the expected parent ion for 52 at m/z 190 (i) and two intense peaks at m/z
112 (ii) and 79 (iii, Scheme II-13). The ion ii is due to the loss of a pyridyl
group to form the stabilized iminium ion. The fragment ion at m/z 79 is due to
the pyridyl cation radical iii, probably formed via McLafferty rearrangement.
The nitrogen in the pyridine ring may pick up a hydrogen atom from the hydroxyl
group adjacent to the pyridine ring. These two major fragments support the
cyclic form 52. The mass spectrum of the second peak (tr = 4.9 minutes) gave
ions at m/z 190 (iv), 106 (v) and 78 (vi). Ion v is due to the acetopyridyl cation
that can form from the β-cleavage of the keto group and is a very stable species.
Ion vi is due to the pyridyl cation (that also can form from 62) because there is
no hydroxyl group adjacent to the pyridine ring. These two fragments support
the ring opened isomer, the ketoamide 21.

\[
\text{Scheme II-13 Mass spectral fragmentation of 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolidin-2-one (52)}
\]

GC-EIMS analysis of 20 (Fig. II-13) gave two major peaks and two minor
peaks. The mass spectrum of one major peak (tr = 4.55 min) gave an ion at m/z
192 (i), as the parent ion, and two intense peaks at m/z 114 (ii) and 79 (iii,
Scheme II-14). Ion ii is due to the loss of a pyridyl group and iii is due to the
pyridyl cation radical. This compound should be assigned as the the cyclic form
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

20. The mass spectrum of the other main peak (t\textsubscript{R} = 4.38 min) gave ions at m/z 192 (iv), 106 (v) and 78 (vi) and consequently this compound should be assigned as the ring opened isomer, 21.

\[
\text{HO} \quad \text{N} \quad \text{CH}_3 \\
\text{192, i} \quad \rightarrow \quad \text{HO} \quad \text{N} \quad \text{CH}_3 \\
\text{114, ii} \quad + \quad \text{79, iii}
\]

\[
\text{HO} \quad \text{N} \quad \text{CH}_3 \\
\text{192, iv} \quad \rightarrow \quad \text{HO} \quad \text{N} \quad \text{CH}_3 \\
\text{106, v} \quad \rightarrow \quad \text{78, vi}
\]

**Scheme II-14** Mass spectral fragmentation of 5'-hydroxycotinine (20)

The assignments for the other fragment ions of 52 and 20 have been reported by Dagne\textsuperscript{16} and will not be repeated again here. More interesting is the GC-EIMS spectrum of 20 (Fig. II-13) which gave four peaks even though the \textsuperscript{1}H NMR spectrum indicated a homogeneous pure compound (Fig II-12). Comparison of the mass spectra of the two minor peaks with the published mass spectra reported by Shigenaga,\textsuperscript{5} demonstrated that they were dehydrated products, the two pyrrolinones 48 and 51. The pyrrolinones may be formed thermally in the GC column by a process which is analogous to what happened with 59 which loses one molecule of methanol to form the same pyrrolinone species. This chemical instability of 20 suggested the possibility of preparing the unstable pyrrolinones, 48 and 51, by thermal dehydration of 20 which will be discussed in more detail later.

2.2.1.3. **Synthesis of 1-Methyl-5-(3-pyridyl)-4-pyrroline-2-one (48) and 1-Methyl-5-(3-pyridyl)-3-pyrroline-2-one (51)**

The two isomeric pyrrolinones, 1-methyl-5-(3-pyridyl)-4-pyrroline-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (51) are reported to be primary microsomal metabolites of β-nicotyrine.\textsuperscript{5} However, these metabolites only accounted for about 10\% of the original substrate.\textsuperscript{5} In an attempt to account
more fully for the fate of these compounds, we were interested in the possible further metabolism of 48 and 51. Therefore synthetic samples of 48 and 51 were required. Shigenaga reported a synthetic route involving the conversion of (S)-nicotine to (S)-cotinine by a published method. (S)-Cotinine was then converted to its α-phenylselenyl derivative 66, by treatment with n-butyllithium followed by phenylselenyl chloride. Subsequent oxidation of 66 to 67 with hydrogen peroxide, followed by base catalyzed elimination should give the pyrrolinone 51. However, from this reaction only a very low yield of 48 was obtained. This product exists in equilibrium with 10-30% of 51 as the minor product as shown by deuterium labeling studies (Scheme II-15). Compound 48 was also found to be unstable and upon standing, the yellow oil turned purple in color, possibly through an autoxidation process yielding 5'-hydroxy-5-pyrrolinone 52 as the product.

**Scheme II-15** Synthesis of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51)

Subsequent attempts by Shigenaga to prepare the Δ^3-isomer 51, gave instead the Δ^4-isomer 48. Since 51 must be the initial product from the elimination of 64 and 48 must form via rearrangement of 51, this result indicates that the more stable or at least more easily isolated form of the two isomers may be 48. Results from in vitro studies on β-nicotyrine metabolism
showed only one peak in the HPLC-diode array analysis which was assigned structure 48 due to its UV and mass spectral characteristics. This metabolic product was also unstable and formed the same autooxidative product, 52.

Pyrroloxinones have been reported to be very unstable,\textsuperscript{21,22,23,24} The stabilities of the tautomeric forms have been compared theoretically and experimentally. Ribo and Vallés calculated the ΔH°\textsubscript{f} for the unsubstituted 2-hydroxy- \textit{N}-methylpyrrole (65) and found that the two carbonyl bearing isomers 66 and 67 are much more stable than the enol form 65. The Δ\textsuperscript{4}-isomer (66) is more stable than the Δ\textsuperscript{3}-isomer (67) based on the lower ΔH°\textsubscript{f} values.\textsuperscript{21} However, these theoretical results are not consistent with the experimental data reported by Baker\textsuperscript{22} and Bordner \textit{et al.}\textsuperscript{23} As reported by Baker, a synthetic mixture of the unsubstituted pyrroloxinones 66 and 67 contained the two carbonyl isomers in a ratio of 9 : 1 (66 : 67). On the other hand, the stabilities of substituted pyrroloxinones are reported to be more complex. When an electron-donating group, such as a methyl group, is present on the 4-position of the pyrrole ring, the Δ\textsuperscript{3}-isomer 68 is the favored form, whereas when an electron-withdrawing group like ethoxycarbonyl is present on the same position, the Δ\textsuperscript{4}-isomer 69 is the only reported isomer. These two compounds were synthesized via dehydration of their hydroxy precursors 70 and 71 (Scheme II-16).\textsuperscript{23}
Scheme II-16  Tautomer formations of synthetic pyrrolinones

In the case of 68, the double bond remained in the Δ^3 position as it was generated while in the case of 69, a rearrangement of the double bond occurred. Steric strain might favor the Δ^4-isomer in both cases since the Δ^3-isomeric structure results in the unfavorable arrangement of three contiguous trigonal carbon atoms in a five-membered ring. This factor, however, is not enough to overcome the conjugation in 68 that would be lost by a rearrangement to the Δ^4-isomer. The conjugation of the N-C=C-C=O(OEt) system in 69, however, permits the rearrangement of its Δ^3-isomer to the Δ^4-isomer. In all of these cases, apparently the enol forms of the pyrrolinones, the hydroxypyrroles, are the most unstable. They have never been detected and several attempts to generate the enol isomers have failed. The resonance of the two carbonyl bearing isomers 66 and 67 could cause a negative charge on the oxygen (i and ii) whereas the enol form 65 only could cause a negative charge on carbon (iii and iv, Scheme II-17).
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

\[
\begin{align*}
\text{Scheme II-17 The resonance of the pyrrolinone isomers} \\
\text{The favored stability of 48 among the three tautomers, 48, 50 and 51, might be explained as discussed above. The theoretical calculation of } \Delta H^0_t \text{ value as reported by Ribo}^{21} \text{ supports the stability of } \Delta^4\text{-isomer. The electron-withdrawing pyridyl group might help to stabilize the } \Delta^4\text{-isomer of this pyrrolinone analog as the case of 69.}
\end{align*}
\]

Shigenaga has also reported that metabolically produced and synthetic pyrrolinone undergo spontaneous autoxidation.\(^5\) This may account for the difficulty encountered in efforts to isolate and fully characterize the pyrrolinone species 48 and 51. According to the mechanism discussed in Scheme II-2, a basic aqueous medium might promote loss of a proton from the enol form 50 and catalyze this autoxidation.

We have attempted to synthesize these two compounds by a different pathway which avoids exposure of the unstable pyrrolinone to basic aqueous medium. We started from (S)-3',3'-dibromocotinine (57). We hoped to eliminate one molecule of hydrogen bromide from the 3' and 4'-position of 57 to form the corresponding 3'-bromopyrrolinone 72. Based on the rearrangement of the pyrrolinone 48 to 51, it is possible that the } \Delta^3\text{-isomer 72 would rearrange}
to the Δ^4-isomer 73. Reductive cleavage of the 3'-bromopyrroline isomers would lead to the desired pyrroline 48 and/or 51 (Scheme II-18).

\[
\begin{align*}
\text{H} & \quad \text{Br}_2 \\
\text{Br} & \quad 50\% \text{HOAc (aq)} \\
\text{CH}_3 & \\
\text{N} & \quad \text{CH}_3
\end{align*}
\]

**Scheme II-18** Attempts to form the pyrroline 48 and 51 from 3',3'-dibromocotinine (57)

In the proposed pathway, elimination of hydrobromide from the geminal dibromide and subsequent reduction of a vinyl or allyl bromide to the corresponding dehalogenated compound is the first key step. Attempts to find a precedent for the elimination reaction failed although similar eliminations from an α,α-dibromoketone in the presence of lithium chloride or lithium carbonate in DMF to form an α,β-unsaturated ketone have been reported by Collington and Jones.

Another approach closer to our proposal has been reported. In the synthesis of the 5'-hydroxypyrroline 52, treatment of 57 with KOMe / MeOH led to the elimination of two moles of HBr to form 5'-methoxypyrroline 58 (Scheme II-19). It was proposed that the base first attacked the 4'-proton to bring about the elimination of the first mole of HBr to form 72. However, the reaction did not stop at this step. After the base catalyzed rearrangement of 72 to 73, the methoxy anion attacked the 5'-position of 73 and caused the elimination of the second mole of HBr to form 58 as the final isolated product (Scheme II-19).
Scheme II-19 Elimination of two moles of HBr from (S)-3',3'-dibromocotinine (57)

By controlling the stoichiometry, it might be possible to control the elimination of HBr in order to form 3'-bromopyrroliidine 72 and/or 73. Since 72 and 73 are vinylic and allylic bromides, it might be possible to obtain the desired pyrroliidines 48 and 51 by Raney nickel catalyzed hydrogenolysis in the presence of potassium hydroxide. Alternatively, reduction of 72/73 using stannic catalysts, such as tributyl stannic hydride (Bu$_3$SnH), following a free radical mechanism, is possible.

This approach to the synthesis of 48/51 was attempted by using one mole of potassium methoxide per mole of 57 in the base dependent elimination reaction. However, the reaction led to a mixture of the starting material, 57, and the final product, 58, suggesting that the reaction had not stopped at the first step as desired. As a part of the same effort, we attempted to control the reaction with a bulky base, potassium tert-butoxide, and a poorer nucleophile, sodium hydride, to block the nucleophilic attack on 73. However, all of these efforts failed to achieve the desired 72 or 73. Instead a mixture of starting material 57 and the methoxypyrroliidine 58 were always obtained.

Literature reports indicated that α,α-dibromoketones, for example, 2,2-dibromobenzocycloheptanones, can be dehydrobrominated with Li$_2$CO$_3$ to form the corresponding α,β-unsaturated ketone in 95% yield. This
In Vitro Studies on the Metabolism of β-Nicotyrine

reaction was tried with 57 (Scheme II-20) and was monitored by UV. We expected that the new carbon-carbon double bond between C4'- and C5'- would result in a shift of the UV spectrum to a longer wavelength, about $\lambda_{\text{max}} = 280 - 290$ nm. This conclusion is based on the published UV data of Δ4'-pyrrolinone 48, which displays a $\lambda_{\text{max}} = 284$ nm. This reaction was monitored by UV every 15 minutes for one hour, but no changes were observed. After standing overnight, however, a new absorption at $\lambda_{\text{max}} = 281$ nm was found and the colorless reaction mixture had changed to dark purple. After another 24 hours this new UV band disappeared. It may be reasonable to assume the 281 nm absorbing chromophore is due to the desired compound with the carbon-carbon double bond in conjugation with the pyridine ring. If present, this product was not stable. This behavior is consistent with the behavior of the pyrrolinones 48 and 51 which have been reported to be unstable and undergo autoxidation in an alkaline medium.5

Scheme II-20 Attempted elimination of HBr from 3',3'-dibromocotinine (57) with lithium carbonate

Since it was known that the pyrrolinones 48 and 51 will undergo autoxidation under alkaline conditions, an effort to eliminate methanol from 61 under acidic conditions with trifluoroacetic acid (TFA) was examined. UV analysis of the reaction mixture showed a new absorption band at $\lambda_{\text{max}} = 281$ nm, the same as that observed in the lithium carbonate reaction, which might be consistent with 48. A white crystalline product was obtained. However, the melting point (125 °C) and $^1$H NMR spectrum showed it to be 5'-hydroxycotinine (20) (CD$_3$OD, δ 8.88, C2-H; 8.76, C6-H; 8.37, C4-H; 7.92, C5-H; 2.79-2.61, C3'-H; 2.62 N-Me; 2.43-2.40 C4'-H). The results suggest that 48 and/or 51 perhaps had formed, but had undergone hydrolysis under the acidic

91
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

conditions during work-up to form the much more stable derivative 20 (Scheme II-21).

\[
\begin{align*}
\text{MeO}^+ & & \text{CR}_3^+ & & \text{H} & & \text{H}_2\text{O} \\
\text{59} & & \text{59} & & \text{48} & & \text{20} \\
\text{H}_2\text{O} & & \text{H}_2\text{O} & & \\
\text{HO} & & \text{Pyrrolinone} & & \\
\text{Pyridine} & & \text{Pyridine} & & \\
\text{Pyridine} & & \text{Pyridine} & & \\
\end{align*}
\]

**Scheme II-21** Elimination of 5'-methoxycotinine (59) with TFA

We also tried to eliminate one molecule of water from 20 with trifluoroacetic anhydride (TFAA). However, instead of the expected pyrrolinones, an unknown product was obtained from this reaction. This reaction will be discussed in more detail in a later section.

GC-EIMS analysis of 5'-methoxycotinine (59, Fig. II-9) and 5'-hydroxycotinine (20, Fig. II-13) pointed to their thermal instability since peaks corresponding to 48/51 were observed. Consequently, we attempted to prepare these unstable pyrrolinones 48 and 51 by pyrolysis. Our first try involved heating 59 in xylene under refluxing. GC-MS analysis of the reaction mixture failed to reveal product formation, probably because of the further polymerization or other side reactions of the pyrrolinones. We next ran the pyrolysis reaction of 59 in a sublimation tube. After heating under anaerobic conditions, a colorless oily condensate was obtained on the cold finger. The \(^1\)H NMR spectrum showed that the sublimate was a mixture of 48 and 51 together with some starting material 59 (Table II-6 and Fig. II-14). Attempts to purify the
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

pyrrolinones by chromatography, however, failed presumably because of the instability of the pyrrolinones.

Table II-6. $^1$H NMR (CDCl$_3$) data assignment of the products from the sublimation of 5'-methoxycaotinine (59)

<table>
<thead>
<tr>
<th>Comp</th>
<th>$^1$H NMR Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>8.67 (1H, s, C2-H), 8.58 (1H, dd, C6-H), 7.69 (1H, m, C4-H), 7.32 (1H, m, C3-H), 3.26 (3H, s, N-Me), 2.63 (2H, m, C3'-H), 2.57 (3H, s, O-Me), 2.51 (1H, m, C4'-H), 2.21 (1H, m, C4'-H)</td>
</tr>
<tr>
<td>51</td>
<td>8.50 (2H, d, C2- and C6-H), 7.65 (1H, m, C4-H), 7.30 (1H, m, C5-H), 7.03 (1H, dd, C3'-H), 6.30 (1H, dd, C4'-H), 5.01 (1H, t, C5'-H), 2.83 (3H, s, N-Me)</td>
</tr>
<tr>
<td>48</td>
<td>8.60 (2H, d, C2- and C6-H), 7.65 (1H, dd, C4-H), 7.35 (1H, m, C5-H), 5.38 (1H, t, C4'-H), 3.22 (2H, d, C3'-H), 3.02 (3H, s, N-Me)</td>
</tr>
</tbody>
</table>

Finally we attempted the pyrolysis reaction of 20 in a pyrolysis tube. The reaction flask was heated to only 135 °C to vaporize the starting material which then passed into the pyrolysis tube which was heated to 280 °C. The products, a purple colored condensate, were collected with a cold trap. GC-EIMS analysis (Fig II-15) showed the pyrolysates to be a mixture of 48 and 51. GC-EIMS analysis showed the ratio of 48 to 51 was about 3:1. In an aprotic solvent, such as chloroform, and under anaerobic conditions, the two pyrrolinones 48 and 51 appeared to be reasonably stable and could be stored for about a week. However, we were not able to prepare the pyrrolinones 48/51 by this method for our further metabolic studies because it only gave a very low yield, about 10%. In addition, the two pyrrolinones existed in an equilibrium mixture from which the individual compounds could not be separated.

In order to save one step in the synthetic route, we also tried the pyrolysis of 61, the precursor of 20, in the same way but we failed. No attempts to optimize the reaction conditions led to useful yields of the desired products.
Fig. II-14. 1H NMR spectrum of the sublimation products of 5-methoxycololine (59) in CDCl₃.
Fig. II-15. GC-EI-MS data of the pyrolysis products of 5'-hydroxycotinine (20).
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2.2.1.4. Synthesis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74)

So far, even though we were able to obtain the two pyrrolinones 48 and 51 as an unstable mixture in chloroform solution, the need to isolate the products in pure form for metabolic studies still eluded us. We next tried to prepare the two pyrrolinones by dehydration of 20 with trifluoroacetic anhydride (TFAA). Instead of the expected pyrrolinones, however, an unknown product was obtained from this reaction. In addition to the pyridyl and N'-methyl proton signals, the 1H NMR spectrum (Fig. II-16) of the crude product showed two peaks at δ 6.75 and 6.36. These signals are similar to the aromatic proton signals for the 3'- and 4'-position of a pyrrole ring, such as the 3'- and 4'-protons of β-nicotyrine (δ 6.74 and 6.28), but are clearly different from the vinyl protons at the 3'- and 4'-positions of a pyrrolinone like the 5'-methoxy derivative 58 (δ 7.25 and 6.50) or 5'-hydroxy derivative 52 (δ 7.23 and 6.28). These NMR data were consistent with a 2'-substituted pyrrole derivative, namely the trifluoroacetoxy derivative of β-nicotyrine, 75. A mechanism to account for the formation of such a product is shown in Scheme II-22. Reaction of TFAA with the 5'-hydroxyl group of 20 forms the acylated intermediate 20a which would eliminates CF₃COOH to give 48. Subsequent O-acylation of 48 yields 75. This acylated product can not undergo tautomerization or autoxidation. However, 75 should undergo hydrolysis to give the 2'-hydroxypyrrole 50, which will then give the pyrrolinones 48 and 51. Consequently, we decided to take advantage of this chemistry. This is a stable precursor that would undergo hydrolysis to the pyrrolinones 48 and 51.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Fig. II-16. 1H NMR spectrum of 2-trifluoroacetoxy-1-methyl-2-(3-pyridyl)pyrrole (75) in CDC6.
Since the hydrolysis by-product from 75, trifluoroacetic acid, is a strong acid which could be toxic to biological systems and could not be used in later metabolism studies, another analog, 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) was considered to be a better candidate as the precursor of the pyrrolinones 48 and 51 (Scheme II-23). The reaction of 5'-hydroxycotinine with acetic anhydride was carried out and a yellow oil was obtained. This product was fully characterized as the expected acetoxypyrrole derivative 74.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

The $^1$H NMR spectrum (Fig II-17) showed two doublets for the vinyl proton signals at δ 6.21 and 5.95, and two signals for the methyl group protons, one at δ 3.45 (the N'-methyl protons), the other at δ 2.34 (the O-methyl protons in acetyl group). All assignments are shown in Table II-9. GC-EIMS analysis (Fig II-18) gave two peaks. The major peak had a retention time of 4.4 minutes and displayed a molecular ion at m/z 216 (C$_{12}$H$_{12}$N$_2$O$_2$, I), consistent with the chemical formula of 74, and a base peak at m/z 174 (ii), which is due to the loss of an acetyl group (Scheme II-24). The minor peak had a retention time of 3.7 minutes and displayed a molecular ion (100%) at m/z 174. The retention time and the fragments of this minor peak were the same as those observed with the pyrrolinone 48. These pieces of evidence indicated that the masked 5'-hydroxy-β-nicotyrine 74 decomposed to the pyrrolinone during GC analysis. The UV spectrum of 74 gave an absorption at $\lambda_{\text{max}} = 290$ nm (Fig. II-19), similar to the UV spectrum of β-nicotyrine and consistent with the presence of a pyrrole ring. CHN analysis was also consistent with 74.

Table II-7. $^1$H NMR (CDCl$_3$) data assignment of 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74)

<table>
<thead>
<tr>
<th>δ (ppm)</th>
<th>J (Hz)</th>
<th>Coupling</th>
<th>Integrate</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.66</td>
<td>2.1</td>
<td>d</td>
<td>1H</td>
<td>C2-H</td>
</tr>
<tr>
<td>8.51</td>
<td>1.7, 4.8</td>
<td>q</td>
<td>1H</td>
<td>C6-H</td>
</tr>
<tr>
<td>7.67</td>
<td>2.1, 7.8</td>
<td>dt</td>
<td>1H</td>
<td>C4-H</td>
</tr>
<tr>
<td>7.31</td>
<td>1.7, 4.8, 7.8</td>
<td>qd</td>
<td>1H</td>
<td>C5-H</td>
</tr>
<tr>
<td>6.21</td>
<td>3.9</td>
<td>d</td>
<td>1H</td>
<td>C3'-H</td>
</tr>
<tr>
<td>5.95</td>
<td>3.9</td>
<td>d</td>
<td>1H</td>
<td>C4'-H</td>
</tr>
<tr>
<td>3.45</td>
<td></td>
<td>s</td>
<td>3H</td>
<td>N'-CH$_3$</td>
</tr>
<tr>
<td>2.34</td>
<td></td>
<td>s</td>
<td>3H</td>
<td>O-CH$_3$</td>
</tr>
</tbody>
</table>
FIG. II-17. $^1$H NMR spectrum of 2-acetoloy-1-methyl-2-(3-pyridyl)pyrrole (74) in CDCl$_3$. 

![NMR Spectrum Diagram]
Fig. II-18. GC-EIMS data of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74).
Fig. II-19. UV spectrum of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in MeOH.
Scheme II-24 Mass spectral assignments for 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74)

An alternative synthetic approach to 74 involving the reaction of acetic anhydride with 5'-methoxycotinine (59) was pursued. Since 59 is the precursor of 20, this approach would save one step in the synthetic route. Indeed, this reaction gave 74 in 77% yield. The $^1$H NMR and GC-EIMS spectra were identical to those of the product obtained from 20. When the reaction of 59 was attempted with TFAA, however, the expected trifluoroacetoxy derivative 75 was not obtained. However, an unknown product was isolated.

$^1$H NMR spectrum (Fig II-20) of this unknown product did not show any vinyl proton signals but only a singlet peak integrating for two protons at $\delta$ 4.40, which should be due to a methylene group (Table II-10). This observation suggested a double bond had formed between C4' and C5' with both carbon atoms bearing a substituent. The GC-EIMS spectrum (Fig II-21) showed a peak with retention time of 4.0 minutes and molecular ion at m/z 270, consistent with a molecular formula C$_{12}$H$_9$N$_2$O$_2$F$_3$, isomeric with 75. The base peak of this compound was at m/z 201 which is due to loss CF$_3$ (Scheme II-26). A tentative assignment for this product is 1-methyl-5-(3-pyridyl)-4-trifluoroaceto-4,5-pyrrolin-2-one (76). The $^{13}$C NMR spectrum (Fig. II-22) showed peaks at $\delta$ 159.6 (CF$_3$CO), 125.0 and 123.4 (vinyl 4'-C and 5'-C), 106.8, (CF$_3$), and 28.1
Chapter 2 In Vitro Studies on the Metabolism of β-Nicotyrine (methyleneyl 3'-C) (Table II-11). High resolution mass spectra of the molecular ion at m/z 270 (Fig. II-23) and the base peak at m/z 201 (Fig. II-24) also were consistent with the proposed structure (Scheme II-25).

Scheme II-25 Reactions of 5'-methoxycytosine (59) with TFAA and acetic anhydride

Table II-8. $^1$H NMR (CDCl$_3$) assignments of 1-methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrrolin-2-one (76)

<table>
<thead>
<tr>
<th>δ (ppm)</th>
<th>Coupling</th>
<th>Integrate</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.76</td>
<td>q</td>
<td>1H</td>
<td>C6-H</td>
</tr>
<tr>
<td>8.61</td>
<td>d</td>
<td>1H</td>
<td>C2-H</td>
</tr>
<tr>
<td>7.85</td>
<td>dt</td>
<td>1H</td>
<td>C4-H</td>
</tr>
<tr>
<td>7.60</td>
<td>m</td>
<td>1H</td>
<td>C5-H</td>
</tr>
<tr>
<td>4.40</td>
<td>s</td>
<td>2H</td>
<td>C3'-H</td>
</tr>
<tr>
<td>2.98</td>
<td>s</td>
<td>3H</td>
<td>N'-CH$_3$</td>
</tr>
</tbody>
</table>
FIG. II-20. 1H NMR spectrum of 1-methyl-2-(3-pyridyl)-4-phenyloraceto-4-pyrorrin-2-one (76) in CDCl₃.
Fig. II-21. GC-EIMS data of 1-methyl-2-(3-pyridyl)-4-trifluoroaceto-4-pyrroline-2-one (76).
FIG. II-22. $^{13}$C NMR spectrum of 1-methyl-2-(3-pyrindyl)-4-thiouracil-2-one (76) in CDCl₃.
Fig. II-23. High resolution mass spectrum of 1-methyl-2-(3-pyridyl)-4-trifluoroaceto-4-pyrroline-2-one (76), molecular ion at m/z 270.062.
FIG. II-24. High resolution mass spectrum of 1-methyl-2-(3-pyridyl)-4-trifluoroacetoxy-4-pyrollin-2-one (76).
Scheme II-26  Mass spectral assignments for 1-methyl-5-(3-pyridyl)-4-
trifluoroaceto-4-pyrrolin-2-one (76)

Table II-9. $^{13}$C NMR (CDCl$_3$) assignments of 1-methyl-5-(3-pyridyl)-4-
trifluoroaceto-4-pyrrolin-2-one (76)

<table>
<thead>
<tr>
<th>$\delta$</th>
<th>174.6</th>
<th>159.5</th>
<th>151.6</th>
<th>148.6</th>
<th>136.3</th>
<th>125.0</th>
<th>123.4</th>
<th>106.8</th>
<th>35.8</th>
<th>29.7</th>
<th>28.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7</td>
<td>2'</td>
<td>2</td>
<td>6</td>
<td>4+3</td>
<td>5</td>
<td>4'</td>
<td>8</td>
<td>5'</td>
<td>9</td>
<td>3'</td>
</tr>
</tbody>
</table>
Scheme II-27 The formation mechanism of 1-methyl-5-(3-pyridyl)-4-trifluoroaceto-4-pyrrolin-2-one (76)

2.2.1.5. Hydrolysis of 2-Acetoxy-1-methyl-5-(3-pyridyl)-pyrrole (74)

Once we prepared the masked pyrrolinone 74, we needed to explore the proposed hydrolysis of this new compound to yield the pyrrolinones 48 and 51, the β-nicotyrine metabolites of interest. We examined the hydrolysis of 74 in aqueous solution at different pH values: (a) 5% hydrochloric acid (pH<1); (b) 10% acetic acid (pH 3.5); (c) phosphate buffer (pH 7.4); and (d) trisodium phosphate (pH 12). These hydrolysis reactions were monitored by $^1$H NMR, GC-EIMS and HPLC diode array.

(a) Under Strongly Acidic Conditions, in 5% HCl/H$_2$O and DCI/D$_2$O

The 2'-acetoxypyrrole 74 undergoes rapid hydrolysis in the strong acidic medium. As soon as the starting materials were mixed, the $^1$H NMR spectrum
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

(Fig. II-25a) showed seven methyl proton signals at δ 3.52, 3.06, 2.88, 2.74, 2.65, 2.42 and 2.09. By comparison with the standards, the signal at δ 3.52 and 2.42 were identified as the N-methyl group and the methyl of the acetyl group of the starting material. The signal at δ 3.06 is due to the N-methyl group of the Δ^4- pyrroline 48, the signal at δ 2.88 is due to the N-methyl group of the Δ^3- pyrroline 51, the signal at δ 2.74 is due to the N-methyl group of the 5'- hydroxypyrroline 52, and the δ 2.65 signal is due to the N-methyl group of 5'- hydroxycotinine (20). The signal at δ 2.09 was assigned to the methyl group of the acetic acid which is the by-product of the hydrolysis. After 10 minutes, the signals at δ 3.52 and 2.42 had disappeared; the signals at δ 3.06 and 2.88 also decreased with time; the signal at δ 2.74 remained in the same proportion as the beginning; the signal at δ 2.09 did not change with time. An unidentified peak at δ 2.47 appeared after 10 minutes but did not increase after that time. The most interesting change was observed over the next hour (Fig. II-25b). The small peak at δ 2.65 increased in size to become the major peak. All other peaks had disappeared except the peaks at δ 2.47 and 2.74. These data indicated that the starting material 74 undergoes hydrolysis in the strong acidic medium rapidly. The initial products, the two pyrrolines 48 and 51 underwent autoxidation to form the 5'-hydroxypyrroline 52 as a minor product. The presence of 5'-hydroxycotinine (20) as the major product indicated that under acidic condition 48 is susceptible to hydrolysis. The ratio of these three products, 20 : 52 : the unknown was found to be 90 : 5 : 5.

The 1H NMR data of the hydrolysis of 74 showed that 48, the Δ^4- pyrroline, was initially the major isomer present in the reaction mixture. However, as the hydrolysis proceeded, 48 disappeared quickly while the Δ^3- isomer 51 remained with not much change. The reason for these interesting results may be explained by the fact that 48 is converted to the hydrate, 5'- hydroxycotinine (20). Due to the nitrogen lone pair, the hydration of 48 is favored over the hydration of 51 which would be expected to give 4'- hydroxycotinine (77). Thus, even though 48 is the dominant intermediate, it is transformed to 20 very rapidly in the strongly acidic medium. Since a standard sample of 77 was not available, we cannot be sure that the unknown peak at δ 2.47 is due to 77. Due to the rapid hydrolysis of 48 to the hydrate, 20 in the
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

In strongly acidic medium, the equilibrium between 48 and 51 is shifted in the direction of 48. By the same reason, the autoxidation of the two pyrrolinones 48 and 51 is only observed to a small extent (Scheme II-28).

Scheme II-28  Hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in HCl/H2O

GC-EIMS spectra (Fig. II-26) of the final hydrolysis reaction mixture showed three peaks with the following retention times and m/z values: 3.5 minutes (174, 119, 78) which is assigned to 48; 4.2 minutes (192, 114, 79) which is assigned to 20 and 4.3 minutes (163, 106, 78) which is assigned to the ring opened isomer of 20, i.e. compound 21. GC-EIMS peaks corresponding to 52 and the unknown product were not observed probably due to the small amounts present.

A kinetic plot for the decomposition of 74 under strongly acidic condition is shown in Figure II-27. The curves clearly display the initial formation and subsequent hydrolysis of 48.
FIG. II-25A. \textit{H} NMR spectrum of the hydrolysate products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DCl/D$_2$O at time = 0.

Chapter 2
In Vitro Studies on the Metabolism of \(\beta\)-Nicotyrine
DC1/D2O at 1 = 70 minutes.

FIG. 11-25. 1H NMR spectrum of the hydrolys products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in

Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine
Fig. II-26. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in HCl/H₂O.
Chapter 2 In Vitro Studies on the Metabolism of β-Nicotyrine

Fig. II-27. Kinetic plots for the hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DCI/D₂O (obtained from the integration of the N-methyl signals observed in timed ¹H NMR spectra. The percentages observed are relative to total amount of starting material used in the experiment.)

(b) Under Weakly Acidic Conditions, in 5% HOAc/H₂O and DOAc/D₂O

Under weakly acidic conditions, the hydrolysis of 74 was very slow and reached completion after 8 days. At the beginning of the reaction, unlike under strongly acidic conditions, ¹H NMR spectra (Fig. II-28a) showed a quite neat spectrum of the starting material 74, with methyl proton signals at δ 3.52, which is due to the N-methyl group of 74, and 2.41, which is due to the methyl of the acetyl group. Besides 74, only two very small signals at δ 3.04 and 2.08 showed up, those were assigned to the N-methyl protons of 48 and the methyl protons of acetic acid. As the reaction proceeded, four peaks associated with N-methyl protons appeared. By comparison with the standards, they were assigned to the N-methyl protons of 48 (δ 3.04), 51 (δ 2.86), 52 (δ 2.73), 20 (δ 2.64) and an unknown (δ 2.47). The peak due to 48 was the first one to appear,
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine
but with time the ratio of the signals for the N-methyl groups of 48 to 51
decreased and 51 eventually become the dominant species. The amount of 52
remained small. The peak due to 20 increased with time and eventually
became the major product (Fig. II-28b). The final hydrolysis products of 74 in
this weakly acidic medium were the same as observed in the strongly acidic
condition. The ratio of 20 : 52 : unknown was 90 : 5 : 5.

Hydrolysis of 74 in the strongly acidic medium, leads to the rapid second
hydrolytic step of the conversion of the pyrroline to 5'-hydroxycotinine (20).
In the weakly acidic medium, the secondary hydrolysis still dominates, however,
at a much reduced rate compared to the initial hydrolysis of 74. Even though
the hydrolysis is slow, autoxidation of the pyrroline is not competitive and,
therefore, 20 is still the major final product.

The other difference between the behavior of 74 in strongly and weakly
acidic conditions was the rapid exchange of the pyrroline ring protons at low pH.
The signals for the 3'- and 4'-protons disappeared very quickly with the rate of
loss of the 4'-proton being faster than that for the 3'-proton. Both signals
disappeared before the peaks due to the N-methyl group and methyl of the
acetyl group were lost as a consequence of hydrolysis. These observations
are accounted for by acid catalyzed exchange (Scheme II-29).

![Scheme II-29](image)

**Scheme II-29** Proton-deuterium exchange of vinyl protons in 2-acetoxy-1-
methyl-5-(3-pyridyl)pyrrole (74)
Fig. 11-28a. The NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyli)-2-pyridine (74) in D2O/D2O at $t = 0$. [Graph or chart]
DOAc/D2O after 8 days.

FIG. II-23b. 1H NMR spectrum of the hydrolysate products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in

120
Fig. II-29. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in HOAc/H₂O.
Chapter 2  In Vitro Studies on the Metabolism of \( \beta \)-Nicotyrine

GC-EIMS analysis (Fig. II-29) of the acetic acid reaction mixture showed four peaks with the following retention times (m/z): 3.75 min (174, 119, 78) assigned to 48; 4.02 min (190, 112, 78) assigned to 52; 4.36 min (192, 114, 79) assigned to the cyclic isomer of 20; and 4.59 min (163, 106, 78) assigned to the ring opened isomer of 20, compound 65. Compound 48 and the ring opened isomer of 65 could be formed on the GC column, thus the GC-EIMS spectra confirmed the conclusion from \(^1\)H NMR analysis regarding the formation of 52 and 20 from the hydrolysis of 74 under weakly acidic condition. Figure II-30 shows the kinetic plot for the various species observed in this experiment.

Fig. II-30. Kinetic plots for the hydrolysis of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in DOAc/D\(_2\)O (obtained from the integration of the N-methyl signals observed in timed \(^1\)H NMR spectra. The percentages observed are relative to total amount of starting material used in the experiment.)
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

(c) Under Neutral Conditions, in 0.1 M pH 7.4 Phosphate Buffer

The hydrolysis of 74 in pH 7.4 phosphate buffer was very slow. This in part may be due to the poor solubility of 74 in water which meant that the study was conducted in a heterogeneous reaction mixture. The oily starting material slowly disappeared with the formation of the water soluble pyrrolinones 48 and 51 and the hydroxyl compounds 52 and 20. The final hydrolysis mixture was a light brown clear solution. Besides the solubility problem, the hydrolysis itself was very slow. Even after adding 10% of DMSO to help dissolve the substrate in the aqueous buffer, the starting material 74 still persisted for 50 hours.

In the absence of DMSO, 1H NMR spectra (Fig. II-31) showed the presence of the substrate 74 for 4 days. The two hydroxyl products 52 and 20 and the pyrrolinones, 48 and 51, appeared slowly during this period. The pyrrolinones 48 and 51 were reasonably stable in this neutral medium. They undergo hydrolysis to 20, and autoxidation to 52 but were detectable for more than two weeks. The peaks due to the aromatic protons of 74 also did not exchange under these conditions. The kinetic plots for the hydrolysis of 74 under the neutral conditions are summarized in Figure II-32.

GC-EIMS analysis (Fig. II-33) of the reaction mixture at day 8 showed four peaks. The retention times and m/z values are as follows: 3.62 minutes (174, 119, 78) assigned to the pyrrolinone 48; 4.00 minutes (190, 112, 78) assigned to 52; 4.31 minutes (192, 114, 79) assigned to the cyclic isomer of 20; and 4.46 min (163, 106, 78) assigned to the ring opened isomer 65. These GC-EIMS spectra confirmed the conclusion reached from the 1H NMR spectral data.
Fig. II-31. 

$^1$H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer of pH 7.4 after 4 days (in D$_2$O).
Fig. II-33. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer of pH 7.4.
(d) Under Strongly Basic Conditions, in pH 12.5% Na₃PO₄/H₂O and Na₃PO₄/D₂O

Since it is already known that the pyrrolineones 48 and 51 are very unstable under basic conditions and undergo autoxidation to form 52, the hydrolysis studies of 74 in aqueous base were carried out under both anaerobic and aerobic conditions. The reactions were monitored by ¹H NMR. Under aerobic conditions, ¹H NMR spectral analysis (Fig. II-34) showed the rapid loss of starting material 74 which could not be detected even in the first scan. Three other peaks were observed at δ 3.22, 3.00 and 2.83. The signal at δ 3.00 is assigned to the N-methyl group of the pyrrolineone 48 and that at δ 2.83 is assigned to 51. The signal at δ 3.22 is due to an unknown compound. Nevertheless, since it was not found under anaerobic conditions (see below), it
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

is likely to result from the oxidation of the pyrrolinones. The pyrrolinones were found to undergo other complex reactions as well including autoxidation and secondary hydrolysis resulting in 52 and 20 and several additional unknown products. After 7 days, besides the known peaks due to 48, 51, 52, 20, and the original unknown peak at δ 3.22, the area between δ 2.2 to 3.1 became an unidentifiable region of complex signals.

At the beginning of the aerobic study under basic conditions, GC-EIMS analysis (Fig. II-35) showed three peaks which were due to the two pyrrolinones, 48 and 51 and a small amount of unreacted starting material 74. After 1 hour, GC-EIMS analysis (Fig. II-36) showed three peaks with the following retention times and m/z values: 4.53 minutes (189, 173, 145, 119, 103, 78); 4.74 minutes (190, 112, 79, assigned to 52), and 9.58 minutes (362, 256, 173, 145, 119, 78). The ion at m/z 189 may be a fragment (C₁₀H₉N₂O₂) resulting from cleavage of a dimer, such as 78. The fragments of this product were related to those observed with 52 including ions at m/z 173, 145, 103 and 78 (See Scheme II-12). The peak displaying a parent ion at m/z 362 could be another dimer of 52, such as 79. The fragments of this peak were also found in the spectrum of 52, including ions at m/z 173, 145, 119, 78. A free radical process has been proposed for the autoxidation of the pyrrolinones (see Scheme II-2). The free radicals might combine to form dimers, such as 78 and 79.

These results indicate that 74 under basic conditions hydrolyzed very rapidly to the two pyrrolinones, 48 and 51. These initial products then underwent further reactions to form oxidized products, including 52 and, perhaps, the dimers 78 and 79. The hydrolysis of 74 under aerobic basic conditions is summarized in Figure II-37.

![Chemical structures](image-url)
Fig. II-34. \(^1\)H NMR spectrum of the hydrolysis products of 2-acetoxyl-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% NaPO\(_4\)/D\(_2\)O.
Fig. II-35. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% Na₃PO₄/H₂O, time = 0.
Fig. II-36. GC-EIMS data of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% Na₃PO₄/H₂O, time = 1 hour.
Fig. II-37. Kinetic plots for the hydrolysis and subsequent reactions of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in aerobic 5% of Na$_3$PO$_4$/D$_2$O (obtained from the integration of the N-methyl signals observed in timed $^1$H NMR spectra. The percentages observed are relative to total amount of starting material used in the experiment.)

On the contrary, under basic anaerobic conditions, the substrate 74 hydrolyzed quite slowly and 3 days were required to complete the primary hydrolysis. $^1$H NMR analysis (Fig. II-38) showed that with the disappearance of the peak due to the substrate 74, two other peaks at $\delta$ 3.00 and 2.83 due to the two pyrrolinones, 48 and 51, appeared. The two pyrrolinones could exist for 10 days and underwent very slow further hydrolysis to form a small amount of 5'-hydroxycytosine (20). Because no 52 was found in the anaerobic basic hydrolysis, the autoxidation of the pyrrolinones by the air can be confirmed by the counter-evidence. The hydrolysis of 74 under anaerobic basic condition is summarized in Figure II-39.
FIG. 11.38. 1H NMR spectrum of the hydrolysis products of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (T4) in anerobic 5% Na3PO4/D2O.
The different hydrolysis velocities under the different conditions may be explained by the catalyzing medium and chemical equilibria. Because of the poor solubility of the lipophilic 74 in aqueous solutions, only a trace amount of 74 will be dissolved in the aqueous solutions. However, as the hydrolysis of 74 to the pyrrolinones proceeds, the dissolution equilibrium shifts and helps the dissolution of 74 in aqueous solution. In strong acid, the protonated pyridinium derivative of 74 will form and help its dissolution. In addition, the acid catalyzes both the hydrolysis of 74 to the pyrrolinones 48 and 51 and the secondary hydrolysis of the pyrrolinones to 20, which drives the dissolution equilibrium forward. Therefore, the velocity of the hydrolysis of 74 in strong acid was very fast. However the acid does not catalyze the autoxidation of the pyrrolinones, thus 20 results as the major final product in the acidic hydrolysis.
Fig. 4.40. HPLC-dilute array chromatogram of the hydrolysate of 2-acetoxy-1-methyl-2-(5-pyridyl)pyrrole (74) in anerobic 5% of Na₂PO₄/H₂O.

Time (min)
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Under neutral conditions, neither the hydrolysis of 74, nor the secondary hydrolysis is catalyzed. The autoxidation of the pyrrolirones will not happen under the anaerobic conditions. Therefore, the hydrolysis and the dissolution equilibria are shifted very slowly. The formation of the two final products, 20 and 52, becomes competitive.

Under strongly basic conditions, the strong base will catalyze both the hydrolysis of 74 and the autoxidation of the pyrrolirones. Under aerobic conditions, the pyrrolirones both undergo further hydrolysis to 20 and autoxidation to 52, as well as the subsequent complex oxygen centered free radical related reactions and polymerizations, which exhaust the hydrolysis intermediates, the pyrrolirones 48 and 51, and help the dissolution equilibrium to shift. On the other hand, under the anaerobic conditions, the hydrolysis intermediates, the pyrrolirones 48 and 51, only can undergo further hydrolysis, but not autoxidation or other subsequent oxygen centered free radical related reactions. Therefore, the rate of the decrease of the concentrations of the pyrrolirones 48 and 51 and shifting of the dissolution equilibrium should be slower than under the aerobic condition. In fact, the hydrolysis under aerobic, strongly basic conditions was observed as a very rapid reaction whereas the hydrolysis under anaerobic conditions was quite slow. So one can come to the conclusion that the autoxidation and the subsequent oxygen centered free radical related reactions are much faster than the secondary hydrolysis under strongly basic conditions.

The hydrolysis products of 74 in the anaerobic, strongly basic condition were also detected by HPLC UV-diode array (Fig. II-40). HPLC showed two peaks at retention time 19.7 and 20.5 minutes. The UV absorption of the peak at 20.5 minutes was $\lambda_{\text{max}} = 232$ nm and 284 nm, same as that reported by Shigenaga.5 The long wavelength absorption should be due to the conjugation of the 4',5'-carbon carbon double bond with the pyridine ring. Therefore, this peak should be assigned as the $\Delta^4$-isomer 48. The other peak with retention time of 19.7 minutes, only showed an absorption at $\lambda_{\text{max}} = 262$ nm, which is due to the pyridine ring. Since the 3',4'-double bond is isolated from the pyridine
Chapter 2  In Vitro Studies on the Metabolism of \( \beta \)-Nicotyrine

ring in 51, the absorption cannot shift to a longer wavelength, therefore, this peak should be assigned as the \( \Delta^3 \)-isomer 51.

The other interesting fact was the ratio and the stabilities of the two pyrrolineone. In the synthetic processes, 48 always appears to be more stable than 51. However, from the hydrolysis experiments, the results were different between the pyrolysis and the hydrolysis. GC-EIMS and \( ^1 \)H NMR data of the products from the pyrolysis of 5'-hydroxycotinine (20) and 5'-methoxycotinine (59), showed that \( \Delta^4 \)-isomer 48 was the major product. On the other hand, in the hydrolysis studies of 74, no matter what the conditions, 51 was found to be the major isomer. These phenomena might be explained by thermodynamic and kinetic considerations. From the pyrolysis, 48 was the first formed structure, so it is kinetically favored, whereas 51 is thermodynamically favored because of the resonance of the double bond and the carbonyl group of the lactam. The original hydrolysis product should be the enol form 50, which undergoes the first tautomerization to give the ketone form, 48, which then undergoes a second tautomerization to form the isomer 51. Therefore, 48 is the kinetically favored isomer of the hydrolysis. Since the pyrolysis is a relatively fast reaction and the products were condensed under cooling, with dry and anaerobic conditions, there were no free proton sources in this system and the initially formed 48 from the elimination of 52 and 20 did not have a chance to undergo the secondary tautomerization, or the hydrolysis or autoxidation. Compound 48 is therefore the major product from the two pyrolysis reactions. Since hydrolysis is a slower reaction, the initially formed 48 has enough time and available protons to equilibrate to the more thermally stable tautomer 51. Therefore, 51 becomes the major product of the hydrolysis. The other reason might be due to the secondary hydrolysis of 48 to 20, that reduces the percentage of 48. Because of the electron-donating effect of the nitrogen, this reaction might have a low activation energy and be a fast process. There is a possibility that 51 could hydrolyze to form 4'-hydroxycotinine (77). However, we found no evidence for the formation of this compound.

Thus, from our experimental data, we conclude that the \( \Delta^4 \)-isomer of the pyrrolineone 48 is the kinetically favored tautomer while the \( \Delta^3 \)-isomer 51 is the
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine
thermodynamically favored tautomer. These facts might help to explain the
stabilities of the pyrrolinone isomers.

In summary, under strongly acidic conditions, 2'-acetoxypyrrole (74) was
hydrolyzed very quickly to generate intermediates that were converted rapidly to
5'-hydroxycotinine (20). Under mildly acidic conditions, a similar process
occurred but at a slower rate to lead again to 5'-hydroxycotinine (20). Under
neutral conditions, the hydrolysis occurred very slowly to form the two
pyrrolinones and eventually to give 5'-hydroxycotinine (20) and the
autoxidation product 5'-hydroxypropanolone 52. Under basic conditions, the
hydrolysis of 74 went very fast, as well as the autoxidation of the hydrolysis
intermediates, 48 and 51, in the presence of oxygen. Two dimers were
identified tentatively as 78 and 79 by GC-EIMS. From all of these results, it is
apparent that the 2'-acetoxypyrrole 74 hydrolyzed to the two pyrrolinones 48
and 51 which, under neutral conditions, are relatively stable. Therefore, the 2'-
acetoxypropanolone 74 may prove to be a useful chemical model for biological
studies relating to the behavior of the unstable pyrrolinones, 48 and 51.

2.2.1.6. ESR Studies on the Hydrolysis of 2-Acetoxy-1-methyl-
5-(3-pyridyl)pyrrole (74)

(a) Introduction

As discussed above, the pyrrolinones 48 and 51 undergo an
autoxidation, whether they are the metabolic intermediates of β-nicotyrine or the
hydrolysis products of the masked precursor, the acetoxypropanolone 74. This
autoxidation is presumed to involve a free radical process. However, there is
not any evidence to support this hypothesis. Therefore, we pursued studies
designed to yield evidence to evaluate this presumed mechanism.

A free radical process has been proposed for the autoxidation of the two
pyrrolinones 48 and 51 to yield the 5'-hydroxypropanolone 52. The reaction
pathway (Scheme II-2) includes oxygen and carbon centered radicals derived
from the substrate. Reactive radicals such as these and potential byproducts
such as superoxide (O_2^·), hydroxyl (HO·), protonated superoxide (HOO·) and
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyline

others which might be formed from the pyrrolinone autoxidation, could covalently bind to the biological macromolecules and cause chemical damage.

A free radical is a molecule that has an odd number of electrons. They can occur in both organic and inorganic molecules and in general are highly reactive and, therefore, transient species. They can be involved in many reactions critical for the normal operation of biological processes. Free radicals can be generated in vivo as by-products of normal metabolic processes and may be produced when an organism is exposed to ionizing radiation, to drugs capable of redox cycling, or to xenobiotics that can form free radical metabolites in situ. A common mechanism of free radical generation starts with the reduction of oxygen to form superoxide (O$_2$-·), which can combine with a proton to form hydroperoxy radical HOO$^\cdot$. A second electron reduction, followed by proton addition leads to hydrogen peroxide (H$_2$O$_2$) and, following an additional electron transfer, leads to hydroxyl radical (HO$^\cdot$) (Scheme II-30). The catalytic action of enzymes involved in electron transport processes involves one-electron transfers which also yield free radical intermediates. Many xenobiotics have been shown to reduce oxygen to these active oxygen species. These xenobiotics are converted to radical species by intracellular metabolic processes. Because of the ubiquity of molecular oxygen in aerobic organisms and its high electronegativity, oxygen centered free radicals are often mediators of various reactions. Excellent articles have been published regarding free radical generation by antibiotics, phagocytic cells, radiation, and xenobiotics, including air pollutants, tobacco smoke, pesticides, solvents, anaesthetics and the general class of aromatic hydrocarbons.

\[
\begin{align*}
O_2 + e^- &\rightarrow O_2^- \\
O_2^- + H^+ &\rightarrow HOO^-\\
HOO^- + e^- + H^+ &\rightarrow HOOH \\
HOOH + e^- &\rightarrow HO^- + HO^- \\
HO^- + e^- + H^+ &\rightarrow H_2O
\end{align*}
\]

Scheme II-30  The reduction of oxygen
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

These free radical sources or initiators share many common mechanisms involved in the mediation of cell damage\textsuperscript{37}. Free radical reactions can proceed for prolonged periods or may be terminated by a free radical scavenging species, some of which are essential to cellular integrity. Depletion of endogenous compounds as free radical scavengers puts cellular components at risk from free radical damage which include macromolecular targets including DNA, proteins, and membrane lipids. Because of the reactivity of unsaturated bonds and sulfur-containing molecules with free radicals\textsuperscript{38}, proteins consisting of amino acid containing unsaturated systems, such as tryptophan, tyrosine, phenylalanine, and histidine, or thiols, such as methionine and cysteine, can undergo free radical-mediated amino acid modification. The enzymes depending on these amino acids for reactivity will be inhibited by exposure to free radicals or radical-generating agents\textsuperscript{39,40}. Cytoplasmic and membrane proteins can also be cross-linked into dimers or larger aggregates after exposure to oxidizing agents which can initiate free radicals\textsuperscript{41,42}.

Cell mutation and death from ionizing radiation are primarily due to free radical reactions with DNA. Moreover, HO\textsuperscript{•} has been indicated as the major agent responsible for the radiation induced cytotoxicity in both prokaryotic and eukaryotic cells\textsuperscript{43,44}. Cytotoxicity in large part is a consequence of chromosomal aberrations arising from either nucleic acid based modifications or DNA strand scission\textsuperscript{45}. Cell death and mutations arising from free radicals generated during normal metabolism and environmental sources, such as photochemical and air pollutants have also been ascribed to reactions with DNA\textsuperscript{46,47}.

The most important radical reaction in cells involves radical addition to unsaturated bonds, such as those present in fatty acids and aromatic rings. The unsaturated bonds of membrane cholesterol and fatty acids can readily react with free radicals and undergo peroxidation. Peroxidation of fatty acids containing three or more double bonds will produce malondialdehyde, which can cause cross-linking and polymerization of membrane components. This can alter intrinsic membrane properties, such as deformability, ion transport, enzyme activity and aggregation state of cell surface determinants\textsuperscript{48,49}.

139
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Because malondialdehyde is diffusible, it will also react with nitrogenous bases of DNA.\textsuperscript{50} Cytosolic proteins can undergo modification by cytoplasmic free radicals. In the case of hemoproteins, such as oxyhemoglobin, either superoxide or hydrogen peroxide can react with the iron substituent to form methemoglobin.\textsuperscript{51} This suggests that a wide spectrum of hemoproteins can be damaged by oxygen-derived free radicals.

In summary, free radicals are highly reactive species and can cause a wide spectrum of damage to biological systems. Our proposed mechanism of autoxidation of the primary metabolites of β-nicotyrine, the pyrrolinone species \textbf{48} and \textbf{51} involves a free radical process. This free radical process might play an important role in the toxicity to Clara cells observed with β-nicotyrine. Therefore, direct experimental evidence for this proposed free radical pathway has been sought.

Several methods of direct detection may be used to characterize free radicals. These include electron spin resonance (ESR, also known as electron paramagnetic resonance, EPR), UV detection in conjunction with flash photolysis, and pulse radiolysis techniques. However, it is difficult to generate detectable concentrations of these fleeting intermediates. The magnetic properties of some free radicals, e.g., the hydroxyl and superoxide anion radicals, render their detection in solution impossible by ESR. In order to overcome these limitations, the ESR spin trapping technique has been employed.

The spin trapping technique was elaborated in 1968 by Janzen\textsuperscript{52,53} and Lagercrantz,\textsuperscript{54,55} and developed rapidly the last twenty years.\textsuperscript{56} The term spin trapping is commonly applied to the method of detecting free radicals wherein an unsaturated compound is made available for a free radical addition reaction to occur, thereby providing a new free radical with a life time long enough to be detected by ESR. These unsaturated compounds used to trap the free radicals are called spin traps. The most commonly used trapping agents are C-nitroso and nitrone containing compounds. The normal addition products, the spin adducts, are aminoxy (or nitrooxide) free radicals which are stable enough to be detected by ESR. Some typical nitrone compounds used as spin traps are
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

DMPO (80, 5,5-dimethyl-1-pyrroline-N-oxide), PBN (81, C-phenyl-N-tert-butyl nitrone), POBN (82, C-N'-oxidepyridyl-N-tert-butynitrone); and typical C-nitroso compounds are MNP (83, 2-methyl-2-nitrosopropane), NB (84, nitrosobenzene) and phenyl substituted nitrosobenzene derivatives, like ND (85, nitrosodurene) and TBNB (86, tris-tert-butylnitrosobenzene). Most of these compounds are commercially available. DMPO and PBN are the most widely used in biological situations because their adducts are most stable. In many instances these spin adducts have lifetimes in the order of hours.56 The ESR hyperfine splitting constants of their adducts are established.57

(b) ESR studies on the hydrates of 2'-acetoxypyrrole 74

Because of the chemical instability of the pyrrolinones 48 and 51, we have elected to examine the autoxidation pathway with the aid of the masked pyrrolinone derivative, the 2'-acetoxypyrrole 74. In order to simulate biological conditions, hydrolysis of compound 74 was first attempted in phosphate buffer at pH 7.4. Unfortunately, the low solubility of 74 in pH 7.4 buffer led to a very slow rate of hydrolysis of the heterogeneous mixture as we discussed in Section 2.2.2.2, (c) and (d). The slow hydrolysis coupled with the expected short life time of the expected free radicals (10⁻³ to 10⁻¹² sec),58 precluded the direct detection of radicals in this experiment because before the radicals can accumulate enough to reach the detection limit, they have already decayed. Consequently, we elected to pursue this study using the spin traps DMPO and PBN.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

(c) ESR Studies with the Spin Traps DMPO and PBN

The mixture of pH 7.4 phosphate buffered solution of 2'-acetoxyprrole 74 with the aqueous solution of DMPO under aerobic conditions was monitored once every hour. At the beginning, this mixture did not gave an ESR signal because of the slow hydrolysis and low concentration of the free radicals formed and trapped by DMPO. However, after two hours, a ten line ESR signal was observed (Fig. II-41). Analysis of the published hyperfine splitting constants suggested that among the ten lines, four lines with a 1 : 2 : 2 : 1 intensity ratio are due to the adduct 87 of DMPO with a hydroxyl radical (\(a_N = 15.0 \text{ G}, a^\beta_H = 15.0 \text{ G}\)). The other six equally intense lines may be suggested to be due to adduct 88 of DMPO with a carbon centered radical (\(a_N = 15.9 \text{ G}, a^\beta_H = 23.0 \text{ G}\)). Since the spin constant I of \(^{14}\text{N}\) is 1, the 2I + 1 rule of quantum theory predicts that the adjacent nitrogen will split the unpaired electron spin into three lines with a hyperfine splitting constant \(a_N = 15.0 \text{ Gauss}\). According to the same principle, the spin constant I of \(^1\text{H}\) is 1/2 and therefore 2I + 1 = 2. So each line of the unpaired electron spin will be split by the β-hydrogen to two lines. Since the hyperfine splitting constant \(a^\beta_H\) is also 15.0 Gauss, the resulting overlapping lines combine to four lines (Scheme II-31 and 32).

\[\text{Scheme II-31} \quad \text{DMPO radical adducts formed from } \cdot \text{OH and } \cdot \text{CR}_3\]
FIG. 11.41. ESR signal of hydrolysates of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer, trapped with DMPo.

\[ \text{DMPO-CH}_3 \]

\[ \text{DMPO-OH} \]

\[ g = 2.0083 \]
\[ \Delta H = 23G \]
\[ g = 2.0061 \]
\[ \Delta H = 15G \]
\[ g = 2.0061 \]
\[ \Delta H = 15G \]
\[ g = 2.0061 \]
\[ \Delta H = 15G \]
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

\[ \text{Scheme II-32} \quad \text{Spin splitting of DMPO free radical adducts} \]

When DMPO traps a carbon centered free radical, the hyperfine splitting constant \( a_N \) is still about 15 Gauss, but the hyperfine splitting constant \( a^H \) is 23 Gauss, so the doublet of triplet splitting no longer overlaps and all six lines are observed. Therefore, the ESR spectrum of the DMPO-OH adduct (87) and the DMPO-CR\(_3\) adduct (88, \( R = H \) or an alkyl group) combine to give ten lines. From the hyperfine splitting constants \( a^H \) value of this ESR spectrum, we can not know the exact structure of the trapped carbon centered free radical. The data, however, provided evidence for the proposed free radical mechanism.

Although superoxide should be the primary free radical generated from the autoxidation of 48/51 (see Scheme II-2), no ESR signal due to the DMPO-superoxide adduct 89 was observed. Such an adduct should give a 12 line (2 \( \times 2 \times 3 \), \( a_N = 13.1 \) G, \( a^H = 10.4 \) G, \( a^H = 1.3 \) G) \(^{59}\text{ spectrum}. Since it is known that superoxide is highly active and the decomposition of \( O_2^- \) to HO\(^-\) (see Scheme II-31) could be completed within one second in an aqueous solution,\(^{60}\) before it is trapped by DMPO, it could already have decayed to HO\(^-\). On the other hand, the DMPO-O\(_2^-\) (92) adduct also can decompose to the DMPO-OH adduct (87),\(^{60}\) therefore, the appearance of the DMPO-OH adduct instead of the DMPO-O\(_2^-\) adduct is understandable. The other expected signal due to the DMPO-OOH adduct (90), which should give a similar 12 lines (2 \( \times 2 \times 3 \), \( a_N = 14.3 \) G, \( a^H = 11.7 \) G, \( a^H = 1.3 \) G) \(^{61}\) was not observed, either because 90 also decomposed to DMPO-OH in an aqueous solution (half life of approximately 2 minutes) or the HO\(_2^-\) radical could dismute to HO\(^-\) (this can occur within one second at pH 7.4).\(^{60}\)
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Attempts to confirm these results were pursued with PBN, another spin trap reagent that has been used extensively. As was the case with DMPO, the mixture of a buffered solution of 2'-acetoxyprrole 74 with an aqueous solution of PBN under aerobic conditions did not give an ESR signal immediately. However, after 24 hours, a spectrum containing six equally intense lines was observed (Fig. II-42). The hyperfine splitting constant of α-nitrogen, $a_N$, is 15.2 Gauss and the hyperfine splitting constant of β-hydrogen, $a_H^β$ is only 2.8 Gauss. From a comparison with the reported hyperfine splitting constants ($a_N = 15.2$ G, $a_H^β = 2.75$ G), it could be attributed to a HO· radical adduct with PBN, PBN-OH (91, $a_N = 15.2$ G, $a_H^β = 2.75$ G), or a carbon centered radical trapped by PBN, PBN-CR$_3$ (92, R = H or alkyl groups, typically, $a_N = 14-16.2$ G, $a_H^β = 2.64-4.35$ G in a pH 7 phosphate buffer). Since normally 91 is not stable in an aqueous solution, at pH 7.4 (life time is only 38 seconds), while 92 is quite stable (life times of hours). On the other hand, 91 can also be split by the HO hydrogen with a hyperfine constant 0.21, that will give a narrow doublet splitting to each line and cause a total of 12 lines. The ESR spectrum obtained after 24 hours only showed 6 lines without the HO-coupling, which suggests a PBN-CR$_3$ adduct (Scheme II-33 and 34).

Scheme II-33  PBN trapped free radicals and its adducts
Irradiated with PBN.

**FIG. 11-42.** ESR Signal of Hydrolys of 2-Acetoxy-1-Methyl-2-(3-Pyridyl)Pyrolo (74) in Phosphatse Buffer.

\[ \text{Diagram of chemical structure} \]

\[ g = 2.0083 \]
\[ a_{g}H = 2.86 \]
\[ a_{N} = 15.26 \]
In the proposed free radical mechanism involved in the autoxidation of the pyrrolinones 48/51 (Scheme II-2), the oxygen centered alkoxy free radical ii should also occur as a reaction intermediate. The DMPO-OR adduct (93, Scheme II-35) normally gives hyperfine splitting constants of \( a_N = 12.8 - 13.6 \) G, and \( a_B^H = 6.5 - 9.6 \) G, and the PBN-OR adduct (94) normally gives hyperfine splitting constants of \( a_N = 13.5 - 14.9 \) G, and \( a_B^H = 1.7 - 2.95 \) G, both of which should give a 6 line signal. From the above data, however, there was no evidence to show the existence of alkoxy radical adducts with either DMPO (DMPO-OR) or PBN (PBN-OR). This fact might be explained by the instability of alkoxy radicals. Since oxygen is more electronegative than carbon, the unpaired electron prefers to locate on a carbon atom to form a carbon centered radical, such as iii or iv (Scheme II-2).

Scheme II-34 Spin splitting of PBN free radical adducts

Scheme II-35 Alkoxy radical and possible spin trap adducts
Fig. II-43. ESR signal of hydrolysates of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer, added ethanol and trapped with DMP.

\[ \text{\textit{2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74) hydrolysate}} \]

\[ \text{ESR signal} \]

\[ g = 2.0078 \]

\[ \text{afH} = 22.8^\circ \]

\[ \text{afN} = 15.7^\circ \]
Fig. 11.4. ESR signal of hydrolysate of 2-acecloxy-1-methyl-2-(3-pyridyl)pyrrole (74) in phosphate buffer, added.

G = 2.080
δH = 3.6G
G = 15.5G
(d) ESR Detection of β-Nicotyrine, 5'-Hydroxypyrrolinone 52 and 5'-Hydroxycotinin (20)

In our proposed mechanism, the free radical process is only involved in the autoxidation of the pyrrolinones, but not in the oxidation of β-nicotyrine by cytochrome P-450. Since the substrate β-nicotyrine possesses an electron rich pyrrole ring, it also may undergo autoxidation involving radical intermediates. In order to determine if β-nicotyrine is a substrate to autoxidation under biological conditions, experiments parallel to those conducted with 74 were conducted. However, no ESR signals were observed during the 5 days incubation period with β-nicotyrine and DMPO at pH 7.4. This result ruled out the possibility that the oxidation of β-nicotyrine undergoes a free radical mediated autoxidation under these conditions. Negative results also were obtained with the 5'-hydroxypyrrolinone 52 and 5'-hydroxycotinin (20) in similar studies. These results provide further evidence that, in this series, the free radicals are only involved in the autoxidation of pyrrolinones 48 and 51.

(e) Studies with the Radical Scavenger Ethanol

In order to confirm HO• production, ethanol, a specific HO• scavenger, was added into the reaction mixtures of pH 7.4 phosphate buffered solution of 2'-acetoxyppyrole 74 with the aqueous solution of DMPO or PBN. The DMPO containing system showed an ESR signal with six equally intense lines: aN=15.8 G, aH=22.8 G (Fig. II-43). The PBN containing system also showed a signal with six equally intense lines: aN = 15.6 G, aH = 3.5 G (Fig. II-44). By comparison with published data, the hyperfine constants of these ESR signals matched those of the 1-hydroxyethyl (-CHOHCH3) adducts with DMPO (95, aN = 15.8 G, aH = 22.8 G in 7.4 phosphate buffer and PBN (96, aN = 15.5 G, aH = 3.7 G, in a 3 : 2 mixture of water to ethanol). The hydroxyl radical, HO•, formed in this reaction abstracted a hydrogen atom from the ethanol to generate the -CH(OH)CH3 radical (Scheme II-36). In the absence of 74, the mixture of ethanol and the spin traps, either DMPO or PBN, in the buffer did not show any ESR signals. Since the hydroxyl radical could be generated from the decomposition of superoxide under aqueous conditions (see Scheme II-32), the evidence for the presence of HO• is offered as indirect support for the
hypothesis that superoxide was formed following the hydrolysis of 74 as a consequence of the autoxidation of the resulting pyrrolinones 48 and 51.

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{HO}^\cdot \rightarrow \text{CH}_3\text{CHOH} + \text{H}_2\text{O}
\]

\[
\begin{align*}
\text{N} & \quad \text{CH}_3\text{CHOH} \quad \text{N} \\
\text{O} & \quad \text{H} \\
\text{80} & \quad \text{95}
\end{align*}
\]

\[
\begin{align*}
\text{CH} & = \text{N}\quad \text{X} \\
\text{N} & \quad \text{CHOH} \\
\text{81} & \quad \text{96}
\end{align*}
\]

Scheme II-36  Ethanol induced free radical adducts of DMPO and PBN

\[
\begin{align*}
\text{N} & \quad \text{H} \\
\text{O} & \quad \text{CHOH} \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]

Scheme II-37  Spin splitting of ethanol induced free radical adducts of DMPO and PBN

(f) Conclusions from the ESR Studies

From the above results, one can come to the following conclusions:

1). The autoxidation of the pyrrolinone species 48 and 51 proceeds via a free radical pathway.

2). The biologically active species hydroxyl radical (HO-) may be produced during the autoxidation of the pyrrolinone species 48 and 51. It should form from the decomposition of the originally formed superoxide anion.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

The superoxide anion forms from dioxygen in the air by a single electron transfer from the pyrrolinones to the dioxygen.

3). Neither the tobacco alkaloid β-nicotyrine nor its metabolites, the 5'-hydroxypyrrolinone 52 and 5'-hydroxycotinine (20), produce free radical species in pH 7.4 phosphate buffer.

2.2.2. In Vitro Metabolism

In Shigenaga's studies, the one β-nicotyrine metabolite was identified as the pyrrolinone 48.5 The amount of this metabolite only accounted for ca 10% of the substrate consumed. Possible additional metabolites might include some hydrophilic species, such as the 5'-hydroxypyrrolinone 52 and 5'-hydroxycotinine (20) since it is known that the pyrrolinones 48 and 51 are able to undergo spontaneous hydrolysis to 20 and autoxidation to 52. Other possibilities include the ring opened forms of the pyrrolinone rings present in 48, 51 and 52, and the the pyrrolidinone ring present in 20.68 In Shigenaga's work, since the incubation mixtures were extracted with methylene chloride, only the lipophilic metabolites would be extracted. Potential hydrophilic metabolites, 52 and 20, which are difficult to extract with CH2Cl2, might remain in the aqueous phase and go undetected. Reactive metabolic intermediates might form adducts with macromolecules and precipitate with the protein fraction.69 Additionally, conjugated highly water soluble species might form.70,71

As part of our efforts to explore more fully the metabolic fate of β-nicotyrine, the following studies have been pursued:

1. Water soluble, non-extracted metabolites present in rabbit liver and lung microsomal incubations have been investigated.

2. Species variation in the metabolism of β-nicotyrine has been investigated.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2.2.2.1. HPLC Analysis

Many HPLC analytical methods for the determination of (S)-nicotine metabolites, including the hydrophilic 5'-hydroxycotinine (20) and 3'-hydroxycotinine (19) have been reported. McManus et al. used an HPLC-MS method with a polystyrene-divinylbenzene column and a linear elution gradient consisting of methanol-water as the mobile phase.72 Kyerematen et al. used an HPLC-radiometric assay with a cyano-RP analytical column and a mobile phase consisting of methanol-water-acetonitrile buffered with triethylamine and acetic acid.73,74 Cundy et al. used an HPLC-radiometric assay with an SCX micropartical cation-exchange column or Partisil-10 ODS micropartical reverse phase column with methanol-water as the mobile phase.75 O'Doherty et al. used a C18 column eluted with methanol-water containing sodium heptane sulfonate and colorometric reagents for detection.76 Shulgin et al. used a silica column eluted with acetonitrile containing 2-methoxyethylamine.77

Based on the lipophilic and hydrophilic properties of our target compounds and the available equipment, we elected to explore HPLC-diode array and GC-EIMS based analyses. Since all of our target compounds absorb in the ultraviolet region, HPLC UV-diode array analysis was chosen as a primary assay. We tried several different columns including an SCX cation-exchange column as well as amino, cyano and phenyl silica columns with different mobile phases including methanol-water and acetonitrile-water. Finally, we found that a reverse phase C18 column with acetonitrile-water buffered with acetic acid and triethylamine will separate and detect both 52 and 20, as well as the lipophilic substrate β-nicotyrine and the two pyrrolinones 48 and 51. In order to achieve reasonable separation times, a gradient was developed in order to analyze the incubation mixture. Unfortunately, the gradient system developed led to a "ghost peak" with $\lambda_{\text{max}} = 230$ nm at a retention time 18-21 minutes which overlapped with the peaks corresponding to the pyrrolinones 48 and 51. When we changed to a C8 column, this "ghost peak" no longer showed up. Therefore, a final HPLC UV-diode array analytical system was established which consists of a binary gradient mobile phase and a C8 column. The column was eluted with a buffer (Buffer A) containing water /
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

acetonitrile / triethylamine / acetic acid (90:10:0.1:0.5, v/v) for 10 minutes, then
linearly ramped to 40:60 of acetonitrile to Buffer A over 10 minutes. After eluting
for 10 minutes under these conditions, the mobile phase was ramped to 100%
of Buffer A over 5 minutes and maintained for another 10 minutes. The total run
time was 45 minutes.

2.2.2.2. GC-EIMS Analysis

In order to identify the β-nicotyrine metabolites, either LC-MS or GC-MS
analysis should be employed. Based on the instruments available in our
laboratory, capillary GC connected with an El-MS detector was involved in our
in vitro studies. Shigenaga separated and identified the two pyrroliones 48
and 51 by a GC-EIMS method which includes a methylphenylsilicone column
(12 M x 0.2 mm diameter) and temperature conditions as follows: injection port
= 225 °C, ion source = 150 °C and mass analyzer = 180 °C. His GC separation
utilized a 70-225 °C temperature gradient with a 4 minute solvent delay and 20
°C/min linear ramp. The similar method was also utilized by Jacob et al who
were able to identify (S)-cotinine (3) and 3′-hydroxycotinine (19) as the
metabolites of (S)-nicotine. In our studies, we utilized the same capillary GC
column. Based on the difference in properties among our target compounds,
e.g. polar components, the hydroxy derivatives 20 and 52, maybe also 19, and
non-polar components, β-nicotyrine and the pyrroliones 48 and 51, we
modified our temperature conditions as following: injection port = 265 °C, ion
source = 265 °C and mass analyzer = 180 °C with an initial temperature 100
°C, solvent delay of one minute followed by a 25 °C/min linear ramp. A
reasonably good separation was obtained from this GC method. Typical
retention times for the target compounds are: (S)-nicotine, 2.2 min, (S)-cotinine,
2.7 min, β-nicotyrine, 3.1 min, 51, 3.2 min, 48, 3.5 min, the acetoxypyrrrole 74,
3.7 min, 52, 4.0 min, 20, 4.2 min, and 19, 5.4 min. The El-MS spectra of each
component were also obtained.
Fig. II-45. HPLC-diode array tracing of β-nicotyrine incubated with rabbit liver microsomes at time = 0 (top) and 75 minutes (bottom).
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Fig. 14.6. HPLC-diode array tracing of β-nicotyrine incubated with rabbit liver microsomes at time = 75 minutes, spiked with β-nicotyrine.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2.2.2.3. Identification and Confirmation of the Metabolites of β-Nicotyrine Found in the In Vitro Studies

Incubations were carried out under the conditions reported by Shigenaga under which β-nicotyrine was incubated with rabbit liver and lung microsomes containing NADPH regenerating system at 37 °C. In addition to "total" incubation mixture, incubation mixtures without either the NADPH regeneration system, the microsomes, or the substrate were included as controls. The reactions were stopped at time = 0, 15, 30, 45, 60, 75, 90 and 120 minutes by adding either 5% trichloroacetic acid, ice-cold methanol or ice-cold acetonitrile. After removing the precipitated protein, the supernatants were analyzed by HPLC UV-diode array. Among the control samples, only β-nicotyrine (t_R = 23.5 minutes) was detected except in the case of the control containing no substrate. The metabolic samples showed complex chromatographic tracings. Besides the biological background, at least ten unknown peaks were observed (Fig. II-45). Based on the available standards, six peaks with retention times of 8.5 minutes, 11 minutes, 13.5 minutes, 20.5 minutes, 21.5 minutes and 23.5 minutes were identified. For comparison, the top tracing in Figure II-45 shows the tracing from the control sample which was not incubated (time = 0) while the bottom tracing in Figure II-45 shows the tracing from the incubation mixture at time = 75 minutes. Another HPLC-diode array tracing from the control sample incubated for 75 minutes containing no substrate which also allows identification of the peaks due to biological background is shown in Figure II-52.

(a) β-nicotyrine

HPLC tracing of the metabolic sample incubated for 75 minutes showed a very weak peak with a retention time at 23.5 minutes which is the same retention time as the standard β-nicotyrine (Fig. II-45). The UV spectrum showed an absorption at \( \lambda_{\text{max}} = 285 \) nm (solid line) which corresponds to the UV spectrum of synthetic β-nicotyrine (dotted line). When standard β-nicotyrine was added to this metabolic sample, the peak increased (Fig. II-46). These data indicated that only a very small amount of β-nicotyrine was recovered after the 75 minutes incubation period. The recovered β-nicotyrine was calculated as
Chapter 2 In Vitro Studies on the Metabolism of β-Nicotyrine

representing only 0.5% of the initial substrate. The two hour incubation mixture showed no β-nicotyrine. This result is in good agreement with reported results.2,5

(b) 1-Methyl-5-(3-pyridyl)-4-pyrroline-2-one (48) and 1-Methyl-5-(3-pyridyl)-3-pyrroline-2-one (51)

The HPLC tracing of the metabolic sample incubated for 75 minutes showed two major unknown peaks at 20.5 and 21.5 minutes. The retention times corresponded to those of the two pyrrolineones 48 and 51 obtained from the basic hydrolysis of the 2'-acetoxyyprrole derivative 74 (See Section 2.2.2.2). The UV spectrum of the peak at 20.5 minutes displayed $\lambda_{max} = 259$ nm (solid line) which is the same as that of the "synthetic" 51 obtained from the hydrolysis of 74 (dotted line, Fig. II-47). The UV spectrum of the peak at 21.5 minutes displayed $\lambda_{max} = 232$ nm and 282 nm (solid line) which are the same as for the β-nicotyrine metabolite reported by Shigenaga and also the "synthetic" 48 obtained from the basic hydrolysis of 74 (dotted line, Fig. II-48). Since we do not have pure standards of 48 or 51, we were not able to perform coelution experiments. The GC-ELMS data from the ethyl acetate extract of this incubation mixture, besides the complex background, displayed three peaks with retention times of 2.45, 3.34 and 3.47 minutes in the total ion chromatogram. The mass spectra of the peaks indicated they were identical to those of β-nicotyrine ($t_R = 2.45$ min), "synthetic" 51 ($t_R = 3.34$ min) and 48 ($t_R = 3.47$ min, Fig. II-49). Unfortunately, we were not able to obtain any quantitative data on these metabolites due to lack of pure synthetic standards.

(c) 5'-Hydroxycotinine (20)

When the incubation mixture was quenched with 5% trichloroacetic acid, the HPLC tracing showed another minor peak with a retention time of 11 minutes and UV $\lambda_{max} = 258$ nm (solid line). Both the retention time and the UV spectrum corresponded to 5'-hydroxycotinine (20). Standard 20 overlapped with this unknown peak (Fig. II-50). Additionally, the UV spectrum of this unknown metabolite was identical with the UV spectrum of standard 20 (dotted line).
Fig. II.47. UV spectra of 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51) dotted; standard; solid: metabolite.
FIG. 11.48. UV spectra of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48): dotted: standard; solid: metabolite.
Fig. II-49. GC-EIMS data of β-nicotyrine incubated with rabbit liver microsomes at time = 75 minutes.
Fig. II-50. HPLC-diode array tracing of β-nicotyrine incubated with rabbit liver microsomes at time = 75 minutes, spiked with 5-hydroxycololine (20).
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

When the incubation mixture was quenched with methanol or acetonitrile, the peak corresponding to 20 was not observed. From the studies of the hydrolysis of the 2'-acetoxypyrrole derivative 74, it was known that the pyrrolinones 48 and 51 undergo secondary hydrolysis to form 20 under acidic condition. Consequently, 20 might not be a true metabolite of β-nicotyrine, but rather may be formed under the acidic work-up by the hydrolysis of 48 and 51. These results provide additional evidence for the formation of the pyrrolinones 48 and 51 from β-nicotyrine. These results also indicate that the primary metabolites 48 and 51 might be hydrolyzed in vivo to 20.

(d) 5-Hydroxy-1-methyl-5-(3-pyridyl)pyrrolidin-2-one (52)

The HPLC tracing of the incubation mixture also showed a minor unknown peak at 13.5 minutes with \( \lambda_{\text{max}} = 260 \) nm (solid line). Both the retention time and the UV spectrum are similar to synthetic 5-hydroxy-1-methyl-5-(3-pyridyl)-pyrrolidin-2-one (52, dotted line of UV spectrum). When this sample was spiked with the standard 52, this minor peak increased (Fig. II-51). However, GC-EIMS analysis of the ethyl acetate extract did not show a peak corresponding to 52, perhaps due to its low concentration.

(e) Nicotinamide

HPLC tracing showed another major unknown peak with a retention time of 8.5 minutes and a UV absorption with \( \lambda_{\text{max}} = 261 \) nm (solid line). The retention time and the UV spectrum did not match any of our known standards. The GC-EIMS (Fig. II-48) ion chromatogram of the ethyl acetate extract gave a peak with a retention time of 2.85 minutes. The mass spectrum of this peak showed a molecular ion at m/z 122, which most likely is due to \( \text{C}_6\text{H}_6\text{N}_2\text{O}^+ \). One fragment at m/z 78 is very likely to be a pyridyl moiety (\( \text{C}_5\text{H}_4\text{N} \)). The mass difference from the parent ion is equivalent CH\(_2\)NO, which is likely to be a formamide moiety. Another fragment at m/z 106, which is due to PyCO\(^+\) as we discussed before, suggested nicotinamide (97) as the structure of this material. Comparison with the GC-EIMS chromatogram and mass spectrum of synthetic nicotinamide confirmed this assignment. The HPLC retention time and diode
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

array spectrum of 97 (Fig. II-52) established that the unknown peak with retention time 2.85 minutes was in fact nicotinamide.

The source of nicotinamide clearly is not the substrate β-nicotyrine or any β-nicotyrine metabolites. A possible source is NADP+ (98), the oxidative form of NADPH (99), the cofactor of cytochrome P-450 (Scheme II-39). Consistent with this proposal a control sample, which contained only microsomes and the NADPH regenerating system, when incubated for one hour under the normal conditions led to the same peak which therefore is assigned to be nicotinamide.

![Scheme II-38 Formation of nicotinamide](image)

The incubation of β-nicotyrine with rabbit lung microsomes was also performed in the same way and the same results were observed. Therefore, we will not repeat the discussion of these results.

2.2.2.4. In Vitro Studies on the Metabolism of β-Nicotyrine with Rat Liver Microsomes

The preliminary in vitro studies on the metabolism of β-nicotyrine reported by Shigenaga employed rabbit liver and lung preparations. He found that lung microsomes were somewhat more efficient than liver microsomes on a per mole basis, but the liver microsomes were richer in cytochrome P-450 than lung. Since it is known that β-nicotyrine metabolites are toxic to cytochrome P-450 rich Clara cells isolated from rabbit lung, possible species differences in metabolic activity were examined.
Fig. II-51. HPLC-diolode array tracing of p-nicotyrine incubated with rabbit liver microsomal.

At time = 75 minutes, spiked with 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrorin-2-one (52).
and the UV absorbance of nicotineamide (solid) and the peak at time = 8.5 minutes (solid).
Chapter 2 In Vitro Studies on the Metabolism of β-Nicotyrine

The incubation of β-nicotyrine with the rat liver microsomes led to a very poor turnover of substrate compared to rabbit liver microsomes. After a one hour incubation with the rabbit liver microsomes, β-nicotyrine was completely metabolized. However, with the rat liver microsomes, after a one hour incubation, only recovered β-nicotyrine was detected by HPLC UV-diode array analysis. GC-EIMS analysis of the CHCl₃ extract of a 3-hour incubation mixture of β-nicotyrine with rat liver microsomes showed about 10% of 48 together with 90% of the unchanged β-nicotyrine. This result is in agreement with the preliminary reported results from the in vivo metabolism of β-nicotyrine in which β-nicotyrine was metabolized efficiently in all of the test animals except the rat. These differences might be caused by different isoenzyme compositions in rat as compared to rabbit liver. In any event, further studies should take a more careful look into species selectivity with regard to the fate of β-nicotyrine.

2.2.2.5. Summary of the In Vitro Studies

Analysis of the aqueous incubation mixtures by HPLC-diode array and GC-EIMS revealed that the minor tobacco alkaloid β-nicotyrine was metabolized by microsomes prepared from rabbit and rat livers and rabbit lungs to unstable intermediates, the two pyrrolinone isomers 48 and 51. One more metabolite, the 5'-hydroxypyrrolinone 52 was found as a direct metabolite of β-nicotyrine in rabbit liver and lung microsomes. The other component, 5'-hydroxycotinine (20), which was reported as a urinary metabolite of (S)-cotinine, was also found to be generated from the acid quenched incubation mixture of β-nicotyrine with rabbit liver and lung microsomes. However, since this compound also is found from the acid catalyzed hydrolysis of the 2'-acetoxyppyrrole 74, a masked form of the pyrrolinone 48 and 51, this product could be an artifact.

2.3. Conclusions from the In Vitro Studies

From the in vitro studies on the metabolism of β-nicotyrine, it was found that β-nicotyrine is first oxidized in a reaction presumably catalyzed by
cytochrome P-450 to two pyrrolinone intermediates, 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51), which are present in equilibrium. These unstable intermediates undergo autoxidation to 5'-hydroxypyrrrolinone 52, most likely through a free radical pathway, and hydration, particularly under acidic condition, to give 5'-hydroxycotinine (20).

Efforts to obtain quantitative information regarding the kinetics of the formation of the pyrrolinones 48 and 51, as well as the hydroxy metabolites 20 and 52, failed because of the instability of the pyrrolinones in the aqueous conditions.

2.4. Materials and Methods

2.4.1. Chemicals

Synthetic reactions were carried out under a nitrogen atmosphere. All of the starting materials for the syntheses and the spin trapping reagents 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and N-tert-butyl-α-phenylnitrone (PBN) were purchased from Aldrich Chemical Co (Milwaukee, WI). Tris, HEPES, potassium chloride, EGTA and the HPLC solvents acetonitrile, triethylamine and acetic acid are commercial products of the Fisher Scientific Company (Pittsburg, PA). NADPH, NADP+, MgCl₂, D-glucose-5-phosphate, glucose-6-phosphate dehydrogenase, and phosphate monobasic, dibasic and tribasic are commercial products of Sigma Chemical Company (St. Louis, MO). All other chemicals were reagent grade or HPLC grade. β-Nicotyrine (2) and β-nicotyrine tartrate, 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) and 5'-hydroxycotinine (20) were synthesized in our laboratory. The water was purified by Milli-Q Plus Ultra-Low Organics Water system made by Millipore Corporation (Bedford, MA).

2.4.2. Analytical Instruments

Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. ¹H and ¹³C NMR spectra were recorded on a
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

Brucker WP 270 MHz instrument linked to an Aspect 2000 computer. Chemical shifts are reported in parts per million (ppm) relative to Me₄Si as an internal standard in CDCl₃ or to 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid, sodium salt (TSP) in D₂O and CD₃OD. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or b (broad). Electron Spin Resonance (ESR) studies were performed on a Brucker ER 200 D instrument. Ultraviolet (UV) spectra were recorded on a Beckman DU 50 spectrophotometer. High resolution mass spectra were obtained by Electron Ionization (EI) on a VG 7070 HF double focusing mass spectrometer. GC-EI mass spectral analyses were performed on an HP 5970 mass selective detector linked to an HP 5890 GC. The GC column was an HP-1 100% dimethylpolysiloxane capillary column, 12.5 m x 0.2 mm x 0.33 μm. The GC temperature program for the synthetic samples, methylene chloride extracts of the incubation samples was Program 1 in which the oven temperature was held at 100 °C for 1 minute after injection and then temperature-programmed to 275 °C at a linear rate of 25 °C/minute. The final temperature was held for 2 minutes. HPLC UV-diode array analyses were performed on a system with two Beckman 114M pumps connected with a Beckman 421A controller to deliver the mobile phase. The HPLC was connected to a Hewlett-Packard 1040A Diode Array detector system. An Altech Econosil, 10 μ particle size, 25 cm x 4.6 mm, C8 analytical column was employed and was eluted first with mobile phase A containing water, acetonitrile, acetic acid and triethylamine with a ratio of 90:10:0.5:0.1 (v/v) for 10 minutes, then, employing a 10 minutes ramp to 40:60 of acetonitrile to the above mobile phase A. After eluting for 10 minutes with this mixture, the mobile phase was ramped back to 100% A over 5 minutes and the elution was continued for another 10 minutes. The total eluting time was 45 minutes. The flowrate was 1 mL/min. The effluent was monitored simultaneously at 260 and 282 nm. Typical retention times were as follows: β-nicotyrine (2), 23 minutes; the pyrrolinone 48, 20 minutes; the pyrrolinone 51, 19 minutes; (S)-cotinine (3), 15 minutes, the 5'-hydroxypyrrolinone 52, 12 minutes; 5'-hydroxycotinine (20), 10 minutes, 3'-hydroxycotinine (19), 7 minutes, nicotinamide (97) 6.5 minutes.
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2.4.3. Source of Tissues

The liver of 6 weeks old Sprague-Dawley male rats (200-250 g), and the livers and lungs of New Zealand White male rabbits (3.5-4 kg) were used for the preparation of microsomes.

2.4.4. Syntheses

2.4.4.1. 5-Methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (58)

Potassium hydroxide pellets (0.42 g, 7.5 mmol) were added to a well-stirred solution of 3’,3’-dibromocotinine (57)\textsuperscript{15} free base (1 g, 3 mmol) in 14 mL of methanol at -10 °C. The mixture was cooled in a dry ice-isopropanol bath to keep the temperature below -5 °C. When all of the KOH pellets had dissolved, the reaction mixture was allowed to warm to room temperature. After 24 hrs the KBr precipitate formed was removed by filtration and the methanol was removed by rotary evaporation at reduced pressure. To the residue was added chloroform (10 mL) and the remaining insoluble KBr was separated by filtration. The chloroform solution was washed three times with water until the water layer was colorless and then was dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}. The chloroform was removed by rotary evaporation at reduced pressure to yield 0.6 g (98%) of crude 5-methoxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (58) as a light brown oil. The crude product was purified by vacuum distillation: b.p. 123-128 °C/0.17 mm Hg; \textsuperscript{1}H NMR (CDCl\textsubscript{3}): δ 8.68 (1H, d, C2-H), 8.60 (4H, m, C6-H), 7.75 (1H, m, C4-H), 7.30 (1H, m, C5-H), 6.89 (1H, C5’-H), 6.40 (1H, d, C4’-H), 3.22 (3H, s, O-Me), 2.63 (3H, s, N-Me); GC-EIMS retention time (m/z): 3.8 min; 204 (30%), 173 (100%), 146 (15%), 126 (86%), 104 (15%), 78 (23%). UV (in MeOH): \( \lambda_{max} = 216 \text{ nm (e=7300)} \), 260 nm (\( \varepsilon=4500 \)). Anal. Calcd. for C\textsubscript{11}H\textsubscript{12}N\textsubscript{2}O\textsubscript{2}: C, 64.69; H, 5.92; N, 13.72. Found: C, 62.74; H, 6.02; N, 13.30. The difference can be accounted for by 0.4 mol of H\textsubscript{2}O (Calcd. for C\textsubscript{11}H\textsubscript{12}N\textsubscript{2}O\textsubscript{2}:0.4 H\textsubscript{2}O: C, 62.49; H, 6.10; N, 13.25).
2.4.4.2. 5'-Methoxycoctinine (59)

A solution of 5-methoxy-1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (58, 10.2 g, 0.1 mol) in 300 mL of methanol containing 2 g of 10% Pd/C was stirred vigorously under 1 atm of hydrogen at room temperature for 24 hrs. After filtering off the catalyst and removing the solvent under vacuum, 10.3 g (100%) of 5'-methoxycoctinine (59) was obtained as a pale yellow oil which solidified when stored in the refrigerator. GC-EIMS retention time (m/z): 3.7 min; 175 (100%), 149 (10%), 128 (100%), 100 (18%), 78 (30%). The analytical sample was recrystallized from ethyl acetate to give white cubic crystals: m.p. 75-76 °C; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.67 (1H, d, C2-H); 8.59 (1H, dd, C6-H); 7.70 (1H, td, C4-H); 7.32 (1H, m, C5-H); 3.27 (3H, s, O-Me); 2.69-2.60 (2H, m, C3'-H); 2.58 (3H, s, N-Me); 2.52-2.41 (1H, m, C4'-H); 2.32-2.15 (1H, m, C4'-H); UV (in MeOH): \(\lambda_{max}=216\) nm (\(\varepsilon=7300\)), 260 nm (\(\varepsilon=4500\)). Anal. Calcd. for C\(_{11}\)H\(_{14}\)N\(_2\)O\(_2\): C, 64.06; H, 6.84; N, 13.58. Found: C, 64.16; H, 6.86; N, 13.64.

2.4.4.2. 5'-Hydroxycoctinine (20)

A solution of the above 5'-methoxycoctinine (59, 1.03 g, 5 mmol) and trimethylsilyl iodide (TMSI, 1.3 g, 6.5 mmol, 0.925 mL) in 2 mL of dry CHCl\(_3\) was stirred at room temperature for 24 hrs. After the addition of 0.82 mL of methanol (0.54 g, 20 mmol) the solvent was removed under vacuum at 50 °C. The residue was treated with saturated sodium bicarbonate and the neutralized solution was extracted with CHCl\(_3\). The extract was washed with saturated sodium sulfite and dried over sodium sulfate. After removing the solvent, 1.73 g of a light brown oil was obtained which gave 0.87 g (90%) of crystalline 5'-hydroxycoctinine (20) when treated with diethyl ether: m.p. 122-123 °C (125-127 °C\(^{16}\)); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.70 (1H, d, C2-H); 8.54 (1H, q, C6-H); 7.77 (1H, m, C4-H); 7.38 (1H, q, C5-H); 6.70 (1H, b, OH); 2.66 (3H, s, N-Me), 2.62-2.34 (4H, m, C3'- and C4'-H); GC-EIMS showed two peaks at retention times (m/z): 4.4 min; 192 (10%), 174 (20%), 163 (35%), 114 (100%), 106 (96%), 78 (94%) assigned to the cyclic isomer, 59. The second peak: retention time (m/z): 4.6 min; 192 (2%), 163 (60%), 106 (100%), 78 (60%) assigned to the ketamide isomer, 21, which formed in the GC column by pyrolysis; UV (in MeOH): \(\lambda_{max}=206\) nm (\(\varepsilon=7000\)), 258 nm (\(\varepsilon=4000\)).
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2.4.4.4. 5-Hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52)

This is the same procedure employed for the synthesis of 5'-hydroxyco tinine (20). The product, 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52), was obtained as yellow crystals in 80% yield; m.p. 146-147 °C (145-146 °C\textsuperscript{16}); \textsuperscript{1}H NMR (D\textsubscript{2}O): \textdelta 8.54 (2H, d, C2- and C6-H), 7.81 (1H, dt, C4-H), 7.49 (1H, m, C5-H), 7.19 (1H, d, C3'-H), 6.25 (1H, d, C4'-H), 2.71 (3H, s, N-Me); GC-EIMS analysis showed two peaks at retention times (m/z): 4.7 min; 190 (70%), 161 (30%), 145 (5%), 133 (26%), 112 (100%), 104 (25%), 79 (97%) and 5.3 min; 190 (10%), 162 (55%), 147 (15%), 132 (95%), 106 (85%), 78 (100%). The peak with the retention time of 4.7 min corresponds to 52, and the peak with the retention time of 5.3 min corresponds to the ring opened isomer, 62; UV (in MeOH): \lambda_{max}=213 \text{ nm} (\varepsilon=9000), 262 \text{ nm} (\varepsilon=3900).

2.4.4.5. 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrrole (74)

(a). From 5'-Methoxycotinine (59)

A solution of 5'-methoxycotinine (59, 1.03 g, 5 mmol) in 5 mL (100 mmol) of acidic anhydride was heated to 80 °C in oil-bath for 6 hrs and then allowed to remain at room temperature overnight. The reaction was monitored by GC-EIMS. When the starting material (retention time 4.4 min) had totally disappeared, the GC-EIMS showed only one peak with a molecular ion of 216 (retention time 4.5 min). After removal of the unreacted acetic anhydride, the brown oily residue was purified first by passing through a neutral alumina column with CHCl\textsubscript{3} to remove the traces of acetic anhydride. Having removed the CHCl\textsubscript{3}, the residue was purified on a silica gel column (1:20, w/w), with CHCl\textsubscript{3} as the solvent. The first fraction gave 0.83 g (76.5%) of 2-acetoxypyrrrole-1-methyl-5-(3-pyridyl)pyrrrole (74) as a pale yellow oil: GC-EIMS retention time (m/z): 4.5 min; 216 (10%), 174 (100%), 145 (10%), 131 (10%), 104 (10%), 78 (10%); \textsuperscript{1}H NMR (CDC\textsubscript{3}): \textdelta 8.66 (1H, d, C2-H), 8.52 (1H, q, C6-H), 7.68 (1H, q, C4-H), 7.32 (1H, q, C5-H), 6.21 (1H, d, C3'-H), 5.95 (1H, d, C4'-H), 3.45 (3H, s, O-Me), 2.35 (3H, s, N-Me); UV (in a HPLC mobile phase containing water : acetonitrile : triethylamine : acetic acid = 50:50:0.1:0.5, pH
Chapter 2 In Vitro Studies on the Metabolism of β-Nicotyrine

3.8): $\lambda_{\text{max}} = 290 \text{ nm (} \varepsilon_{\text{max}} = 8000 \text{)}$. Anal. Calcd. for C$_{12}$H$_{12}$N$_2$O$_2$: C, 66.65; H, 5.59; N, 12.95. Found: C, 65.62; H, 5.53; N, 12.81. The difference is due to the presence of 0.15 mol of H$_2$O (Calcd. for C$_{12}$H$_{12}$N$_2$O$_2$·0.15 H$_2$O: C, 65.83; H, 5.59; N, 12.79).

(b). From 5'-Hydroxycotinine (20)

To a solution of 20 (37.6 mg, 0.2 mmol) in 2 mL of CHCl$_3$, 0.3 mL (3 mmol) of acidic anhydride was added. The reaction mixture was heated under refluxing for 6 hrs and the reaction was monitored by GC-EIMS. After removing the solvent, the brown oily residue was purified by preparative thin layer chromatography (PTLC) using 500 micron silica gel GF-254. The plate was developed with 50:3 of CHCl$_3$ : MeOH (v/v). The product ($R_f$ 0.4) was isolated by washing the silica gel with MeOH to gave 28 mg (65 %) of the yellow oil. GC-EIMS and $^1$H NMR (CDCl$_3$) spectra gave the same data as the reaction with 5'-methoxycotinine (59).

2.4.4.6. 1-Methyl-5-(3-pyridyl)-4-trifluoraceto-4-pyrrolin-2-one (76)

To a solution of 1.92 g of 5'-methoxycotinine (59, 1.92 g, 9.3 mmol) in 10 mL of dry CH$_2$Cl$_2$ cooled to 0 °C with an ice-water bath was added 5.4 mL of TFAA. The reaction mixture was stirred at 0 °C for 1.5 hrs and then heated under refluxing overnight. After removing the solvent, the product, 1-methyl-5-(3-pyridyl)-4-trifluoraceto-4,5-pyrrolin-2-one (76) was purified by preparative thin layer chromatography (silica gel GF 254) with ethyl acetate as the mobile phase: $R_f$ 0.5; $^1$H NMR (CD$_3$OD): δ 8.76 (1H, d, C2-H), 8.61 (1H, s, C6-H), 7.85 (1H, m, C4-H), 7.60 (1H, m, C5-H), 4.40 (2H, s, C3'-H), 2.98 (3H, s, N-Me); GC-EIMS retention time (m/z): 4.0 min; 270 (25%), 241 (<5%), 201 (100%), 173 (10%), 145 (40%), 119 (75%), 78 (25%). High resolution El-MS for m/z 270 (parent ion): calcd. for C$_{12}$H$_9$N$_2$O$_2$F$_3$: 270.0616124, found: 270.061874; for m/z 201 (base peak, M+ - CF$_3$): calcd. C$_{11}$H$_{9}$N$_2$O$_2$: 210.0664027, found: 201.066513; $^{13}$C NMR (CDCl$_3$, internal standard: CDCl$_3$, δ 77): δ 174.599 (CF$_3$CO), 159.547 (NH$_2$CO), 151.610 (2-C), 148.550 (6-C), 136.310 (3- and 4-
In Vitro Studies on the Metabolism of $\beta$-Nicotyrine
C), 126 000 (5-C), 123.357 (4'-C), 106.810 (CF$_3$), 77 (CDCl$_3$), 35.826 (5'-C), 29.65 (N-CH$_3$) 23.092 (3'-C).

2.4.4.7. 1-Methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51)

(a). From Sublimation of 5'-Methoxytocinine (59)

5'-Methoxytocinine (59, 20.6 mg, 0.1 mmol) was placed in a sublimation apparatus which was evacuated and then refilled with nitrogen for three times. The sublimator was gently heated with an oil-bath to 120 °C to give a condensate on the cold finger as a colorless oil. The $^1$H NMR spectrum in CDCl$_3$ showed signals which could be assigned according to the published data$^5$, and quantitatively estimated by integration of the N-methyl protons as follows: 54.5% of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48), 25.5% of 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51), and 20% of recovered 5'-methoxytocinine 59. The assignments of $^1$H NMR spectra are summarized in Table II-8.

(b). From Pyrolysis of 5'-Hydroxytocinine (20)

5'-Hydroxytocinine (20, 87.2 mg, 0.454 mmol) was placed in a flask connected with a 50 cm long pyrolysis tube wrapped with electro-thermal wire and a cold trap. The whole system was purged with nitrogen, then the pyrolysis tube was heated to 280 °C. The system was evacuated and then refilled with nitrogen for four times. The flask containing 5'-hydroxytocinine then was gently heated with an oil-bath up to 135 °C while the pyrolysis tube was kept at 280 °C. The process was carried out under a vacuum (0.12-0.14 mm Hg) for 1.5 hrs and resulted in a mixture of the two pyrrolinones, 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one, (48), and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51) together with a dark purple colored material of unknown composition (9 mg, 11.4 %): $^1$H NMR (CDCl$_3$) of 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48): $\delta$ 8.67 (m, 2H, C2- and C6-H), 7.69 (dt, 1H, C5-H), 5.38 (t, 1H, C4'-H), 3.21 (d, 2H, C3'-H), 3.03 (s, 3H, N-Me); 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51): $\delta$ 8.51 (m, 2H, C2- and C6-H), 7.69 (dt, 1H, C4-H), 7.30 (m, 1H, C3-H), 7.03 (dd, 1H, C3'-H), 6.30
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine
(dd, 1H, C4'-H), 5.01 (t, 1H, C5'-H), 2.83 (s, 3H, N-Me). High resolution El-MS of
the condensate (M+): Calcd. for C10H10N2O: 174.0793±131, found:
174.078354.

(c). From Hydrolysis of 2-Acetoxyprrole-1-methyl-5-(3-
pyridyl)pyrrole (74)

2-Acetoxyprrole-1-methyl-5-(3-pyridyl)pyrrole (74, 2.2 mg, 0.01 mmol)
was dissolved in 1 mL of 0.02 M aqueous trisodium phosphate, pH 11.8. This
basic solution was immediately added to 9 mL of 0.1 M aqueous monosodium
phosphate (pH 5.1) to give a final pH value of 7.4. Both solvents were saturated
with nitrogen. The sample was immediately analyzed by HPLC UV-diode array.
The chromatography showed only two peaks. Retention time: 19.7 min, \( \lambda_{\text{max}} = 
210 \text{ nm and 257 nm and 20.5 min, } \lambda_{\text{max}} = 230 \text{ nm and } 280 \text{ nm. This sample}
was extracted with ethyl acetate and analyzed by GC-EIMS retention time (m/z):
4.2 min (174, 119, 96, 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one, 51); 4.4 min
(174, 119, 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one, 4B); 4.7 min (190, 112, 79, 5-
hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one, 52).

2.4.4.8. β-Nicotyrine Free Base and Its Tartrate Salt

β-Nicotyrine was prepared by a dehydrogenation of (S)-nicotine
catalyzed by 10% palladium on carbon following the published method\(^5\) except
the reaction temperature was modified to 240 °C. β-Nicotyrine free base was
purified by vacuum distillation (92-94 °C / 0.35 mmHg) and the yield was
increased to 22%. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.67 (1H, q, C2-H), 8.50 (1H, q, C6-H),
7.65 (1H, q, C4-H), 7.28 (1H, m, C5-H), 6.74 (1H, d, C3'-H), 6.28 (1H, , C4'-H),
6.21 (1H, m, C2'-H) 3.84 (3H, s, N-Me). GC-EIMS retention time (m/z): 4.2 min;
158 (100%), 143 (5%), 130 (21%), 116 (9%), 105 (4%), 90 (6%), 79 (6%), 78
(5%). β-nicotyrine tartrate was prepared in 95% EtOH as the published
method.\(^5\) It was yield 89% as pale yellow needle crystals, m.p. 88-88 °C (86-88
°C); \(^1\)H NMR (D\(_2\)O): \(\delta\) 8.60 (1H, d, C2-H), 8.44 (1H, dd, C6-H), 7.90 (1H, dt,
C4-H), 7.48 (1H, q, C5-H), 6.81 (1H, t, C4'-H), 6.27 (1H, dd, C2'-H), 6.14 (1H, m,
C2'-H), 4.51 (2H, s, OH), 3.68 (3H, s, N-Me), 3.33 (2H, m, CH). GC-EIMS
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

retention time (m/z): 4.2 min; 158 (100%), 143 (5%), 130 (21%), 116 (9%), 105 (4%), 90 (6%), 79 (6%), 78 (5%).

2.4.5. Hydrolysis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74)

2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74, 10 mg) was dissolved in 0.5 mL of the following solutions: (a) 10% DCI / D₂O (pH<1); (b) 5% DOAc / D₂O (pH 3.5); (c) 0.1 M phosphate buffer (in D₂O, pH 7.4); and d). 5% of Na₃PO₄ / D₂O (pH 12). The reaction mixtures were stored at room temperature and monitored by ¹H NMR. The quantitative data were obtained from the integration of N-methyl groups: δ 2.41 (2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole, 74), 2.64 (5'-hydroxycoctinine, 20), 2.73 (5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrroline-2-one, 52), 2.83 (1-methyl-5-(3-pyridyl)-3-pyrroline-2-one, 51) and 3.02 (1-methyl-5-(3-pyridyl)-4-pyrroline-2-one, 48). Parallel experiments were operated in H₂O solutions and extracted with ethyl acetate which were analyzed by GC-ElMS. The GC-EIMS data are summarized in Table II-10.

Table II-10. GC-EIMS analysis of the hydrolysis products of 74

<table>
<thead>
<tr>
<th>Solvent</th>
<th>tᵣ (min)</th>
<th>m/z</th>
<th>Compd</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl/H₂O</td>
<td>3.6</td>
<td>174, 145, 119, 78</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
<td>192, 175, 114, 106, 79</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>191, 163, 106, 78</td>
<td>62</td>
</tr>
<tr>
<td>HOAc/H₂O</td>
<td>4.0</td>
<td>190, 161, 145, 132, 112, 104, 79</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>174, 163, 145, 119, 114, 106, 86, 79</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>192, 163, 134, 106, 78</td>
<td>21</td>
</tr>
<tr>
<td>Phosphate buffer (pH 7.4)</td>
<td>3.5</td>
<td>174, 145, 132, 119, 96, 78</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>174, 145, 132, 119, 89, 78</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>190, 161, 145, 133, 112, 79</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>4.4</td>
<td>192, 174, 145, 114, 106, 79</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>163, 134, 106, 78</td>
<td>21</td>
</tr>
<tr>
<td>Na₃PO₄/H₂O (5%)</td>
<td>4.5</td>
<td>189, 188, 173, 145, 131, 119, 103, 78</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>190, 161, 145, 133, 112, 79</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>9.6</td>
<td>362, 305, 256, 173, 145, 119, 103, 78</td>
<td>79</td>
</tr>
</tbody>
</table>

176
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2.4.6. ESR Studies of the Hydrolysis of 2-Acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74)

2.4.6.1. Purification of DMPO

The commercial DMPO was a brown, oily liquid that shows a broad UV absorption between 200-270 nm and which gives an ESR signal similar to the spin adducts of carbon-centered radicals. The water used in the purification of DMPO was obtained by reverse osmosis and further purified by Milli-Q Plus Ultra-Low Organics Water system. DMPO was purified under anaerobic conditions including the use of nitrogen purged water. The commercial DMPO (0.8 g) in 8 mL of the purified and deoxygenated water was filtered through a short active charcoal column. ESR detection did not obtain any signals from this purified DMPO solution. It was stored at -20 °C and darkness. The concentration of the purified aqueous DMPO was 0.3 M as detected by UV spectrometry.

2.4.6.2. Spin Trapping with DMPO or PBN and ESR Measurement

The stock solutions of the substrates and the free radical trapping reagents used were as follows: 2-acetoxy-1-methyl-5-(3-pyridyl)-pyrrole (74) 6 mM in 0.1 M phosphate buffer (pH = 7.0); β-nicotyrine: 13 mM in 0.1 M phosphate buffer (pH = 7.0); DMPO: 0.3 M in water and PBN: saturated in water. The samples were siphoned into 2 inch long capillary tubes (I.D. 1 mm) and sealed. Unless otherwise stated, the ESR parameters were set at X-band, 100 kHz modulation frequency; microwave power 20 mW; modulation amplitude 2.0 G; time constant 1.25 s; scan time, 500 s; receive gain 8.0 x 10^5; central field 3483 G; scan width 100 G. The line-width of the ESR was measured by a E-500 gauss meter.

The control samples used in these experiments were as follows:

1. A 6 mM of 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) in 0.1 M phosphate buffer (pH = 7.0). (Sample 1)
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

2. A 0.3 M solution of DMPO in water. (Sample 2)

3. A saturated solution of FBN in water. (Sample 3)

4. A 13 mM solution of β-nicotyrine in 0.1 M phosphate buffer (pH = 7.0) mixed with the 0.3 M solution of DMPO in water (1:1, v/v). (Sample 4)

5. A 13 mM solution of β-nicotyrine in 0.1 M phosphate buffer (pH = 7.0) mixed with the saturated solution of PBN in water (1:1, v/v). (Sample 5)

6. A mixture of 50 µL of 0.1 M phosphate buffer (pH = 7.0) and 25 µL of 0.3 M solution of DMPO in water plus 25 µL 100% ethanol. (Sample 6)

7. A mixture of 20 µL of 0.1M PBN in 100% of ethanol and 20 µL of 0.1 M phosphate buffer (pH = 7.0). (Sample 7)

The experimental samples were as follows:

1. A mixture of 6 mM 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) in 0.1M phosphate buffer (pH = 7.0) and 0.3 M solution of DMPO in water (1:1, v/v). (Sample 8)

2. A mixture of 6 mM 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) in 0.1M phosphate buffer (pH = 7.0) and saturated solutions of PBN in water (1:1, v/v). (Sample 9)

3. A mixture of 50 µL of 6 mM 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) in 0.1 M phosphate buffer (pH = 7.0) and 25 µL of 0.3 M solution of DMPO in water plus 25 µL 100% ethanol. (Sample 10)

4. A mixture of 20 µL of 6 mM of 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) in 0.1 M phosphate buffer (pH = 7.0) and 20 µL of 0.1 M of PBN in 100% ethanol. (Sample 11)

The samples were examined by ESR at the following times with the results as summarized in Table II-11.
Table II-11. ESR detection times and signals

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (hour)</th>
<th>ESR Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0, 1, 2</td>
<td>No signal</td>
</tr>
<tr>
<td>2</td>
<td>0, 1, 2</td>
<td>No signal</td>
</tr>
<tr>
<td>3</td>
<td>0, 1, 2</td>
<td>No signal</td>
</tr>
<tr>
<td>4</td>
<td>0, 1, 2</td>
<td>No signal</td>
</tr>
<tr>
<td>5</td>
<td>0, 1, 2</td>
<td>No signal</td>
</tr>
<tr>
<td>6</td>
<td>0, 1, 2</td>
<td>No signal</td>
</tr>
<tr>
<td>7</td>
<td>0, 1, 2</td>
<td>No signal</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>10 lines</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
<td>6 lines</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>6 lines</td>
</tr>
<tr>
<td>11</td>
<td>0.5</td>
<td>6 lines</td>
</tr>
</tbody>
</table>

Table II-12. Hyperfine splitting constants of the ESR signals

<table>
<thead>
<tr>
<th>Sample</th>
<th>$a_N$ (G)</th>
<th>$a_{\beta H}$ (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 (4-line)</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>(6-line)</td>
<td>15.9</td>
<td>23</td>
</tr>
<tr>
<td>9 (6-line)</td>
<td>15.2</td>
<td>2.8</td>
</tr>
<tr>
<td>10 (6-line)</td>
<td>15.8</td>
<td>22.8</td>
</tr>
<tr>
<td>11 (6-line)</td>
<td>15.5</td>
<td>3.6</td>
</tr>
</tbody>
</table>

2.4.7. Preparation of Tissues

2.4.7.1. Preparation of Rabbit Liver Microsomes

After carbon dioxide (dry ice) asphyxiation, the livers were perfused in situ via the portal vein with 250 mL ice cold 1.15% KCl. Livers were minced with scissors and the pieces were homogenized in a glass Potter-Elvehjem homogenizer with a teflon pestle in 3 volumes (w/v) of 0.25 M sucrose buffer at
Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine

pH 7.4 with 0.05 M Tris/HCl. The homogenate was centrifuged at 1,000 g for 15 minutes and the resulting supernatant fraction was centrifuged at 10,000 g for 20 minutes. The secondary supernatant was centrifuged at 100,000 g for 65 minutes. The pellet was rinsed and reconstituted in 1:1 [w (g)/v (mL)] of 0.1 M of Tris/HCl buffer at pH 7.4 containing 0.1 mM of EGTA. The suspended mixture was recentrifuged at 100,000 g for 65 minutes. The final pellet was rinsed with the same buffer to remove all of the fat and homogenized in 3:1 volume (w(g)/v(mL)) of the same buffer and stored at -70 °C. Protein concentration was determined by the Lowry method.80

2.4.7.2. Preparation of Rat Liver Microsomes

After CO2 asphyxiation, the livers were perfused in situ via the portal vein with 100 mL of ice cold 0.25 M sucrose buffered at pH 7.4 and with 0.05 M Tris/0.05 M HCl. Livers were minced with scissors and the pieces were homogenized in 3 volumes of the same buffer to the weight of the livers with a Teflon-glass homogenizer. The homogenates were subjected to centrifugation at 10,000 g for 20 min at 4 °C and the resulting supernatant fraction was centrifuged at 100,000 g for 75 min at 4 °C. The pellets were rinsed with the same buffer to remove the fat and reconstituted in 1:1 [w (g)/v (mL)] of 0.15 M KCl buffered at pH 7.4 with 0.02 M KH2PO4/K2HPO4 and this mixture was centrifuged a second time at 100,000 g for 75 min. The resulting pellets were homogenized in this buffer at a ratio of 1 mL / 1g liver. Protein concentration was determined by the Lowry method.

2.4.7.3. Preparation of Rabbit Lung Microsomes5

After carbon dioxide (dry ice) asphyxiation, the lungs were perfused via the pulmonary artery with 25 mL ice cold 1.15% KCl then the lung microsomes were prepared by the same procedure as for the preparation of liver microsomes. The final pellet was homogenized in 3:1 volume [w (g)/v (mL)] of 0.1 M Tris/HCl buffer at pH 7.4 containing 0.1 mM EGTA and used for the incubation with β-nicotyrine tartrate immediately. Protein concentration was determined by the Lowry method.
2.4.8. Incubations

2.4.8.1. Incubations with Rabbit Liver and Lung Microsomes

Mixtures containing 3-4 mg/mL of rabbit lung microsomes or 5-6 mg/mL of rabbit liver microsomes, β-nicotyline tartrate (154 μg/mL, 0.5 μmol), and an NADPH regenerating system (0.5 mM NADP+, 0.5 mM NADPH, 8 mM glucose-6-phosphate, 1 unit/mL glucose-6-phosphate dehydrogenase and 4 mM MgCl₂) in a final volume of 4 mL of 0.1 M Tris/HCl buffer, pH 7.4, containing 0.1 mM of EGTA, were incubated at 37 °C for 2 hours in a metabolic shaker. The incubation mixtures were added to an equal volume of ice cold acetonitrile or methanol, or to 1/2 volume of 5% aqueous trichloroacetic acid to precipitate the proteins. The quenched mixtures were centrifuged at 16,000 g for 5 minutes. The supernatants were centrifuged with filtration at 16,000 g for another 5 minutes to further remove the precipitated proteins. The final supernatants were analyzed by HPLC UV-diode Array. In order to identify the components, synthetic compounds were added to the individual samples to look for increased peak size.

2.4.8.2. Incubations with Rat Liver Microsomes

Mixtures containing liver microsomes (6 mg of protein/ml), β-nicotyline tartrate (154 μg, 0.5 μmol), EGTA (1 mM) in 0.1 M HEPES buffer, pH = 7.4, and an NADPH regenerating system (0.5 mM NADP+, 8 mM glucose 6-phosphate, 1 unit/mL glucose-6-phosphate dehydrogenase, and 4 mM MgCl₂) in a total of 4 mL of 0.1 M of Tris/HCl buffer at pH 7.4 containing 0.1 mM of EGTA were incubated at 37 °C for 0 hr, 1 hr and 2 hr in a metabolic shaker. The resulting mixtures were separated into two parts: part 1 was extracted with CH₂Cl₂ (2 mL), the extracts were vortexed for 1 min and the phases separated by dry-ice freezing. The CH₂Cl₂ layers were analyzed by GC-EIMS. To part 2 was added ice-cold methanol and the precipitated proteins were removed by centrifuge action at 16,000 g for 5 minutes. The supernatants were centrifuged with filtration at 16,000 g for another 5 minutes to further remove the coagulated proteins. The final supernatants were detected by HPLC UV-diode array.
References


Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine


Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine


Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine


Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine


Chapter 2  In Vitro Studies on the Metabolism of β-Nicotyrine


Chapter 3  In Vivo Studies on the Metabolism of \( \beta \)-Nicotyrine

3.1. Background

Studies on the in vivo metabolism of \( \beta \)-nicotyrine were reported by Jenner and Gorrod in 1973.\(^1\) However, no effort was made to identify the metabolites of \( \beta \)-nicotyrine. Shigenaga has reported results on the in vitro formation of metabolites of \( \beta \)-nicotyrine with rabbit liver and lung microsomes.\(^2\) He identified the pyrrolinone 48 and confirmed that the metabolism of \( \beta \)-nicotyrine was catalyzed by an NADPH dependent process, presumably by cytochrome P-450. In our in vitro studies, besides the reported pyrrolinone species 48, the 5'-hydroxypyrrolinone 52 was also found as a metabolite in rabbit lung and liver microsomal preparations. The pathway leading to 52 may involve a free radical process as suggested by ESR results discussed in Chapter 2. In addition to 48 and 52, 5'-hydroxycotinine (20), a hydrolysis product of the pyrrolinone 48, was also detected and identified from the acid quenched incubation mixture. The possible formation of these metabolites in vivo is addressed in this chapter. It should be pointed out that rough estimates of the percentage of the metabolites of \( \beta \)-nicotyrine identified so far in vitro account for only 10% of the \( \beta \)-nicotyrine consumed.\(^2\)

The major effort conducted in this area concerned the isolation, characterization and syntheses of the metabolites of \( \beta \)-nicotyrine produced in vivo and the interpretation of the significance of the results. For the most part, these studies were pursued in the white rabbit, a species which has been employed extensively in previous metabolic studies of nicotine alkaloids.\(^3\) In
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

order to proceed with the in vivo studies, it was first necessary to obtain an estimate of the acute toxicity of β-nicotyrine.

3.2. Results and Discussion

3.2.1. Toxicity of β-Nicotyrine and Dosage Studies

It is known that (S)-nicotine is highly toxic to rodents. The LD$_{50}$ in mice is 0.3 mg/kg (i.v.), 9.5 mg/kg (i.p.), and 230 mg/kg orally.$^4$ The acutely fatal dose of (S)-nicotine for an adult human is estimated to be 60 mg of the free base.$^5$ The pyrrolidine ring of (S)-nicotine will be protonated at physiological pH and interaction of (S)-nicotine with the acetylcholine receptor is likely to involve this protonated species.$^6,^7$ The symptoms of (S)-nicotine poisoning include nausea, salivation, abdominal pain, vomiting, diarrhea, cold sweat, headache, dizziness, disturbed hearing and vision, mental confusion, and marked weakness. The blood pressure falls. Breathing becomes difficult. The pulse becomes weak, rapid and irregular. Collapse may be followed by terminal convulsions. Death may result within a few minutes from respiratory failure.

β-Nicotyrine should be less toxic than (S)-nicotine because the aromatic lone pair electrons on the nitrogen in the pyrrole ring is no longer basic and will not be protonated under physiological conditions. Consequently, β-nicotyrine will not likely act on the acetylcholine receptor of the cholinergic neurons. Estimates of the acute toxicity of β-nicotyrine have not been reported. Therefore, in order to carry on the in vivo studies with rodent animals safely, we needed to estimate this toxicity. In order to compare the toxicity with that of (S)-nicotine, mice were chosen for these studies. As expected, doses of up to 100 mg/kg by intraperitoneal injection were not lethal. Since this dose was probably high enough to allow us to detect the urinary metabolites of β-nicotyrine, it was chosen for our in vivo metabolic studies.

3.2.2. Extraction of Urine

The potential metabolites of β-nicotyrine, 20 and 52, and possibly other metabolites, are polar and water soluble and therefore difficult to separate from
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

the other polar components of urine. In addition, 20 and 52 are expected to have similar polarities based on their very similar structures. The issues will be:

1) To separate the polar potential metabolites derived from β-nicotyrine from other polar components of urine.

2) To separate the metabolites of β-nicotyrine with similar polarities from each other.

3) To identify these metabolites.

Several studies on the isolation of the urinary metabolites of (S)-nicotine and (S)-cotinine have been reported. O'Doherty et al. used colorimetric reagents in their HPLC analysis,8 while Cundy et al. used high-performance liquid-radiochromatography with a Partisil-10 ODS column or Partisil-10 SCX column and a UV detector.9 These methods, however, failed to separate the anticipated metabolites of β-nicotyrine. Nwosu et al. used an SCX column in an HPLC assay with a UV detector.10 It was possible to separate 3'-hydroxycotinine from (S)-nicotine and (S)-cotinine. This method, however, did not resolve 20 and 52. Kyerematen et al. used an IBM Optima cyano RP cartridge connected in series with a cyano RP steel column. A water-methanol-acetate buffered eluent and monitoring with both a radioactivity flow detector and a M440 UV absorbance detector were used. It was possible to detect the ketamide 21 derived from the 5'-hydroxycotinine (20), but 20 itself was not detected.11,12 GC methods, such as reported by Neurath et al.,13 were not pursued since we anticipated thermal instability problems and the poor solubility of the urinary metabolites in organic solvents required by GC analysis. In the hope of developing an assay that would be effective for both lipophilic and hydrophilic metabolites, the development of a new extraction and analysis procedure was pursued.

Based on the above considerations, we decided to proceed by removal of the water from the urinary samples by lyophilization followed by continuous soxhlet extraction with a proper organic solvent which would concentrate the metabolites from the complex urinary residue. Since the synthetic hydroxyl
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

derivatives of β-nicotyrine, 20 and 52, are solids at room temperature, we anticipated that they would not be volatilized by the lyophilization step. The in vitro metabolic intermediates, the pyrrolineone species 48 and 51, are highly unstable and would be expected to undergo further metabolism to more stable metabolites, such as 20 and 52.

Considering the solubilities in organic solvents of the potential urinary metabolites, as well as the solubilities of the other urinary components, we proceeded to screen a series of solvents for the continuous extraction. These included hexanes, benzene, chloroform, ethyl acetate, acetonitrile and methanol. Finally, we found benzene to be best suited since it was effective in extracting all of the target compounds, β-nicotyrine, 20 and 52, while still providing an acceptable background signal in the UV detection. We also tested the extraction time from 30 minutes to 3 hours and found that at 1 hour the recoveries were 80% for 20, 90% for 52, and 100% for β-nicotyrine. Based on this study, we decided to extract the target compounds with benzene for 2 hours. Since we had available a good HPLC UV-diode array assay from our in vitro studies, we decided to employ the same HPLC UV-diode array assay for our in vivo studies. In order to perform both the reverse phase HPLC assay and GC-MS analysis, the analytical samples, after removing the benzene, were reconstituted in methanol, a good solvent for these techniques.

3.2.3. HPLC UV-diode Array Analysis of β-Nicotyrine Treated Rabbit and Mouse Urinary Samples

The HPLC UV-diode array analysis of the extracted urine samples used the same procedures as in our in vitro studies. A C8 column was employed and was eluted first with mobile phase A containing water, acetonitrile, acetic acid and triethylamine with a ratio of 90:10:0.5:0.1 (v/v) for 10 minutes, then, employing a 10 minutes ramp to 40:60 of acetonitrile to the above mobile phase A. After eluting for 10 minutes with this mixture, the mobile phase was ramped back to 100% A over 5 minutes and the elution was continued for another 10 minutes. The total eluting time was 45 minutes.
Fig. III-1. HPLC tracing of urine samples: (a) 24 Hour urine sample obtained from untreated rabbit; (b) 24 Hour urine sample obtained from rabbit treated i.p. with β-nicotyrine.
FIG. III.2. HPLC tracing of 24 hour urine sample obtained from mice treated with p-nicotine (2).

Time (min)
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

Figure III-1 shows the HPLC tracings of the urine samples obtained from an untreated rabbit as a control sample (top) and a β-nicotyrine treated rabbit (bottom). Comparing the two tracings, one can see the following results:

1) The region from 3 to 7 minutes shows a very complex set of peaks but with no major differences.

2) A major peak appears at a retention time of 8 minutes in the treated sample which was not observed in our in vitro studies.

3) In the region from 9 to 15 minutes, two peaks of moderate intensity are present in the treated sample. One has a retention time of 10 minutes, which is consistent with 20, while the other has a retention time of 13.5 minutes, which is consistent with 52. The UV spectra of these two peaks also are consistent these assignments. These two peaks were also confirmed by spiking with the synthetic standards. The details will be discussed in a later section.

4) The region from 15 to 30 minutes is complex, but no obvious differences could be detected between the treated and untreated samples.

The HPLC-diode array tracing of the mouse urine obtained from the β-nicotyrine treated mice in toxicity studies displayed the same patterns (Fig. III-2).

3.2.3.1. In Vitro Metabolites Not Observed in Rabbit Urine Samples

With the aid of synthetic samples, we searched for β-nicotyrine, which should show up at 23 minutes, and the pyrrolinone species 48 and 51, which should show up at 19 and 20 minutes. However, we did find these compounds. The absence of β-nicotyrine is consistent with the results reported by Jenner and Gorrod that β-nicotyrine is metabolized extensively in vivo in experimental animals.\(^1\) The absence of the pyrrolinone species 48 and 51 is consistent with their chemical instability as we have discussed in Chapter 2. However, the characterization of 20 and 52 would be indirect evidence of the formation of the pyrrolinones 48 and 51 in vivo as metabolic intermediates because they are the known degradation products of 48 and 51.
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

3.2.3.2. Characterization of the Metabolites Derived from β-Nicotyrine in Rabbit Urine

1) 5'-Hydroxycotinine (20) and 5-Hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52)

From the HPLC UV-diode array analysis, two minor peaks were found with retention times of 10 minutes, corresponding to that of 20, and 13.5 minutes, corresponding to that of 52. When the urine sample was spiked with synthetic 20, the peak at retention time 10 minutes increased in intensity (Fig. III-3c). Furthermore, the diode array UV absorption of this unknown peak was identical with that of the standard 20 (Fig. III-4a). The second peak at retention time of 12 minutes increased as expected when the urine sample was spiked with synthetic 52 (Fig. III-3d). Additionally, the diode array UV absorption of this unknown peak was identical with that of the standard 52 (Fig. III-4b). Although 5'-hydroxycotinine has also been reported as an in vivo metabolite of (S)-cotinine, the characterization of 52 as a urinary metabolite of β-nicotyrine is reported for the first time.

2) Characterization of the Principal Urinary Metabolite of β-Nicotyrine

The principal HPLC peak eluting at 8.0 minutes could not be related to the results obtained from our in vitro studies. The HPLC UV-diode array spectrum of this peak displayed a chromophore with \( \lambda_{\text{max}} = 260 \text{ nm} \), which is similar to that of 5'-hydroxycotinine (20, Fig. III-4a). However, the different retention time rules out 20 as a possible structure. In an effort to obtain more information on this species, we attempted to analyze the crude extract of this urinary sample by GC-EIMS. Figure III-5 shows the total ion current (TIC) tracing of the ethyl acetate extract of the urinary sample obtained from β-nicotyrine treated rabbit (Fig. III-5b) and the control sample which was obtained from an untreated rabbit (Fig. III-5a). One major peak at retention time 5.0 minutes appears only in the tracing of the β-nicotyrine treated urinary sample. The EI mass spectrum of this unknown major peak (Fig. III-5c) shows a parent ion at m/z 192 (72% abundance). The major fragment ions append at m/z (%):
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

The parent ion at m/z 192 is consistent with a molecular formula C_{10}H_{12}N_{2}O_{2}, that is the molecular formula corresponding to hydroxycotinine derivatives. The mass spectral characteristics of this metabolite were clearly different from those of both 5'-hydroxycotinine 20 and the ring opened ketoamide isomer 21.

Therefore, this metabolite had to be an isomer of 20 with one possibility being trans-3'-hydroxycotinine (19a), the major urinary metabolite detected in smokers' urines.\textsuperscript{16,17} In fact, the published EI mass spectrum of 19a\textsuperscript{15} and this principal metabolite of β-nicotyrine were essentially identical. Therefore, we needed to synthesize 3'-hydroxycotinine 19, both the cis- (19b/c) and the trans-isomers (19a/d), to provide authentic samples for comparison.
Fig. III-3. HPLC tracing of rabbit urine samples. (a) 24 Hour urine sample obtained from untreated rabbit; (b) 24 Hour urine sample obtained from rabbit treated i.p. with β-nicotyrine; (c) Sample (b) spiked with synthetic 20; (d) Sample (b) spiked with synthetic 52.
Fig. III-4. (a) UV spectra of synthetic 5'-hydroxycotinine (20, dotted line) and the urinary metabolic (t_R = 10 minutes, solid line); (b) UV spectra of synthetic 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (52, dotted line) and the urinary metabolic (t_R = 13.5 minutes, solid line).
Fig. III-5. GC-EIMS data: (a) TIC of urine sample from untreated rabbit; (b) TIC of urine sample from β-nicotyrine treated rabbit; (c) GC-EIMS spectrum of the unknown metabolite derived from β-nicotyrine.
3.2.4. Synthesis of cis-3'-Hydroxycotinine (19b/c) and trans-3'-Hydroxycotinine (19a/d)

3.2.4.1. cis-3'-Hydroxycotinine (19b/c)

Scheme III-1 Synthesis of cis-3'-hydroxycotinine (19b/19c)

Scheme III-2 Formation of the four regional isomers of isoazolidine 102

Cis-3'-hydroxycotinine was first synthesized by Dagne in 1972. Reaction of 3-pyridylcarboxaldehyde (100) and N-methylhydroxylamine hydrochloride provided the trans-C-(3-pyridyl)-N-methylnitrone (101). This
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

compound was treated with methyl acrylate to produce a mixture of cis- and trans-isoxazolidines 102. Hydrogenation of 102 gave racemic cis-3'-hydroxycotinine (19) as showed in Scheme III-1.

In this synthetic pathway the stereochemical questions of interest include both regio chemistry and diastereochemistry of the isoxazolidines 102. From the condensation of the nitrone 101 and methyl acrylate, two possible regio isomers could be produced, the 3'-methoxycarbonyl (i.e. 102a and 102b) and the 4'-methoxycarbonyl isomers (i.e. 102c and 102d) as shown in Scheme III-2. Only relative geometries are intended in the depictions.

In Dagne's studies, only three of these isomers were reported: 102b, 102c and 102d. The structure assignments were based on $^1$H NMR analysis which displayed three N-methyl peaks and three O-methyl peaks.\textsuperscript{19} He also isolated the three isomers by silica gel chromatography and identified them by combination of $^1$H NMR and mass spectral analysis. Besides the discussion of the NMR assignments, Dagne also assigned the 4'-methoxycarbonyl isomers by mass spectral analysis. The major evidence is the fragment ion at m/z 161 which is the residue derived from the 5-membered ring of the 4'-methoxycarbonyl isomers. Dagne also assigned the fragment ion at m/z 119 to Py-C≡N+CH$_3$ (Scheme III-3). However, in our studies, GC-EIMS (Fig. III-6) analysis of the reaction mixture from the condensation of the nitrone 101 and methyl acrylate showed four peaks with the same required parent ion at m/z 222 corresponding to C$_{11}$H$_{14}$N$_2$O$_3$. The retention times of the four peaks were 4.6, 4.7, 4.9 and 5.0 minutes. The mass spectra of these four peaks are quite similar, but all of the major fragment ions matched those reported by Dagne.\textsuperscript{19} These mass spectral data are summarized in Table III-1.

Scheme III-3  Fragment ions formed from 4'-methoxycarbonylisoxazolidine 102c/d

204
Fig. III-6. GC-EIMS data of the methoxycarboxylisoaxazolidine (102) isomers.
Table III-1. Mass spectra data of the products from the condensation of C-pyridyl-N-methylNitronate and methyl acrylate

<table>
<thead>
<tr>
<th>No</th>
<th>RT (min)</th>
<th>%</th>
<th>Compd</th>
<th>m/z (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.6</td>
<td>14.4</td>
<td>\textbf{102c}</td>
<td>222 (20), 191 (5), 161 (5), 144 (12), 136 (45), 135 (100), 119 (100), 92 (6), 78 (15)</td>
</tr>
<tr>
<td>2</td>
<td>4.7</td>
<td>22.5</td>
<td>\textbf{102d}</td>
<td>222 (30), 191 (37), 163 (15), 161 (27), 144 (25), 135 (100), 119 (56), 78 (20)</td>
</tr>
<tr>
<td>3</td>
<td>4.9</td>
<td>27.0</td>
<td>\textbf{102a}</td>
<td>222 (56), 163 (11), 144 (35), 135 (100), 119 (25), 84 (18), 78 (20)</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>36.1</td>
<td>\textbf{102b}</td>
<td>222 (30), 163 (28), 144 (40), 135 (100), 119 (47), 84 (16), 78 (27)</td>
</tr>
</tbody>
</table>

In an effort to assign the structures to these four isomers, a major consideration was stability. Apparently, the cis-4'-isomer (\textbf{102c}) should be the most unstable because of hindrance. On the other hand, the trans-3'-isomer should be the most stable. Therefore, the peak at 4.6 minutes with the lowest abundance (14.4\%) is tentatively assigned as \textbf{102c} and the peak at 5.0 minutes with the highest abundance (36.1\%) as \textbf{102b}. For the same reason, the peak at 4.7 minutes (22.5\%) is assigned as \textbf{102d} and the peak at 4.9 minutes (27.0\%) as \textbf{102a}. The mass spectral data also support these assignments. Dagne has assigned the fragment ion at m/z 161 to \textbf{102c/d} (Figure III-6a and III-6b). The fragment ion at m/z 119, which occurs in relatively higher abundance for the 4'-isomers than the 3'-isomers as reported by Dagne (93\% vs 50\%),\textsuperscript{19} showed a high abundance in Figure III-6c which is assigned as \textbf{102d}. On the other hand, the fragment ion at m/z 84, which was not reported by Dagne but showed up only in our mass spectra (Figure III-6d and III-6e), should be derived from the 3'-isomer (Scheme III-4).
The other evidence to support our assignments is the ion at m/z 191 which only appeared in the spectra shown in Figure III-6b and III-6c but not III-6d and III-6e. It is due to loss of 31 mass units, a methoxyl group, from the molecular ion. Due to the steric hindrance in the 4'-isomers, the loss of the CH$_3$O· moiety may help to release steric strain (Scheme III-5). Figure III-6c showed a high abundance of the m/z 191 ion which indicates that the trans-isomer of 4'-isoxazolidine 102d can more easily lose than the cis-isomer 102c.

The thermal stabilities of the isoxazolidine isomers also are of interest. Besides the above mentioned four isoxazolidine isomers 102a-d, the TIC shown in Figure III-6a indicates two other major peaks. One is at a retention time of 1.6 minutes with a parent ion at m/z (%) 120 (100) and fragment ions at m/z (%) 119 (93), 105 (10), 92 (17), 79 (23) and 78 (35). The other is at a retention time of 4.0 minutes with a parent ion at m/z (%) 136 (55) and fragment ions at m/z (%) 135 (100), 119 (10), 108 (15), 106 (10), 92 (10), 80 (25) and 78 (23). The retention time and the mass spectrum of the later compound were identical with the starting material 101. This was unexpected since the TLC showed no starting material. Apparently, under GC conditions, the isoxazolidine products are unstable and undergo thermolysis to generate the nitrone 101 (Scheme III-6).
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

Scheme III-6 Thermolysis of the isoxazolidine 102

**Scheme III-7** Proposed fragmentation of N-methyl-C-(3-pyridyl)enamine 103

The second peak observed at a retention time of 1.6 minutes in the TIC of this reaction mixture is likely to be the imine 103 based on the mass spectrum (Scheme III-7). The parent ion at m/z 120 could be C₇H₆N₂ corresponding to N-methyl-C-(3-pyridyl)enamine is the reduction product of the nitrone 101. As displayed in Scheme III-7, the fragment ion at m/z 119 is due to the ion PyC≡N+CH₃ (vi), which has lost one hydrogen from the methylene position of the parent ion i; m/z 105 corresponds to vii, which has lost one molecule of methane from the parent ion i; m/z 92 corresponds to the pyridylmethene ion iii, which has lost CH₂=N⁺; m/z 78 corresponds to the pyridyl ion iv and m/z 79 corresponds to the protonated pyridyl ion v. The formation of 103 under GC-EIMS conditions is similar to other reports on the reduction of tertiary amine N-
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

oxide type systems to tertiary amines. Daghe has reported that (S)-cotinine N-oxide (25) undergoes reduction to (S)-cotinine (3) in the mass spectral analysis. The detection of 101 and 103 by GC-EIMS indicated the thermal instability of isoxazolidine isomers 102.

The mixture of isoxazolidine isomers 102 underwent hydrogenation catalyzed by palladium on carbon. Figure III-7 shows the GC-EIMS analysis of the hydrogenation products. The mass spectrum of the new peak with a retention time 5.2 minutes was identical to the expected product 3'-hydroxycotinine (19). The ¹H NMR data of the recrystallized product from the hydrogenation reaction showed two signals assigned to N-methyl protons: one signal is at δ 2.64, which should be due to the cis-3'-hydroxycotinine; the other is at δ 2.76, which should be due to the trans-3'-hydroxycotinine. The ratio of these two isomers was 9:1. After a second recrystallization, the ¹H NMR spectrum showed the purified product to be >99% cis-3'-hydroxycotinine (19b/c). The GC-EIMS spectrum of this twice recrystallized product gave only one peak with a retention time of 5.3 minutes and a mass spectrum with a parent ion (C₁₀H₁₂N₂O₂) at m/z (%): 192 (72), the molecular formula of 19. The major fragment ions are at m/z (%): 174 (5), 163 (5), 148 (5), 135 (32), 119 (20), 114 (20), 106 (100), 93 (35), 79 (33). This mass spectrum is identical to the published spectrum of cis-3'-hydroxycotinine.

The hydrogenation reaction pathway of the of 3'-methoxycarbonylisoxazolidines, for example, the trans-3'-methoxycarbonylisoxazolidine 102b, involves initial cleavage of the weak nitrogen-oxygen bond in the isoxazolidine ring to form the corresponding α-hydroxymethyl ester 104b, which spontaneously cyclizes with loss of the methoxyl moiety to form the corresponding lactam 19b (Scheme III-8). The hydrogenation step should retain the relative configurations at C3' and C5'. However, from the subsequent rotation of the C3'-C4' bond, the trans-3'-methoxycarbonylisoxazolidine 102b gives cis-3'-hydroxycotinine (19b) only. The cis-3'-methoxycarbonylisoxazolidine 102a gives trans-3'-hydroxycotinine (19a).
Fig. III-7. GC-EIMS data of hydrogenation products of the methoxycarboxylisoxazolidines (102). (a) TIC tracing. Mass spectrum of: (b) 102c; (c) 102d; (d) 102a, (e) 19b/c.
Attempts to rationalize quantitative relationships between the ratios of these isoxazolidine isomers and the 3'-hydroxycotinine isomers suggest that some of 102a is converted to cis-3'-hydroxycotinine (19b). An alternative ring opening pathway, similar to what happens with 5'-hydroxypyrrolinone 52 and 5'-hydroxycotinine (20), might happen in the hydrogenation reaction of 102a. Since this isomer possesses more steric strain energy it may undergo ring opening to the species 106, a process which results in loss of chirality at C3'. The ketone in 106 then is reduced to a hydroxyl group by the hydrogenation reaction to form cis-3'-hydroxycotinine (Scheme III-9). As the overall result, combined with the hydrogenation product of 102b, the hydrogenation of the two 3'-isoxazolidines, 102a and 102b, gave the products as 9:1 ratio of cis- to trans-3'-hydroxycotinine (19b : 19a).
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

Scheme III-9 Formation of cis-3'-hydroxycotinine (19b) from the cis-3'-methoxycarbonylisoazolidine 102a

The 4'-methoxycarbonylisoazolidines 102c and 102d will not produce 3'-hydroxycotinine. Hydrogenation of these isomers should give lactams 105 (Scheme III-10). The GC-EIMS analysis of the hydrogenation products suggested that the two 4'-methoxycarbonylisoazolidines 102c and 102d were either stable to reduction or underwent reaction to undetected products. Lactam formation (Scheme III-10) would not be expected to occur.

Scheme III-10 Proposed hydrogenation of the 4'-methoxycarbonylisoazolidines 102c/d

3.2.4.2. trans-3'-Hydroxycotinine (19a/d)

It is very easy to prepare the trans-isomers 19a/d from the cis-3'-hydroxycotinines (19b/c). Following the published method (Scheme III-11),

212
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

19b, for example, was converted to its mesylate derivative 107 with retention of configuration at C3' and C5'. Compound 107 was subsequently converted to the corresponding acetate 108. Since this conversion is an SN2 reaction, the configuration at carbon-3' would be expected to undergo inversion. The trans-3'-acetoxytocotinine (108) subsequently was hydrolyzed under basic conditions to give the expected trans-3'-hydroxytocotinine (19a). After recrystallization, the 1H NMR spectrum showed only one peak due to the signal for the N-methyl protons at δ 2.76 (the trans-isomer). No signal at δ 2.64 (corresponding to the cis-isomer) was found. The GC retention time of the trans-isomers 19a/d was 5.5 minutes, the same as that of the cis-isomers 19b/c. Furthermore, the EIMS of this diastereomer gave identical characteristics to that of the reported mass spectrum.\(^{18}\)

![Scheme III-11 Synthesis of trans-3'-hydroxytocotinine (19a/d)](image_url)

213
Chapter 3  in Vivo Studies on the Metabolism of β-Nicotyrine

3.2.5. Characterization of the Structure of the Hydroxycotinine Metabolite of β-Nicotyrine

With the availability of 19a/d and 19b/c, we turned our attention to the hydroxycotinine metabolite derived from β-nicotyrine. The GC-EI mass spectrum of this unknown metabolite proved to be identical to the spectrum observed for synthetic 19a/d and 19b/c (Fig. III-8). When spiked with either the cis- or trans-3'-hydroxycotinine standard, the resulting tracing showed the expected increase in the intensity of the unknown metabolic peak. Analogous results were obtained by HPLC diodé array analyses (Fig. III-9). Consequently, we could assign the regiostructure of this metabolite as a 3'-hydroxycotinine. The same compound was present in the urine of β-nicotyrine treated mice (Fig III-2).

Further evidence was sought to help establish the structure of this unknown metabolite. Jacob et al. have reported a silyl ether derivatization procedure that leads to improved chromatographic properties of the trans-3'-hydroxycotinine extracted from smokers’ urine. They found that the tert-butyldimethylsilyl ether 109 of 3'-hydroxycotinine, a relatively high molecular weight derivative, produces a major ion at m/z 249 with electron ionization due to loss of the tert-butyl group from the parent ion. This silyl ether derivative proved to be excellent for selected ion chromatography (SIC). Therefore, we decided to employ this silyl ether derivative to analyze the β-nicotyrine metabolite. The tert-butyldimethylsilyl ether was prepared from both of the two synthetic standard 3'-hydroxycotinines, trans 19a/d and cis 19b/c, and the unknown urinary metabolite derived from β-nicotyrine. All of the reaction products were analyzed by GC-EIMS (Scheme III-12).

\[
\begin{align*}
\text{Pyridine} & \quad + \quad \text{ClSi}^+ \\
\text{19} & \quad \text{DMAA} \\
\rightarrow & \quad \text{109}
\end{align*}
\]

**Scheme III-12** Synthesis of silyl ether 109 of 3'-hydroxycotinine (19)

214
Fig. III-8. GC-EIMS data of 3'-hydroxycotinine. (a) TIC od synthetic 19a/d; (b) TIC of synthetic 19b/c; (c) SIC of ion at m/z 192 from unknown β-nicotyrine metabolite in rabbit's urine. Mass spectra of: (d) Synthetic 19a/d; (e) Synthetic 19b/c; (f) The peak in (c).
Fig. III-9. UV absorption of synthetic 3'-hydroxycotinine (19b/c, dotted line) and unknown urinary metabolite derived from β-Nicotyrine (solid line).
Fig. III-10. GC-El SIC (m/z 249) and spectra of silyl ether 109 derived from:
(a) Synthetic 19a/d; (b) Urinary metabolic of β-nicotyrine; (c) Synthetic 19a/d;
(d) Urinary metabolite derived from β-nicotyrine.
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

Scheme III-13 Fragmentation of the dimethyl-\textit{tert}-butylsilyl ether of 3'-hydroxycotinine 109

The GC-EIMS analysis (Fig. III-10) of the reaction mixture with the synthetic 3'-hydroxycotinines showed that the starting materials had totally disappeared after one hour. A new peak with a retention time at 9.6 minutes appeared. The mass spectrum of this new peak gave major fragment ions at m/z (%): 291 (4), 249 (100), 175 (2), 144 (38), 115 (7), 88 (4) and 75 (35). The molecular ion of 109 at m/z 306 (I) was not detected in the mass spectrum. As we discussed above, 109 is a molecule with high molecular weight, it undergoes fragmentation to lose the \textit{tert}-butyl group or a methyl group from the parent radical cation to form a silyl cation under electronic ionization impact. The ion at m/z 291 (II) is due to loss of a methyl group, and the ion at m/z 249 (III) is due to loss of the \textit{tert}-butyl group. The ion at m/z 175 is due to the ion IV, i.e. loss of the \textit{tert}-butyldimethylsiloxyl group. The ion at m/z 144 is due to the ion V resulting from loss of the Py-C+H=CH₂ moiety from ion III. The ion at m/z 115 is due to the \textit{tert}-butyldimethylsilyl ion VI. The ion at m/z 75 is due to the dimethylsilanol ion VII. The ion at m/z 88 can not be assigned (Scheme III-13).
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

This ion at m/z 249 was chosen for the selective ion chromatography (SIC) analysis. The SIC chromatograms at m/z 249 from the silyl ethers derived from the synthetic trans 19a/d and cis 19b/c showed only one peak at the same retention time, 9.6 minutes. Full scans gave identical mass spectra. The urinary sample was treated and analyzed in the same way. The SIC analysis of GC-EIMS (m/z 249) gave an identical spectrum to those of the synthetic standards trans 19a/d and cis 19b/c. These results confirmed that the major unknown metabolite derived from β-nicotyrine in rabbits was a 3'-hydroxycotinine.

3.2.6. Stereochemistry of 3'-Hydroxycotinine

Although the results with the silyl ethers confirmed the urinary metabolite of β-nicotyrine as a 3'-hydroxycotinine, the relative and absolute stereochemistries remained to be established. It is well-known that the stereochemistry of 3'-hydroxycotinine derived from (S)-nicotine and (S)-cotinine is (3'R,5'S)-trans-3'-hydroxycotinine (19a). The available evidence, however, did not identify the stereochemistry of the 3'-hydroxycotinine derived from β-nicotyrine. Neither HPLC nor GC analysis distinguished between the two diastereomers of 3'-hydroxycotinine. The silyl ether derivative should retain the configuration at the C3' and C5' positions, however, unfortunately HPLC and GC did not resolve these ethers, 109a/d and 109b/c.

Scheme III-14  Chloride derivatization of cis- and trans-3'-hydroxycotinine
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

Jacob et al. reported another GC-EIMS analytical method to identify the stereochemistry of 3'-hydroxycoctinine derived from (S)-nicotine metabolism.\textsuperscript{16} The two diastereomers of 3'-hydroxycoctinine could be converted to their 3'-chloro derivatives, 3'-chlorococctinine (110). The resulting diastereomers, 110a/d and 110b/c, can be resolved by GC analysis. In this way we could identify the relative stereochemistry of 3'-hydroxycoctinine derived from β-nicotyrine. The chlorination of 3'-hydroxycoctinine to 110 may be carried out in different ways. Dagne has reported several different methods to make 3'-chlorococctinine.\textsuperscript{19} The easiest method is the treatment of 3'-hydroxycoctinine with thionyl chloride. In this reaction, the configuration of the stereogenic center at the 3'-position will be inverted. The chloro product from the cis-isomer 19b, for example, will be the trans-3'-chlorococctinine 110b while the chloro product from the trans-isomer 19a will be cis-3'-chlorococctinine 110a. The reaction pathway proceeds through the chlorosulfonyloxy intermediates 111a or 111b. The chlorosulfonyloxy group is displaced by chloride anion to form the 3'-chlorococctinine 110. This step is an S\textsubscript{N}2 reaction, therefore, the configuration at C-3' is inverted (Scheme III-14).

The chlorination of 3'-hydroxycoctinine to 3'-chlorococctinine was carried out on both the synthetic cis- and trans-3'-hydroxycoctinine standards, as well as the rabbit urinary metabolite derived from β-nicotyrine. The chloro derivative of cis-3'-hydroxycoctinine (110b/c) showed a peak at a retention time of 5.4 minutes while the chloro derivative of trans-3'-hydroxycoctinine (110a/d) showed a peak at a retention time of 5.6 minutes. Both 110a/d and 110b/c gave identical mass spectra (Fig. III-11). The parent ion and the major fragment ions were observed at m/z (%): 212 (9), 210 (28), 175 (14), 134 (12), 132 (35), 118 (100), 91 (16), 78 (10). The molecular formula of 110 is C\textsubscript{10}H\textsubscript{11}ClN\textsubscript{2}O and the molecular weight is 210 (\textsuperscript{35}Cl) and 212 (\textsuperscript{37}Cl). Since the abundance of \textsuperscript{35}Cl and \textsuperscript{37}Cl is 3:1, the abundance of 210 to 212 is also 3:1. The fragmentation pattern is summarized in Scheme III-15. The molecular ion i loses a chlorine atom to form the ion at m/z 175 (ii). Loss of the O=C=N-CH\textsubscript{3} moiety from i leads to fragment ions at m/z 153 and 155 (iii) with the expected ratio of 3:1. Loss of the pyridyl group form i results in fragment ion at m/z 132 and 134 (iv), again with the expected ratio of 3:1. The base peak at m/z 118 is
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine assigned to the ion v which formed from ii by loss of the O=C=N-CH₃ moiety or from iii by loss of chlorine. The ion at m/z 91 is due to ion vi and m/z 78 is the pyridyl ion vii.

Scheme III-15 Mass fragmentation of 3'chlorocotinine 110

The minor peaks observed in each of the synthetic standards and the urinary metabolite may be due to the presence of small amounts of the other diastereomer of the 3'-hydroxycotinines. After three recrystallizations of the synthetic 3'-hydroxycotinines, 19a/d and 19b/c, the 1H NMR showed the purity of each compound was > 99%. However, the minor peak showing up in each 3'-chlorocotinine still was observed. Consequently these minor peaks may be formed from the corresponding 3'-hydroxycotinine due to retention of configuration at carbon-3' during the chlorination reaction. One possible source of the minor component may be via an intramolecular displacement reaction (Scheme III-16). As reported by Dagne, the chlorine in the chlorosulfite may attack the 3'-carbon atom from the same side with the chlorosulfite group resulting in retention of configuration. The other possibility is an $S_{N}1$ process since the chlorosulfonyloxy group is a relatively good leaving group.
Fig. III-11. GC-EI SIC (m/z 210) of 3'-chlorocotinine (110) derived from: (a) Synthetic cis-3'-hydroxycotinine (19b/c); (b) Synthetic trans-3'-hydroxycotinine (19a/d); Mass spectra of 3'-Chlorocotinine derived from: (c) Synthetic cis-3'-hydroxycotinine (19b/c); (d) Synthetic trans-3'-hydroxycotinine (19a/d).
Scheme III-16 Proposed intramolecular substitution in the chlorination reaction

The chloro derivative obtained from the urinary metabolite derived from β-nicotyamine gave a peak with a retention time at 5.4 minutes with an identical mass spectrum to the spectra of the two synthetic standards. This tracing is identical with that of the chloro compound from synthetic cis-3′-hydroxycotinine and established that the relative stereochemistry of the major urinary metabolite derived from β-nicotyamine is cis (Fig. III-15).

It was of interest that the stereochemistry of the 3′-hydroxycotinine derived from β-nicotyamine is the cis-isomer 19b/c. We decided to examine more closely the in vivo metabolic and stereochemical composition of the 3′-hydroxycotinines derived from (S)-nicotine and (S)-cotinine. We treated the urine samples obtained from a smoker and rabbits treated i.p. with (S)-nicotine and (S)-cotinine in the same way to generate the chloro derivatives of the urinary metabolites derived from (S)-nicotine and (S)-cotinine. The SIC tracings at m/z 210 from the (S)-cotinine treated rabbit samples showed a major peak at retention time 5.7 minutes and a minor peak at 5.5 minutes; the ratio of the peaks was 9:1. From the (S)-nicotine treated rabbit urinary samples, the major peak appeared at a retention time of 5.7 minutes and the minor peak at 5.5 minutes; the ratio was 8:2. From the smoker’s urine, the same peaks were observed; the ratio was 7:3 (Fig. III-12). All of these peaks gave mass spectra
that were identical to the synthetic 110s. These data confirmed that the configuration of the 3'-hydroxycotinine derived from (S)-cotinine is \textit{trans} (19a). In the case of (S)-nicotine, however, the \textit{cis}-isomer 19b was also present although the \textit{trans}-isomer was still the major rabbit urinary metabolite. A similar result was found in the smoker's urine. The major isomer was the \textit{trans} 19a while about 20-30\% of the \textit{cis} 19b was present. The significance of these results awaits further studies.

3.2.7. Mechanistic pathway Leading to the Formation of \textit{cis}-3'-Hydroxycotinine from \textit{β}-Nicotyrine

The relative configuration of the 3'-hydroxycotinine metabolites derived from \textit{β}-nicotyrine is different from that derived from (S)-nicotine and (S)-cotinine. The metabolic pathway leading to the formation of the \textit{cis}-3'-hydroxycotinine from \textit{β}-nicotyrine is not obvious. One possibility was that \textit{cis}-3'-hydroxycotinine might be derived from the in vivo inversion of \textit{trans}-3'-hydroxycotinine. We therefore examined the in vivo metabolism of \textit{trans}-3'-hydroxycotinine in the rabbit. The urine sample obtained from a rabbit treated i.p. with \textit{trans}-3'-hydroxycotinine was extracted and chlorinated as discussed before. The SIC (m/z 210) analysis of standard \textit{trans}-3'-hydroxycotinine and the urine sample gave identical data that indicated only the \textit{trans}-3'-hydroxycotinine was present in the urine (Fig. III-13).

In vitro studies had established that \textit{β}-nicotyrine is oxidized in a reaction catalyzed by cytochrome P-450 to give the pyrrolineones 48 and 51 and that 48 and 51 subsequently are converted to 5'-hydroxycotinine, an isomer of 3'-hydroxycotinine. This in vitro pathway suggested that \textit{cis}-3'-hydroxycotinine may also be formed from the pyrrolineones 48 and/or 51. The general instability of 48 and 51 precluded the possibility of performing an in vivo experiment with these compounds. However, the masked pyrroline derivative, the 2-acetoxo-1-methyl-2-(3-pyridyl)pyrrole (74) was available. We had established that 74 undergoes chemical hydrolysis to the pyrrolineones 48 and 51 in aqueous medium and therefore serves as a source of 48 and 51 that can be used in in vivo studies on the formation of \textit{cis}-3'-hydroxycotinine (Scheme III-17).
Fig. III-12. GC-EIMS SIC (m/z 210) of 3'-chlorocotinine (110) derived from:
(a) Synthetic trans-3'-hydroxycotinine (19a/d); (b) (S)-Cotinine (3); (c) (S)-Nicotine (1); (d) Smoker's urine.
Fig. III-13. GC-EIMS SIC (m/z 210) of 3'-chlorocotinine (110) derived from: (a) Synthetic trans-3'-hydroxycotinine (19a/d); (b) Urine of rabbit treated i.p. with trans-3'-hydroxycotinine.
Fig. III-14. HPLC tracing of urine samples obtained from: (a) Untreated rabbit; (b) Rabbit treated i.p. with 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74)
Scheme III-17 In vivo metabolism of the 2'-acetoxypryroline 74

HPLC UV-diode array analysis of the urine sample obtained from a rabbit treated with 74 established that this compound is converted to cis-3'-hydroxyxocotinine (Fig. III-14). Besides background peaks, a major peak appeared at a retention time of 7 minutes which could be assigned to 3'-hydroxyxocotinine. Two minor peaks were also present. One, at retention time 8.5 minutes, was assigned to 5'-hydroxyxocotinine (20) while the second, at retention time 11.5 minutes, was assigned to the 5'-hydroxyxoryrolinone 52. Those assignments were based on comparison of HPLC retention times and UV spectra with those of synthetic standards. The GC-EIMS analysis of the same urine sample showed a major peak with a retention time at 5.3 minutes, which corresponded to that of the cis- and trans-3'-hydroxyxocotinines, and an identical mass spectrum with that of the 3'-hydroxyxocotinines. After treatment with SOCl₂, the chloro derivative of this urinary metabolite was analyzed by GC-EIMS. The SIC tracing of m/z 210 of this reaction mixture showed a major peak (>95%) at a retention time 5.4 minutes, as expected for the trans-3'-chloro derivative 110 of cis-3'-hydroxyxocotinine, and a minor peak (<5%) at 5.6 minutes, due to the cis-3'-chloro derivative of trans-3'-hydroxyxocotinine (Fig. III-15). Both of the peaks gave identical mass spectra which also was identical with that observed with synthetic 110. Therefore, the cis-stereochemistry of this 3'-hydroxyxocotinine metabolite was established. This result provided strong support for the proposal that the pyrrolinones 48 and 51 serve as in vivo precursors of the cis-3'-hydroxyxocotinine metabolite of β-nicotyrine. Based on these results we
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

propose that β-nicotyrine is first oxidized in vivo to the pyrrolinones 48 and 51 which then are converted to cis-3'-hydroxycotinine.

The exact sequence leading from 48 and 51 to 19b/c remains to be established. Formally the conversion involves the net addition of a mole of water to the pyrrolinones. We already had established that the Δ^4.5-pyrrolinone 48 undergoes hydrolysis to yield 5'-hydroxycotinine (20). However, one would expect the Δ^3.4-pyrrolinone 51 would hydrolyze to 4'-hydroxycotinine (77, Scheme III-18). From our prior studies on the hydrolysis of the 2'-acetoxyprrole derivative 74, only 5'-hydroxycotinine (20) was produced. Therefore, we expect that cis-3'-hydroxycotinine (19b/c) is formed from the pyrrolinones via an alternative pathway. One possible pathway that could lead to cis-3'-hydroxycotinine from β-nicotyrine is summarized in Scheme III-19. Similar to that which was summarized in Scheme II-2, the 3'-hydroxy-Δ^4.5-pyrrolinone 55 could be an antioxidative product of the pyrrolinones 48 and 51 through a free radical pathway. Subsequently, 55 could be reduced to form cis-3'-hydroxycotinine 19b/c.

Scheme III-18  Hydrolysis of the pyrrolinones 48 and 51
Fig. III-15. GC-EIMS SIC (m/z 210) of 3'-chlorocotinines (110) derived from: (a) Synthetic cis-3'-hydroxycotinine (19b/c); (b) β-Nicotyrine (2); (c) The urinary metabolite of 2-acetoxy-1-methyl-2-(3-pyridyl)pyrrole (74).
The proposed pathway includes the carbon-carbon double bond reduction of 55 to 19b/c. Although not common, metabolic reductions of \(\alpha,\beta\)-unsaturated ketones to the saturated ketones have been reported. Steriodal drugs often fall into this class. For example, the major plasma and urinary metabolite of norethindrone (112) in women is the 3\(\beta,5\beta\)-tetrahydro derivative 113.\(^{21}\) Another example is the in vivo metabolism of Cl-966 (114). Bjorge et al. reported that the in vivo metabolism of Cl-966 in rats included the reductive conversion to the piperidine analog 115 (Scheme III-20).\(^{22}\) These examples encouraged us to consider the proposed reduction pathway in further detail.
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

\[
\begin{align*}
112 & \rightarrow 113 \\
\text{COOH} & \quad \text{CF}_3 \\
114 & \rightarrow 115 \\
\end{align*}
\]

Scheme III-20 Examples of in vivo reduction of C-C double bonds

In order to evaluate this proposal, the in vivo metabolism of the 3'-hydroxypyrrolineone 55 needed to be examined. Unfortunately, this compound was not available at the time. However, its isomer, the 5'-hydroxypyrrolineone 52 was available. In vivo reduction of 52 to the corresponding 5'-hydroxycotinine (20) would provide indirect evidence in support of the proposed reduction pathway. The in vivo metabolism of 52 was examined in rabbits. HPLC-diode array analysis (Fig. III-16) of the urine sample obtained from a rabbit treated with 52 showed a major peak at retention time 8 minutes which can be assigned to 5'-hydroxycotinine (20) by comparison of the HPLC retention time and UV spectrum with synthetic standard. GC-EIMS (Fig. III-17) analysis of the same urine extract showed a major metabolite peak with a retention time at 5.7 minutes and a minor peak at 5.5 minutes. The retention time and the mass spectrum of the peak at 5.5 minutes were identical to that observed with synthetic 5'-hydroxycotinine (20). The retention time and the mass spectrum of the major peak at 5.7 minutes were identical to those observed with the ring opened isomer 21 of 20 as shown and discussed in Chapter 2 (Scheme III-21). These data confirmed that the major urinary metabolite of the 5'-hydroxypyrrolineone 52 is 5'-hydroxycotinine 20. This result greatly supported our proposed carbon-carbon double bond reduction pathway for the formation of cis-3'-hydroxycotinine.
Fig. III-16. HPLC tracing of urine samples obtained from: (a) Untreated rabbit; (b) Rabbit i.p. treated with 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrrolin-2-one (52).
Fig. III-17. GC-EIMS SIC (m/z 106) derived from: (a) Untreated rabbit urine; (b) Synthetic 5'-hydroxycotinine (20); (c) Urine from 5-hydroxy-1-methyl-(3-pyridyl)-3-pyrroline-2-one (52) treated rabbit; Mass spectra: (d) & (e) Synthetic 21 and 20; (f) & (g) 21 & 20 derived from 20 i.p. treated rabbit urine.
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

Scheme III-21  In vivo reduction of 5'-hydroxypryrrolinone 52

The cis-stereochemistry of the 3'-hydroxycotinine found as the urinary metabolite of β-nicotyrine also may be consistent with the proposed reduction pathway since reduction of the 4',5'-double bond present in 55 would be expected to yield the cis geometry on the basis of steric considerations.

Scheme III-22  Proposed formation mechanism of cis-3'-hydroxycotinine from β-nicotyrine

The proposed in vivo pathway leading to the formation of cis-3'-hydroxycotinine from β-nicotyrine is summarized in Scheme III-22. β-Nicotyrine is first oxidized to the pyrrolinones 48 and/or 51 by a cytochrome P-450 catalyzed reaction. The pyrrolinones undergo further oxidation to the 3'-hydroxypryrrolinone 55 via a free radical process possibly involving the hydroxyperoxide 54. The 3'-hydroxypryrrolinone 55 then is reduced to generate the corresponding cis-3'-hydroxycotinine.
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

3.2.8. Metabolic Relationship Between (S)-Nicotine and β-Nicotyrine

The characterization of cis-3'-hydroxycotinine (19b/c) as the major in vivo metabolite of β-nicotyrine prompted further consideration of the in vivo metabolism of (S)-nicotine. (3'R, 5'S)-trans-3'-Hydroxycotinine (19a) is the major metabolite of (S)-nicotine and (S)-cotinine. This metabolite has been detected in the urine of rats treated with (S)-nicotine and in the urine of the guinea pig and rhesus monkey treated with (S)-cotinine. It also has been reported that 19a has been detected in smokers' urine. Only one report suggests that 19b may be a metabolite of (S)-nicotine. Voncken et al. reported that cis-3'-hydroxycotinine was separated and identified by GC-MS as an (S)-nicotine metabolite in the urine of rats and hamsters dosed with (S)-nicotine as well as in smokers' urine.

Since it is known that cis-3'-hydroxycotinine is derived from β-nicotyrine and that β-nicotyrine may be derived from the (S)-nicotine iminium species, the possible formation of 19b from (S)-nicotine was of interest. As shown in Scheme III-23, (S)-nicotine may first be oxidized by cytochrome P-450 to its iminium intermediate 15, which may be in equilibrium with its conjugate base, the enamine 17. Liver aldehyde oxidase catalyzes the oxidation of 15/17 to (S)-cotinine which is further converted to trans-3'-hydroxycotinine (19a). This pathway has been established by Murphy and confirmed by Dagne and Peterson. In vivo studies on the metabolism of (S)-cotinine showed that 19a, but not 19b, is formed. Therefore, it may be concluded that 19a is derived from (S)-cotinine. In the lungs, owing to the absence of aldehyde oxidase, this pathway may not operate. Instead, the (S)-nicotine metabolic intermediate 15/17 might be oxidized to β-nicotyrine by an oxidase such as MAO-B as reported by Shigenaga. Then β-nicotyrine may be converted to 19b/c (Scheme III-23).
Fig. III-18 GC-EIMS data of β-nicotyrine standard and derived from (S)-nicotine metabolism: (a) TIC of synthetic β-nicotyrine; (b) SIC (m/z 158) of (S)-nicotine metabolite in rabbit’s urine; (c) Mass spectrum of synthetic β-nicotyrine; (d) Mass spectrum of the peak in (b).
Scheme III-23 Proposed biotransformation pathway of (S)-nicotine leading to 19a and 19b/c

Preliminary studies have provided some evidence to support this proposed pathway for (S)-nicotine. GC-EIMS analysis of the urine sample obtained from (S)-nicotine treated rabbits has documented the presence of β-nicotyrine. Figure III-18a is the TIC tracing of synthetic β-nicotyrine and III-18b is the ion chromatogram of m/z 158, the molecular ion of β-nicotyrine, obtained from the urine sample of a rabbit treated with (S)-nicotine. A peak with the same retention time as synthetic β-nicotyrine is present at 3.8 minutes. Figure III-18c is the mass spectrum of synthetic β-nicotyrine and III-18d is the mass spectrum of urine.
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine
spectrum of the peak shown in III-18b. The two mass spectra are very similar. This preliminary result needs further studies. The Future Work section is presented as a guide to future work in this area.

3.3. Summary of in Vivo Studies on the Metabolism of β-Nicotyrine

1. The in vivo studies on β-nicotyrine metabolism were carried on in mice and rabbits by following i.p. administration of β-nicotyrine. The 24 hour urine samples were collected and analyzed by GC-EIMS and HPLC UV-diode array techniques. The results with these two species were similar. Three metabolites were detected and identified by HPLC UV-diode array analysis. The two minor metabolites are 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) and 5'-hydroxycotinine (20). The major metabolite is cis-3'-hydroxycotinine (19b/c). The two in vitro metabolic intermediates, 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51), were not found.

2. The relative stereochemistry of the major in vivo metabolite, 3'-hydroxycotinine, is pure cis. The relative configuration was established by conversion to the known trans-3'-chlorocotinine.

3. The formation of cis-3'-hydroxycotinine has been proposed to proceed via the autoxidation of the unstable pyrrolinone metabolic intermediates 48 and 51 to 3-hydroxy-1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (55) followed by the reduction of 55 to 19b/c. This proposed metabolic pathway has been investigated by in vivo model studies employing 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74) and 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52). 2-Acetoxy-1-methyl-5-(3-pyridyl)-pyrrole (74) is the masked derivative of the pyrrolinones 48 and 51 which undergoes hydrolysis to the two pyrrolinones in aqueous medium. The major in vivo metabolite of 74 was cis-3'-hydroxycotinine (19b/c). The proposed reductive pathway is supported by results from in vivo studies on 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) which undergoes metabolic reduction to 5'-hydroxycotinine (20).
3.4. Future Work

In order to examine the proposed pathway leading to the formation of cis-3'-hydroxycotinine from β-nicotyrine, the following studies should be pursued:

1. Metabolic studies with a synthetic sample of the 3'-hydroxypyrrolinone 55 could demonstrate its conversion to cis-3'-hydroxycotinine (19b/c). If such an experiment were successful, efforts to characterize the putative reductase leading to 19b/c should be pursued.

2. Another interesting area concerns the relationship between (S)-nicotine and β-nicotyrine. The preliminary evidence for the formation of cis-3'-hydroxycotinine 19b from (S)-nicotine requires confirmation. One approach to this would involve in vivo studies with the (S)-nicotine iminium species 15. If 19b/c were identified as an in vivo metabolite of 15, then the proposed metabolic pathway (S)-nicotine --> (S)-nicotine iminium 15 --> β-nicotyrine --> cis-3'-hydroxycotinine 19b/c would be supported.

3. The absolute configuration of metabolically produced 3'-hydroxycotinine needs to be determined. Unlike (S)-nicotine, β-nicotyrine is an achiral molecule. Therefore the absolute configuration at the two stereocenters remains to be determined. Such information could aid in attempts to characterize the metabolic conversion of β-nicotyrine to cis-3'-hydroxycotinine.

3.5. Materials and Methods

3.5.1. Chemicals

Synthetic reactions were carried out under a nitrogen atmosphere. All of the starting materials for the syntheses were purchased from Aldrich Chemical Co. (Milwaukee, WI). Reactions requiring anhydrous conditions were carried out under nitrogen atmosphere. (3'R, 5'S)-trans-3'-Hydroxycotinine perchlorate was a gift of Dr. Peyton Jacob III (San Francisco General Hospital, CA). The
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

HPLC solvents acetonitrile, triethylamine and acetic acid were purchased from the Fisher Scientific Company (Pittsburg, PA). The water was purified by Milli-Q Plus Ultra-Low Organics Water system made by Millipore Corporation (Bedford, MA).

3.5.2. Analytical Instruments

Melting points were determined on a Thomas Hoover melting point apparatus and as uncorrected. 1H NMR spectra were recorded on a Brucker WP 270 MHz instrument linked to an Aspect 2000 computer. Chemical shifts are reported in parts per million (ppm) relative to Me₄Si as an internal standard in CDCl₃ or to 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid, sodium salt (TSP) in D₂O and CD₃OD. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or b (broad). GC-EI mass spectral analyses were performed on an HP 5970 mass selective detector linked to an HP 5890 GC. The GC column was an HP-1 (100% dimethylpolysiloxane) capillary column, 12.5 m x 0.2 mm x 0.33 μm. The GC temperature program for the synthetic samples, organic solvent extracts of the urine samples was Program 1 in which the oven temperature was held at 100 °C for 1 minute after injection and then temperature-programmed to 275 °C at a linear rate of 25 °C/minute. The final temperature was held for 2 minutes. For the stereochemistry studies of 3'-hydroxycotinine, the GC temperature program was Program 2 in which the oven temperature was held at 70 °C for 1 minute after injection and then temperature-programmed to 270 °C at a linear rate of 20 °C/minute. The final temperature was held for 2 minutes. HPLC UV-diode array analysis were performed on a system with two Beckman 114M pumps connected with a Beckman 421A controller to deliver the mobile phase. The HPLC was connected to a Hewlett-Packard 1040A Diode Array detector system. An Alltech Econosil, 10 μ particle size, 25 cm x 4.6 mm, C8 analytical column was employed and was eluted first with mobile phase A containing water, acetonitrile, acetic acid and triethylamine with a ratio of 90:10:0.5:0.1 (v/v) for 10 minutes, then, employing a 10 minutes ramp to 40:60 of acetonitrile to the above mobile phase A. After eluting for 10 minutes with this mixture, the mobile phase was ramped back to 100% A over 5 minutes and the elution was
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

continued for another 10 minutes. The total eluting time was 45 minutes. The flowrate was 1 mL/minute. The eluent was monitored simultaneously at 260 and 282 nm. Typical retention times were as follows: β-nicotyrine (2), 23 minutes; (S)-cotinine (3), 15 minutes, the 5'-hydroxypyrroline 52, 12 minutes; 5'-hydroxycotinine (20), 10 minutes, 3'-hydroxycotinine (19a and 19b), 7 minutes.

3.5.3. Animals

New Zealand White male rabbits (1.8-2.4 kg) and ICR male mice (20-30 g) were used for toxicity and in vivo metabolic studies of β-nicotyrine. The rabbits were also used in the in vivo metabolic studies of (S)-nicotine, (S)-cotinine, compound 52 and 74.

3.5.4. Syntheses

3.5.4.1. 3-(3-Pyridyl)-N-methylnitrone (101)

Pyridine-3-carboxaldehyde (100, 64 g, 0.6 mmol, 56.4 mL) was added to a solution of N-methylhydroxylamine hydrochloride (50 g, 0.6 mol) in 200 mL of absolute ethanol. The reaction mixture was stirred at room temperature for 3 hours and checked with thin layer chromatography (TLC, Silica gel GF-254, eluted with CHCl₃/MeOH, 10:1 as the mobile phase), which showed no more starting material 100 (Rf 0.65) left. Only one spot with a Rf value 0.74 showed on the TLC. A white solid formed which was filtered and the solvent of the mother liquid was removed by rotary evaporator. The combined solid was treated with saturated sodium carbonate and the resulting solution was extracted with CHCl₃. The extract was dried over potassium carbonate. After removing the solvent, the residue was recrystallized from chloroform/hexanes (2:1) to give 55.5 g (68%) of white crystalline material of C-(3-Pyridyl)-N-methylnitrone (101), m.p. 76-78 °C (Ref: 74-76 °C¹⁸). ¹H NMR (CDCl₃): δ 9.00 (1H, t, C2-H), 8.97 (1H, d, C6-H), 8.60 (1H, dt, C4-H), 7.45 (1H, s, vinyl), 7.36 (1H, m, C5-H), 3.95 (3H, s, N-Me); GC-EIMS retention time and m/z (%): 4.0 min; 136 (60%), 135 (100%), 119 (8%), 108 (15%), 106 (10%), 92 (10%), 80 (28%), 78 (25%).

242
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

3.5.4.2. 2-Methyl-3-(3-pyridyl)-5-methoxycarbonyl-isoxazolidine (102)

Methyl acrylate (124.3 g, 1.444 mol, 130 mL) was added into a flask equipped with a magnetic stirrer, reflux condenser and nitrogen introducer. C-(3-Pyridyl)-N-methylene trine (101, 25 g, 184 mmol) was added under stirring. The reaction mixture was heated in an oil-bath at temperature of 105 °C to reflux for 10 hours. The TLC (silica gel GF 254, CHCl₃ / MeOH 10 : 1) showed only one spot (Rᵢ 0.41) and no starting materials left. After cooling to room temperature, the excess methyl acrylate was removed from the reaction mixture by rotary evaporator. A dark brown residue was obtained and used as the starting material for the hydrogenation reaction directly. GC-EIMS found 5 major peaks with the retention times and m/z (%): 4.0 min; 136 (55%), 135 (100%), 119 (10%), 108, (15%), 106 (10%), 92 (10%), 80 (25%), 78 (20%); 4.6 min; 222 (20%), 191 (5%), 161 (5%), 144 (10%), 135 (100%), 136 (45%), 119 (12%), 109, (10%), 104 (10%), 78 (15%); 4.7 min; 222 (30%), 191 (37%), 163 (15%), 161 (27%), 144 (25%), 135 (100%), 119 (56%), 104 (15%), 78 (20%); 5.0 min; 222 (56%), 163 (11%), 144 (35%), 135 (100%), 119 (29%), 116 (18%), 106 (10%), 104 (10%), 84 (18%), 78 (20%); 5.1 min; 222 (30%), 205 (10%), 163 (28%), 161 (5%), 144 (30%), 135 (100%), 119 (47%), 116 (20%), 106 (10%), 104 (12%), 84 (16%), 78 (27%).

3.5.4.3. cis-3'-Hydroxycotinine (19b/c)

A solution of the crude products from the above reaction in 250 mL of absolute ethanol containing palladium on carbon (10%, 3 g) was hydrogenated under atmospheric pressure for 3 days with stirring. After stopping the reaction, the catalyst was filtered out through Celite and the solvent was removed by rotary evaporator. To the residue was added 150 mL of acetone. The resulting acetone solution was filtered to remove the insoluble materials. After removing the solvent, 17.4 g of a white crystalline material of cis-3'-hydroxycotinine (19b/c) were obtained after recrystallization from ethanol, m.p. 146-146.5 °C (Ref. 148-149 °C¹⁸). The overall yield was 55.2% for the two steps. ¹H NMR (CDCl₃):  8 8.55 (1H, d, C2-H), 8.53 (1H, t, C6-H), 7.86 (1H, dt, C4-H), 7.54 (1H, m, C3-H), 4.70 (1H, t, C4'-H), 4.60 (1H, t, C4'-H), 3.00 (1H, m, C3'-H), 2.64 (3H,
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine
s, N-Me), 1.82 (1H, m, C5'-H); GC-EIMS retention time and m/z (%): 5.4 min; 192 (72%), 174 (5%), 163 (5%), 148 (5%), 135 (35%), 119 (20%), 114 (22%), 106 (100%), 93 (39%), 79 (35%).

3.5.4.4. cis-3'-Hydroxycotinine mesylate (107)

Methylsulfonic chloride (2.9 g, 25 mmol, 2 mL) was added dropwise to a solution of cis-3'-hydroxycotinine (19b/c, 3.84 g, 20 mmol) in 20 mL of dry pyridine and cooled to 5 °C with an ice-bath under the protection of nitrogen. The reaction mixture was stirred at the same temperature for 4 hrs and a yellow solid formed. The TLC (silica gel GF 254, CHCl₃/MeOH 10:1) showed two spots, pyridine and the product (Rf 0.67) and no starting materials left. The crude resulting products was treated with 25 mL of cold water and extracted with CHCl₃. The extract was dried over sodium sulfate and decolorized with active charcoal. After removing the solvent, a white solid was obtained. After recrystallization from CHCl₃ / hexanes, 4.63 g (86%) of white crystalline material of cis-3'-hydroxycotinine mesylate (108) was obtained, m.p. 134-134.5 °C (Ref. 133-134 °C).¹H NMR (CDCl₃): δ 8.66 (1H, dd, C2-H), 8.56 (1H, d, C6-H), 7.64 (1H, dt, C4-H), 7.40 (1H, m, C5-H), 5.31 (1H, t, C3'-H), 4.53 (1H, t, C5'-H), 3.34 (3H, s, S-Me), 3.07 (1H, m, C4'-H), 2.70 (3H, s, N-Me), 2.17 (1H, m, C4'-H). GC-EIMS retention time and m/z (%): 5.34 min; 192 (72%), 174 (5%), 163 (5%), 148 (5%), 135 (35%), 119 (20%), 114 (22%), 106 (100%), 93 (39%), 79 (35%).

3.5.4.5. trans-3'-Hydroxycotinine acetate (108)

cis-3'-Hydroxycotinine mesylate (107, 2.16 g, 8 mmol) was added to a solution of sodium acetate (8.2 g, 0.1 mol) in glacial acetic acid (40 mL). The reaction mixture was stirred and heated in an oil-bath to reflux under nitrogen protection for 1.5 hrs. During the reaction, the colorless starting materials changed to a dark purple color. The TLC (silica gel GF 254, CHCl₃/MeOH 10:1) showed only one spot (Rf 0.83) and no starting materials left. After cooling to room temperature, the reaction mixture was poured into 100 mL of ice-water. To the aqueous mixture was added solid sodium carbonate to adjust the pH value to 9. The aqueous solution was extracted with CHCl₃ and dried over potassium carbonate. After removing the solvent, 1.92 g (72%) of yellow oil of
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine

trans-3'-hydroxycotinine acetate (108) was obtained. 1H NMR (CDCl₃): δ 8.62 (1H, dd, C2-H), 8.55 (1H, d, C6-H), 7.47 (1H, dt, C4-H), 7.36 (1H, m, C5-H), 5.48 (1H, t, C3'-H), 4.68 (1H, m, C5'-H), 2.78 (3H, s, N-Me), 2.47 (2H, m, C4'-H), 2.16 (3H, s, O-Me); GC-EIMS retention time and m/z (%): 6.1 min; 235 (<5%), 193 (<5%), 174 (100%), 163 (5%), 146 (10%), 131 (9%), 119 (32%), 106 (68%), 96 (20%), 78 (13%). This crude product was used directly for the preparation of trans-3'-hydroxycotinine.

3.5.4.5. trans-3'-Hydroxycotinine (19a/d)

The crude trans-3'-hydroxycotinine acetate (108) was added to 10 mL of 5% of sodium hydroxide solution. The reaction mixture was heated to 80 °C for 1 hour in an oil-bath under stirring. The TLC (silica gel GF 254, CHCl₃ / MeOH 10:1) showed only one spot (Rf 0.59) and no starting materials left. After cooling to room temperature, the product was extracted with CHCl₃ and dried over potassium carbonate. After removing the solvent and recrystallization from CHCl₃/hexane, 1.0 g of a pale yellow crystalline material trans-3'-hydroxycotinine (19a/d) was obtained, overall yield 65% for the two steps, m.p. 107-108 °C (Ref. 71-72 °C from acetone/pentane¹⁸). 1H NMR (CDCl₃): δ 8.52 (1H, d, C6-H), 8.45 (1H, s, C2-H), 7.71 (1H, dt, C4-H), 7.51 (1H, m, C3-H), 4.91 (1H, q, C3'-H), 4.71 (1H, t, C5'-H), 2.76 (3H, s, N-Me), 2.46 (2H, m, C4'-H); GC-EIMS retention time and m/z (%): 5.4 min; 192 (44%), 174 (5%), 163 (5%), 148 (5%), 135 (32%), 119 (20%), 114 (18%), 106 (100%), 93 (38%), 79 (38%).

3.5.5. Preparation of Urine Samples

The smoker's urine was obtained from a 31 years old white man and the non-smoker's urine was obtained from a 30 years old white man. The urine samples of rabbits and mice were collected for 24 hours before drug administrations as the control samples. After the drug administrations by i.p. injection, another 24 hours of urine were collected as the metabolic samples.

All of the urine samples were centrifuged at 10,000 g for 15 min to remove the suspensoid. The clear supernatants were lyophilized. 2 g of the solid urinary residues were soxhlet extracted with 150 mL of benzene for 2 hrs.
Chapter 3  In Vivo Studies on the Metabolism of \( \beta \)-Nicotyrine

After removing benzene, the extract residue was reconstituted with 2 mL of methanol and subsequently analyzed by HPLC UV-diode array and GC-EIMS. The solutions for intraperitoneal injections were prepared as following:

\[
\begin{align*}
\beta\text{-Nicotyrine tartrate:} & \quad 50 \text{ mg/2 mL of water} \\
5'\text{-Hydroxypyrrolinone 52:} & \quad 50 \text{ mg/1 mL of water} \\
2'\text{-Acetoxypyrrole 74:} & \quad 50 \text{ mg / 1 mL of water} \\
(S)\text{-Cotinine:} & \quad 50 \text{ mg / 1 mL of water}
\end{align*}
\]

3.5.6. Preparation of the Silyl Ether Derivatives of 3'-Hydroxycotinine 109

(a). From the Synthetic 3'-Hydroxycotinines

A solution of 2 mg of \textit{cis-} or \textit{trans-3'-hydroxycotinine} free base (19b/c or 19a/d) in 500 \( \mu \text{L} \) of anhydrous N,N-dimethylacetamide (DMAA) containing 12 mg of \textit{tert-}butyldimethylsilyl chloride and 12 mg of imidazole was vortexed for 1 minute in a capped tube, then placed on a heating block at 80 °C for 1 hour. After cooling to room temperature, 0.4 mL of a 90:10 mixture of toluene : butanol and 1 mL of water were added, the mixture was vortexed for another 1 minute, centrifuged, and the tube placed in dry ice to freeze the aqueous layer. The top organic layer was analyzed by GC-EIMS (Temperature Program 2). Retention time and m/z (%): 9.7 min; 291 (5%), 249 (100%), 218 (<5%), 190 (<5%), 175 (<5%), 144 (35%), 116 (10%), 88 (6%), 75 (35%).

(b). From Metabolic 3'-Hydroxycotinines in Urine Extract

Methanol reconstituted rabbit’s urine extract treated with \( \beta \)-nicotyrine (0.5 mL) was dried under nitrogen and room temperature. The residue was added to 500 \( \mu \text{L} \) of anhydrous DMAA containing 12 mg of \textit{tert-}butyldimethylsilyl chloride and 12 mg of imidazole and vortexed for about 1 minute in a capped tube and then placed on a heating block at 80 °C for 1 hour. After cooling to room temperature, 0.4 mL of a 90:10 mixture of toluene : butanol, and 1 mL of water were added. The mixture was vortexed for another 1 minute, centrifuged, and the tube was placed in dry ice to freeze the aqueous layer. The top organic layer was analyzed by GC-EIMS (Temperature Program 2). Retention time and
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine
m/z (%) of the major peak: 9.7 min, 291 (5%), 249 (100%), 218 (<5%), 190 (<5%), 175 (<5%), 144 (35%), 116 (10%), 88 (6%), 75 (35%). The SIC (m/z 249) also showed only one peak at retention time of 9.7 min.

3.5.7. Preparation of 3'-Chloro Derivatives of 3'-Hydroxycotinine Diastereomers (110)

(a) Synthesis of 3'-Chloro Derivatives of 3'-Hydroxycotinine from the Standard 3'-Hydroxycotinine (Method 1)\textsuperscript{15}

Pyridine (10 drops) and thionyl chloride (2 drops) were added sequentially to a solution of cis- or trans-3-hydroxycotinine (19b/c or 19a/d, 0.55 mg, 0.003 mmol) in 2 mL of methylene chloride in a capped tube. The reaction mixture was vortexed for 1 minute. The solvents was removed by nitrogen stream. 1 mL of 1 M sulfuric acid was added to neutralize the extra base, and 5 mL of 70:30 of toluene : n-butanol was added. The mixture was vortexed for another 1 minute and centrifuged, the top organic layer was discarded. To the bottom aqueous layer 1 mL of 50% potassium carbonate was added and the neutralized aqueous phase was extracted with 200 mL of 90:10 of toluene : n-butanol. After freezing the bottom aqueous layer in dry ice, the top organic phase was analyzed by GC-EIMS (Temperature Program 2). Two peaks were separated with retention times: 7.9 min and 8.0 min. Both peaks gave nearly identical mass spectra with m/z (%): 210 (40%), 175 (20%), 132 (35%), 118 (100%), 91 (20), 78 (10%).

(b) Synthesis of 3'-Chloro Derivatives of 3'-Hydroxycotinine from the Standard 3'-Hydroxycotinine (Method 2)\textsuperscript{18}

Cis- or trans-3-hydroxycotinine (19b/c or 19a/d, 2.25 mg, 0.013 mmol) was added to a capped tube containing 200 μL of thionyl chloride (326 mg, 2.7 mmol). The reaction mixture was heated on a heating block at 80 °C for 2 hrs. After removing the extra SOCl\textsubscript{2} by rotary evaporator, the residue was added to 0.5 mL of saturated Na\textsubscript{2}CO\textsubscript{3}, and extracted with 1 mL of ethyl acetate by vortexing for 1 minute. The bottom aqueous phase was frozen in dry ice and the top organic layer was analyzed by GC-EIMS (Temperature Program 1).
Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine

Two peaks were separated with retention times of 5.4 min and 5.6 min. Both peaks gave identical mass spectra with m/z (%): 210 (20%), 175 (15%), 153 (10%), 132 (25%), 118 (100%), 91 (20%), 78 (8%).

(c) Synthesis of 3′-Chlorocotinine from 3′-Hydroxycotinine as the Rabbit Urinary Metabolite

Methanol reconstituted urine extract (0.5 mL), obtained from the rabbits treated i.p. with β-nicotyrine, 74, (S)-nicotine or (S)-cotinine, was dried under nitrogen and room temperature. The residue was treated in the same way as either Method 1 or Method 2. GC-EIMS (Temperature program 1) retention time and the mass spectra of the 3′-chlorocotinine derived from the urine metabolite derivatives were the same with those derived from the standards.

3.5.8. In Vitro Transformation of trans-3′-Hydroxycotinine to its cis-Isomer

trans-3′-Hydroxycotinine (50 mg) in 1 mL of water was administrated i.p. to the rabbits and 24 hrs urine samples were collected as the metabolic samples. The urines were centrifuged, lyophilized, extracted and chloronated in the same way as described above. GC-EIMS analyses were preformed on these urine samples and a cis-3′-chlorocotinine derived from a standard trans-3′-hydroxycotinine. The SIC (m/z 210) of both standard and urine samples gave the ratio of cis- to trans-3′-hydroxycotinine as 3:7. However, these ratios were changed with the concentration of the samples. In samples diluted to 1/1000, both standard and urine samples only gave one peak which is due to the 3′-chloro derivative of trans-3′-hydroxycotinine.

3.5.9. In Vivo studies of Metabolism of (S)-Nicotine in Rabbits

Two New Zealand White male rabbits (2.0-2.5 kg) were used for these studies. The urines of the rabbits were collected for 24 hrs before drug administrations as the control samples. (S)-Nicotine was administered i.p. every 12 hrs for a total of 6 injections over 72 hrs. The two rabbits received different doses. The doses are shown in Table III-2.
Table III-2. Injection doses of (S)-nicotine for the rabbit studies

<table>
<thead>
<tr>
<th>Rabbit 1 (Green label)</th>
<th>Rabbit 2 (Blue label)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose (mg/kg)</strong></td>
<td><strong>Inject. (mg/mL)</strong></td>
</tr>
<tr>
<td>1</td>
<td>2.5 / 1</td>
</tr>
<tr>
<td>2.5</td>
<td>6.25 / 0.5</td>
</tr>
<tr>
<td>5</td>
<td>12.5 / 1</td>
</tr>
<tr>
<td>5</td>
<td>12.5 / 1</td>
</tr>
<tr>
<td>5</td>
<td>12.5 / 1</td>
</tr>
<tr>
<td><strong>Total 58.75 mg / 5.5 mL</strong></td>
<td><strong>Total 68.75 mg / 5.5 mL</strong></td>
</tr>
</tbody>
</table>

The urinary samples were collected for 96 hrs. They were extracted and analyzed by HPLC UV-diode array and GC-EIMS in the same way as in the β-nicotyrine metabolic studies. The metabolite peaks were confirmed by spiking the with the synthetic standards and reanalyzed by HPLC UV-diode. Retention time (λ_max of UV absorption): 7.4 min (256 nm, nicotinamide), 7.8 min (259 nm, 3'-hydroxycotinine), 9.7 min (258 nm, 5'-hydroxycotinine), 16.3 min (260 nm, (S)-cotinine), and 23.0 min (284 nm and 232 nm, β-nicotyrine); GC-EIMS retention times (m/z): 3.0 min (162, (S)-nicotine), 3.8 min (158, β-nicotyrine), 4.8 min (176, (S)-cotinine), 5.3 min (192, 3'-hydroxycotinine), 5.6 min (192, 20) and 5.7 min (192, 21).
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotine

References


Chapter 3 In Vivo Studies on the Metabolism of β-Nicotyrine


251
Chapter 3  In Vivo Studies on the Metabolism of β-Nicotyrine


21 Gerhards, E. (1971) The metabolism of norethisteron (17α-ethinyl-4-oestren-17β-ol-3-one) and of DL-norgestrel (13-ethyl-17α-ethinyl-4-oestren-17β-ol-3-one) was investigated. Acta Endocrinol. 68, 219.


Chapter 4  Conclusion

Chapter 4

Conclusions

1. The minor tobacco β-nicotyrine has been shown to be toxic to Clara cells isolated from rabbit lungs.\(^1\) This pneumotoxicity depends on the metabolites of β-nicotyrine generated by cytochrome P-450 catalysis.

2. The in vitro metabolism of β-nicotyrine is species dependent. The incubation of β-nicotyrine with rat liver microsomes supplemented by NADPH only gave the starting substrate β-nicotyrine. Consequently β-nicotyrine is not metabolized extensively by rats' liver microsomes. Parallel in vitro studies with microsomes prepared from rabbit livers and lungs showed β-nicotyrine is extensively metabolized.

3. Since the in vitro metabolic intermediates of β-nicotyrine, 1-methyl-5-(3-pyridyl)-4-pyrroline-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (51), are unstable and could not be isolated as pure synthetic standards, a masked derivative, 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74), was synthesized. Hydrolysis of 74 gave these two pyrroline tautomers, 48 and 51, directly. Under strongly acidic conditions 74, via 48/51, generated 5'-hydroxycotinine (20). Under mildly acidic conditions, a similar process occurred but at a slower rate again to 5'-hydroxycotinine (20). Under aerobic and strongly alkaline conditions, the autoxidation is the favored pathway, and 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (52) together with some complex products are formed. Under neutral conditions, the two pathways are competitive and both 20 and 52 are found as the final products.

4. The formation of 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrroline-2-one (52) from the two pyrroline intermediates 48 and 51 was proposed to proceed by a free radical pathway. This free radical process has been documented by ESR analysis with spin trapping by analysis of the hydrolysis of 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74). The ESR studies gave definite
Chapter 4 Conclusion

Signals consistent with the formation of hydroxyl radical HO· and unidentified carbon centered radicals (CR3·).

5. β-Nicotyrine is oxidized in vitro by microsomes prepared from rabbit livers and lungs to two pyrroline tautomeric isomers, 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51), which exist in equilibrium. This metabolism is NADPH dependent. Besides the pyrroline intermediates, two new metabolites were identified, 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) and 5'-hydroxycotinine (20). Compound 20, which is reported as a urinary metabolite of (S)-nicotine and (S)-cotinine, was found only under acidic quenching conditions. Consequently, 20 may not be formed enzymatically, but may be formed by a hydrolysis of the pyrrolineones, 48 and 51.

6. The in vivo metabolism of β-nicotyrine was studied in mice and rabbits by i.p. administration of β-nicotyrine. β-Nicotyrine is metabolized very rapidly in vivo in these two animal species; no β-nicotyrine was found in the urine samples collected in 24 hours. 5-Hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) and 5'-hydroxycotinine (20) were found as two minor urinary metabolites of β-nicotyrine. Furthermore, the in vivo formation of 20 was observed as a reduction product of 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52) in the rabbit.

7. The two in vitro metabolic intermediates, 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one (48) and 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (51), were not found in the in vivo studies. This indicates that these metabolic intermediates are not stable in vivo and undergo further metabolism.

8. cis-3'-Hydroxycotinine (19b/c) was found as the major urinary metabolite of β-nicotyrine in mice and rabbits. The structure was confirmed by HPLC UV-diode array, GC-EIMS analysis and silyl ether derivitization. The stereochemistry of this metabolite is pure cis, which is opposite to the trans (19a/d) derived from (S)-cotinine and the predominantly trans metabolite derived from (S)-nicotine.
Chapter 4  Conclusion

9. It has been shown that 3'-hydroxycotinine forms through the pyrrolinone tautomers, 48 and 51, by in vivo studies with 2-acetoxy-1-methyl-5-(3-pyridyl)pyrrole (74), the latent form of the pyrrolinones. The major in vivo metabolite of 74 was cis-3'-hydroxycotinine (19b/c). A proposed metabolic pathway for the in vivo formation of 3'-hydroxycotinine involves a free radical sequence to form a precursor of 3'-hydroxycotinine, 3-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (55), followed by the reduction of 55 to 19b/c. The reduction mechanism has been indirectly documented by in vivo studies with 5-hydroxy-1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one (52). The reduction product, 5'-hydroxycotinine (20), was found as the major metabolite of 52.

10. The stereochemistry of 3'-hydroxycotinine derived from (S)-nicotine, as well as that found in smoker's urine, appears to be a mixture of the cis- and trans-diastereomers. Considering the results from the previous studies, this stereochemistry supports the following: In liver, (S)-nicotine is oxidized by cytochrome P-450 to the 1-methyl-5-(3-pyridyl)-1,5-dehydropyrrolidinium ion (15), which is present in equilibrium with its enamine free base, 1-methyl-5-(3-pyridyl)-2,3-dihydropyrrole (17). This metabolic intermediate is further oxidized by aldehyde oxidase, which is only present in liver, to (S)-cotinine, a major urinary metabolite of (S)-nicotine. The final hepatic metabolite of (S)-nicotine through (S)-cotinine is trans-3'-hydroxycotinine (19a/d). In lung, because of the absent of aldehyde oxidase, the intermediate iminium species 15 via its conjugated base, the enamine 17, will be oxidized by an oxidase such as monoamine oxidase-B (MAO-B) to β-nicotyrine which then is converted to cis-3'-hydroxycotinine (19b/c). Preliminary studies indicate that β-nicotyrine is present in the urine of rabbits treated with (S)-nicotine. The hepatic pathway is the major one. It is possible, however, that a free radical process involved in the metabolism of β-nicotyrine, a potential lung metabolite of (S)-nicotine, may be of toxicological significance.

References


255
Vita

Xin Liu, the daughter of Ms. Wanru Liu and Professor Wei Gao, was born in Beijing, People's Republic of China on August 7, 1948. She grew up in Beijing, where she was graduated from the Beijing First Ladies Middle School with a gold medal and Beijing 101 High School. At this point the tragic Cultural Revolution in China interrupted her formal education. After waiting for 11 years, she obtained the opportunity to attend Beijing Polytechnique University, where she received the degree of Bachelor of Science in Chemistry in 1982, and the degree of Master of Science in Environmental Engineering in 1985. After working in the Institute of Materia Medica, Chinese Academy of Medicinal Sciences in Beijing for one year, she was awarded the McNamara Fellowship from the Economic Development Institute of the World Bank, and came to Virginia Polytechnic Institute and State University, Blacksburg, Virginia, U. S. A. as a visiting scholar in August, 1986. She started her Ph.D. program in 1988 in Virginia Polytechnic Institute and State University in the division of Organic Chemistry. In October of 1993, she started her postdoctoral research in Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan. She completed the degree of Doctor of Philosophy in Chemistry on June 16, 1995.

Xin Liu