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CYANIDE TRAPPING OF METABOLICALLY GENERATED ELECTROPHILIC INTERMEDIATES DERIVED FROM TERTIARY AMINES

by

Bert Ho

B.A., University of California, Berkeley 1975

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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of the

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To My Parents

for

understanding

loving and caring

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CYANIDE TRAPPING OF METABOLICALLY GENERATED ELECTROPHILIC INTERMEDIATES DERIVED FROM TERTIARY AMINES

BERT HO

Abstract of the Dissertation

Many xenobiotics undergo metabolic oxidative transformations in mammalian enzyme systems to intermediates capable of alkylating nucleophilic sites on cellular macromolecules. The chemically reactive electrophilic intermediates generated by the microsomal mixed function oxidase system may be associated with the toxicity of the xenobiotic. The unstable carbinolamine intermediates from the metabolic α-carbon hydroxylation of tertiary amines may ionize to electrophilic iminium species. These metabolically generated iminium ions undergo nucleophilic attack by cyanide ion to form stable α-cyanoamine adducts. In order to evaluate the generality of this metabolic pathway and its potential relationship to the cytotoxicities caused by tertiary amines, the microsomal metabolism of 1-benzylpyrrolidine (1) and methapyrilene (2) has been examined.

The gas chromatography-mass spectral analyses of incubations of 1-benzylpyrrolidine (1) and specifically deuterium labeled analogs of 1 with rabbit liver microsomal preparations have led to the characterization of 1-benzyl-2-pyrroline (222), 1-benzyl-2-pyrrolidinone (220), and 1-benzyl-5-hydroxy-2-pyrrolidinone (226). Similar incubations conducted in the presence of sodium cyanide led to the characterization of 1-benzyl-2-cyanopyrrolidine (223), cis- and trans-1-benzyl-2,5-dicyano-

pyrrolidine (234a, 234b) and 1-benzyl-5-cyano-2-pyrrolidinone (235). The cyano adducts are believed to result from nucleophilic attack by cyanide ion on metabolically formed iminium species.

Incubations of tritium labeled 1-benzylpyrrolidine with rabbit liver microsomal preparations led to the NADPH dependent incorporation of radiolabel into the macromolecular fraction. Attempts to characterize the possible arene oxide rearrangement product 1-(4-hydroxybenzyl) pyrrolidine (248) as a metabolite of 1 were unsuccessful. These results are consistent with the postulate that metabolically generated iminium ions are capable of alkylating biomacromolecules.

Extension of these studies to the microsomal metabolism of the hepatocarcinogen methapyrilene (2) in the presence of ^{14}C-labeled sodium cyanide have led to the characterization of N-(2-pyridyl)-N-(2-thienylmethyl)-N'-cyanomethyl-N'-methyl-1,2-ethanediamine (341). The structures of the cyanomethyl compound and other methapyrilene metabolic products were assigned on the basis of gc-eims and nmr spectroscopy and in some instances by comparison with authentic compounds. Other metabolic products characterized from microsomal incubations of 2 include 2-(2-thienylmethylamino)pyridine (258), normethapyrilene (327), N-formyl-N-(2-thienylmethyl)-2-aminopyridine (348), and N-(2-pyridyl)-N-(2-thienylmethyl)-N', N'-dimethyl-1-oxo-1,2-ethanediamine (349). Evidence for the presence of methapyrilene N-oxide (351) was also found. Quantitative gc analysis reveals that the majority of metabolized methapyrilene remains unaccounted for.

The syntheses of the suspected metabolites are presented. The chemistry of the key synthetic intermediate, N-(2-pyridyl)-N-(2-thienylmethyl)aminoacetaldehyde (337), is discussed. A second possible cyano

adduct, N-(2-pyridyl)-N-(2-thienylmethyl)-N',N'-dimethyl-1-cyano-1-ethanediamine (344), was found to be too unstable to recover from microsomal preparations. The significance of iminium ion formation in the hepatocarcinogenicity of methapyrilene remains unsettled.

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CHAPTER I

INTRODUCTION

It is well documented that many xenobiotics undergo a variety of metabolic transformations in mammalian systems. In most cases, these biotransformations are associated with detoxification processes. However, it has become apparent in recent years that many foreign compounds are metabolized by the liver and various other tissues to potent alkylating and arylating intermediates. These chemically reactive electrophilic intermediates oftentimes generated by the mixed function oxidase enzyme systems are believed to be responsible for various drug-induced tissue lesions. Although precise molecular details are lacking, covalent binding of these reactive species to macromolecular nucleophilic functionalities appears to be the initial event associated with the formation of these lesions.[1]

There have been intensive efforts in recent years to elucidate the mechanisms responsible for the conversion of various chemical agents to ultimate toxins. The large number of aliphatic amines in therapeutic use today has prompted us to investigate the possible formation of chemically reactive electrophilic intermediates in the metabolism of these compounds.

It is generally believed that tertiary amines undergo metabolic oxidative N-dealkylation by way of intermediate carbinolamines. In addition to cleavage of the carbon-nitrogen bond, the putative carbinolamines might also ionize to form electrophilic iminium intermediates.

The presence of these chemically reactive species can be demonstrated by the coincubation of the substrate with sodium cyanide. As demonstrated in previous studies, the metabolically generated iminium ion may undergo nucleophilic attack by cyanide ion to form isolable α-cyanoamine adducts.[2]

This dissertation will summarize our efforts to assess the generality of this metabolic pathway and its possible significance with regard to the formation of macromolecular alkylating agents. Analysis of the metabolic pathways of tertiary amines will allow us to evaluate the possible relationship between the metabolic formation of chemically reactive intermediates derived from these compounds and associated chemically-induced cytotoxities.

The majority of the pathways in drug metabolism involve the transformation of lipophilic organic substances into more polar and water-soluble derivatives which results in the reduction of the pharmacological activity of the foreign compound and its rapid excretion. Drugs and other lipid-soluble xenobiotics would accumulate in the body and interfere with normal cellular processes if not converted into excretable water-soluble products.[3] Hence, in the early part of this century the term "detoxification" referred in general to those processes in which a foreign compound is metabolically converted to a less biologically active, more polar substance excretable <u>via</u> bile, urine or other body secretions.[4]

However, it was soon recognized that metabolism does not necessarily lead to loss of biological activity. In 1947, R. T. Williams made the following observation:

> Although the term "detoxification" or detoxification mechanism" implies some process or processes whereby toxicity in a compound is reduced or abolished, in its modern usage it includes not only changes involving a reduction in toxicity but also tthose involving an increase in toxicity.[4]

It has become evident that the metabolic products of biotransformation processes oftentimes contribute to, or are responsible for, the biological effects attributed to the administered xenobiotic. In recent years, it has become increasingly evident that the toxic effects exerted by many xenobiotics actually result from the action of metabolic intermediates or products. Furthermore, it is now clear that the enzyme systems responsible for the "metabolic activation" of these toxic compounds are the same metabolic enzymes responsible for "detoxification."[1]

Chemical carcinogens were the first class of xenobiotics demonstrated to undergo biotransformation in vivo to reactive metabolites which bind covalently to tissue macromolecules.[5] Current knowledge indicates that many other kinds of toxicity, including mutagenesis, hepatic and renal necrosis, methemoglobinemia, and fetotoxicities are mediated by metabolically activated intermediates.[6] Although the molecular mechanims of toxicity are not clear, it is generally agreed that the metabolic intermediate which binds to macromolecules is strongly electrophilic in nature.[5] The initial event in organ toxicity appears to be the reaction of electrophilic metabolites with nucleophilic moieties on cellular components (i.e., protein and nucleic acids). The lack of reactivity of these chemically inert substrates and the frequent localization of tissue damage in organs and species having the required metabolizing enzymes support this theory.[1] Chemically inert foreign substances can be converted to chemically reactive intermediates by a variety of metabolic reactions. It appears that the microsomal mixed function oxidase system is responsible for the metabolic activation of the majority of toxic chemical agents.[1]

The enzyme systems responsible for drug metabolism are primarily located in the endoplasmic reticulum of liver cells. Physical disruption of the intact cells leads to the fragmentation of the endoplasmic reticulum into small lipid vesicles called microsomes. The mixed function oxidase activity is localized in the microsomes[7] and requires a source of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and dioxygen (O_2) to catalyze the following reaction in which substrate (R-H) is converted to oxidized product (R-OH):[8]

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

The stoichiometry of the equation indicates that dioxygen undergoes an overall four-electron reduction; two electrons are coupled to the oxidation of substrate and two electron to the oxidation of NADPH to $NADP^+$. One oxygen atom of dioxygen is converted to water and the other is incorporated into the oxidized substrate. The hemoprotein cytochrome P-450 is the terminal oxidase responsible for the oxidative transformation of the substrate. It has been shown that the drug metabolizing activity of the mammalian mixed function oxidase system requires at least three essential components - membrane-bound cytochrome P-450, NADPH-cytochrome P-450 reductase, and phosphatidylcholine.[9]

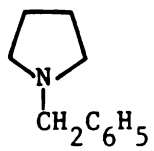
The versatility and nonspecificity of the mammalian hepatic cytochrome P-450 mixed function oxidase enzymes system is unique in biochemistry. The liver microsomal mixed function oxidase system is responsible for the oxidative metabolism of a large variety of endogenous and exogenous substrates such as fatty acids, steroids, drugs, and environmental contaminants.[10] Hepatic cytochrome P-450 actually is a family

of related enzymes. These multiple forms of cytochrome P-450 display different patterns of substrate specificity and different sensitivities to various inducers, such as phenobarbital and 3-methylcholanthrene, and inhibitors, such as SKF-525A and piperonyl butoxide.[11]

A large body of evidence has established the role of cytochrome P-450 microsomal oxidation in the metabolic activation of a vast array of structurally different cytotoxic agents. There has been an intensive effort to fully characterize the different metabolic pathways responsible for the conversion of various chemically inert xenobiotics to ultimate toxins and to elucidate structural information on the chemical nature of the reactive species. Towards these ends, considerable effort has been directed towards examining the oxidative metabolism of structurally different toxic agents such as arylamides, polycyclic aromatic hydrocarbons, nitrosamines, vinylethers, halogenated arenes, estrogens, and vinyl chlorides.[1,5,12,13]

There is a large number of compounds in therapeutic use which contain an aliphatic amine moiety. These and other organic nitrogen compounds can undergo a variety of diverse metabolic transformations. The detailed mechanisms of these pathways have not been elucidated despite intensive research.[14] Because of their widespread use, the potential toxicity of aliphatic amines should be a matter of toxicological concern. Yet, the possible existence of toxic intermediates generated from the metabolic activation of aliphatic amines has not been thoroughly investigated. As will be discussed in Chapter II, there is evidence which indicates the existence of possible reactive intermediates formed from the microsomal oxidative metabolism of tertiary aliphatic amines. In view of this situation, we have decided to examine the hepatic micro-

somal oxidative metabolism of tertiary aliphatic amines to gain some insight into the mechanism of their metabolism. The efforts described in this dissertation are directed towards isolating and identifying the metabolites and possible metabolic intermediates resulting from the microsomal metabolism of two structurally different tertiary amines. The first compound studied, 1-benzylpyrrolidine (1), is a simple model tertiary amine; the second compound is methapyrilene (2) an antihistamine recently shown to be carcinogenic. The focus of these studies is to elucidate the structural identity of possible reactive metabolites and to evaluate their potential toxicity.



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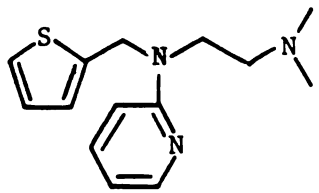

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A brief review of the general nature of various metabolic intermediates is presented in the next chapter. Following this, evidence for the formation of a reactive metabolic intermediate from aliphatic amines will be presented. These discussions will detail the rationale and goals of the efforts described in Chapters IV and V.

CHAPTER II

METABOLICALLY GENERATED ELECTROPHILIC INTERMEDIATES

A. A GENERAL OVERVIEW

In recent years, drug metabolism studies have led to the realization that many xenobiotics are metabolized primarily by the liver to potent alkylating or arylating agents. The observation that many toxic foreign compounds are chemically inert, that tissue lesions are frequently found only in organs having drug metabolizing enzymes, and that these lesions are often found in tissues distant from the site of introduction of the xenobiotic have led to the concept of metabolic activation.[1] A large number of chemical agents with diverse structural features are known to be toxic. A common property of many chemical toxins is their conversion to electrophilic species that ultimately are responsible for tissue damage. In the area of chemical carcinogenesis, the electrophilic species which actually initiates the neoplastic event is referred to as the ultimate carcinogen.[5]

Undoubtedly, the structural modification of informational macromolecules (DNA, RNA) by chemical carcinogens is one of the initial and necessary events in malignant transformations. The resulting molecular lesions are the basis for the alteration of gene expression.[15] The characterization of the structures of these small molecule-biomacromolecular interactions will, no doubt, provide insight into the mechanisms of carcinogenicity and related drug toxicities. The precise chemical structures of the ultimate carcinogens are understandably difficult to elucidate in most cases. Their high reactivity and the minute amounts

produced result in difficulties in the extraction, isolation, and identification of the intact metabolites. The structures of some reactive metabolites have been characterized in a few instances but in most cases, the evidence for the formation of a metabolically generated intermediate is incomplete and indirect.

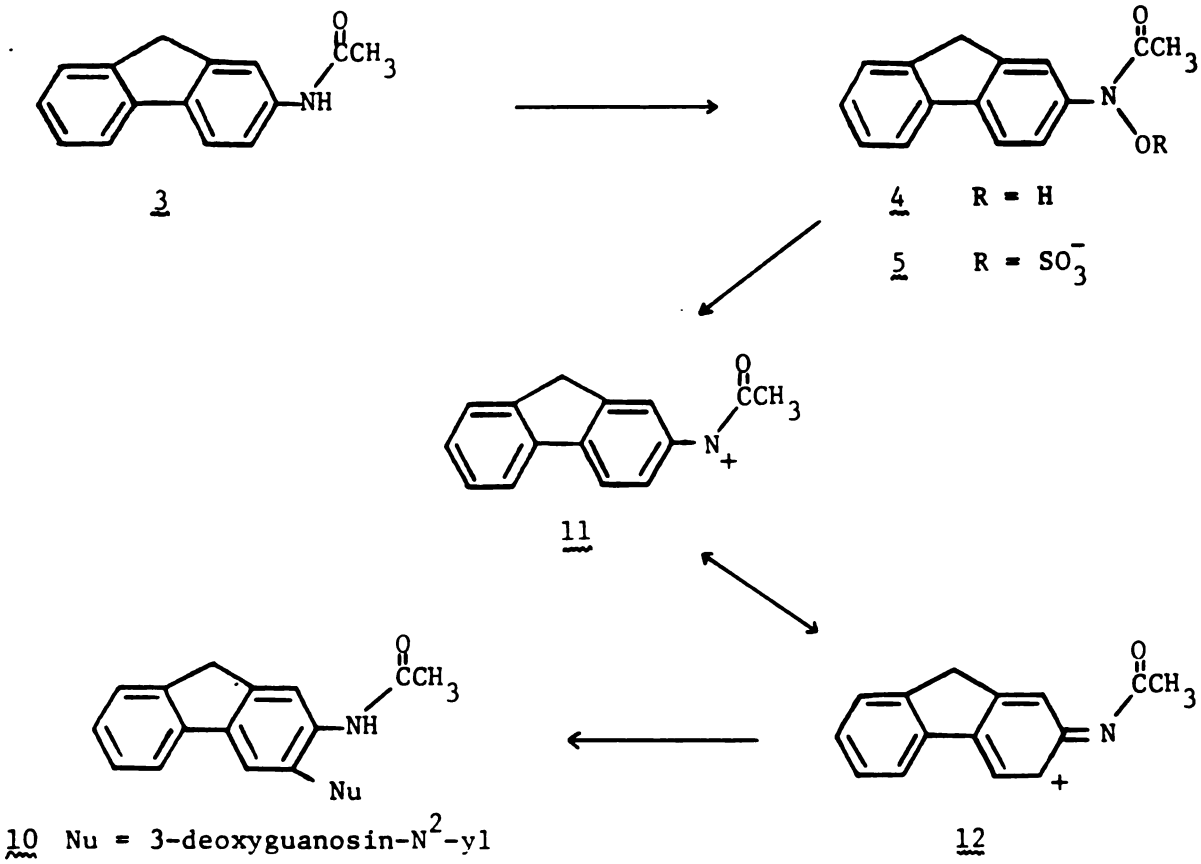
The formation of reactive metabolites can be evaluated by examining the severity of the tissue lesion and the amount of covalently bound metabolite at the site of damage. In this way, covalent binding becomes an in vivo index of formation of the reactive metabolite.[16] The pretreatment of the test animal with inducers or inhibitors of drug metabolism will accordingly affect the rate and extent of metabolism of the toxic agent and the severity of the tissue damage. If the extent of tissue damage and covalent binding are altered in the expected manner by inhibitors and inducers of cytochrome P-450 enzymes, then it is likely that metabolic activation of the parent agent by the mixed function oxidase system is involved in the formation of tissue lesions. Additional information can be gained with in vitro experiments employing microsomal isolates. Once again, covalent binding (in vitro) can be used as an index of reactive metabolite formation. The effect of various additions to or deletions from this controlled system or the effect of pretreatment of the animals with inducers or inhibitors can be carefully evaluated in this way. An extension of these studies involves examining the effects of small molecule nucleophiles such as glutathione or cysteine on the severity of tissue damage and/or the level of covalent binding.[1]

Covalent binding of small molecules to macromolecules should not be taken as prima facie evidence that metabolism is limited to toxicity.

However, macromolecular binding is solid though presumptive evidence for the *in vitro* or *in vivo* formation of an electrophilic (and usually cytotoxic) metabolite.[17]

In several instances, the structural identities of electrophilic metabolites have been reported. There is little doubt that the interaction of electrophilic metabolites with cellular components is the initial prerequisite event that leads to cellular necrosis. However, the detailed mechanism of the ensuing processes which eventually results in cell death remains to be elucidated. The structures of reactive intermediates have been postulated on the basis of degradation products or adducts formed with model nucleophiles. The following examples of metabolically generated intermediates are presented to illustrate the concepts of metabolic activation.

The metabolic activation of the carcinogen 2-acetylaminofluorene (AAF, 3) has been described in detail.[5,18] In a manner similar to a number of carcinogenic aromatic amines and amides, AAF is activated by a series of metabolic transformations. This arylamide initially undergoes N-hydroxylation by the cytochrome P-450 microsomal enzyme system to form the stronger carcinogen, N-hydroxy-2-acetylaminofluorene (N-hydroxy-AAF, 4).[5,19] However, these two carcinogens are not reactive *in vitro*, implying a need for further metabolism. Studies with rat liver preparations showed that one or more soluble sulfotransferases, in the presence of 3'-phosphoadenosine-5'-phosphosulfate (PAPS), can transform N-hydroxy-AAF to its O-sulfate ester. This highly reactive ester, N-sulfonoxy-2-acetylaminofluorene (5), is believed to be the major ultimate carcinogenic metabolite of AAF in rat liver.[20,21,22]



3

4 R = H

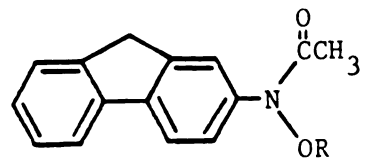
5 R = SO_3^-

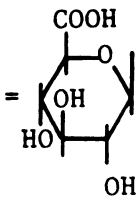
11

10 Nu = 3-deoxyguanosin-N^2-yl

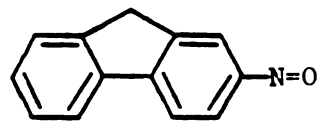
12

In extrahepatic tissues where N-hydroxyl-AAF sulfotransferase activity has not been detected, other possible electrophilic species are believed to be the ultimate carcinogenic metabolite of AAF or N-hydroxy-AAF. The O-glucuronide of N-hydroxy-AAF 6, a major metabolite, is weakly electrophilic. N-hydroxy-AAF also undergoes a peroxidase catalyzed transformation which ultimately gives rise to the electrophile N-acetoxy-2-acetyl aminofluorene (7) and 2-nitrosofluorene (8). A soluble acyltransferase catalyzes the rearrangement of the N-acetyl group of 4 to its N-hydroxy moiety to give rise to the potent electrophile N-acetoxy-2-aminofluorene (9).[5,18]



6 R = 

7 R = 



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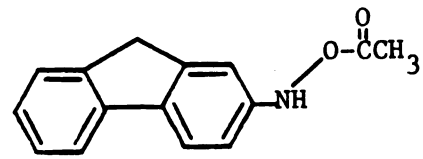

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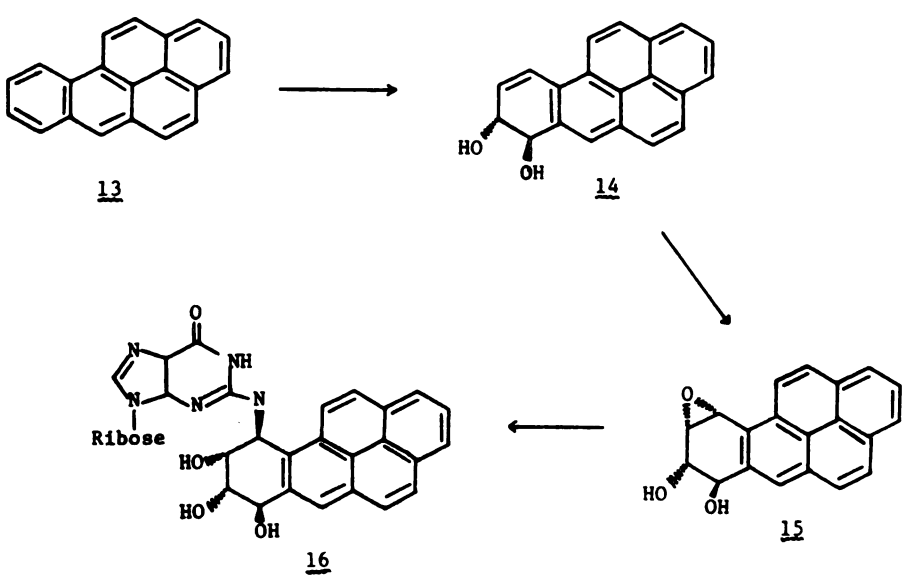
In in vivo experiments with rat liver, the AAF-nucleic acid adducts have been identified as guanine adducts. Characterization of the major adduct 10 shows that substitution occurs at the C-2 amino group of deoxyguanosine residues.[23] The guanine substituent at C-3 of the acetylaminofluorine molecule suggests the presence of the resonance-stabilized onium species 11 ↔ 12.[24] Thus the metabolic activation of 2-acetylaminofluorene is initiated by the cytochrome P-450 catalyzed N-hydroxylation of the amide nitrogen, followed by conjugation or isomerization to generate a good leaving group. The resulting esters are highly reactive and can easily form the resonance stabilized electrophilic species 11 ↔ 12 which are highly susceptible to attack at either the amido nitrogen or C-3 by nucleophilic functionalities.

Out of the family of carcinogenic polycyclic aromatic hydrocarbons, metabolism of the ubiquitous benzo[a]pyrene (13) has probably been most extensively studied. Generally, the aromatic hydrocarbons are metabolized by the hepatic cytochrome P-450 mixed function oxidases. In vitro and in vivo studies have demonstrated that benzo[a]pyrene is metabolically activated to intermediates that can interact with DNA,

RNA, and protein.[25] Like many other polycyclic aromatic hydrocarbons, benzo[a]pyrene undergoes initial oxidative metabolism to form reactive arene oxides which spontaneously isomerize to arenols, undergo hydration to dihydrodiols (a reaction catalyzed by microsomal epoxide hydrase), or are conjugated with glutathione.[12,26]

Results from in vitro experiments gave rise to the theory that the activation of benzo[a]pyrene to an ultimate carcinogen required not only arene oxide formation but also the presence of a dihydrodiol moiety.[27] The equivalency in carcinogenic potency of benzo[a]pyrene and its trans 7,8-dihydrodiol 14 was evidence that this dihydrodiol was a probable intermediate towards the ultimate carcinogen.[28] Subsequent studies led to the identification of (+)-7R,8S-dihydroxy-9R,10R-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene (15) as the ultimate carcinogenic metabolite of benzo[a]pyrene. This reactive diol epoxide metabolite is the major product resulting from the stereoselective epoxidation of the dihydrodiol (-)-7R,8R-dihydroxy-7,8-dihydrobenzo[a]pyrene (14) which in turn is formed by the catalytic action of mixed function oxidase and epoxide hydrase on the substrate benzo[a]pyrene.[29]



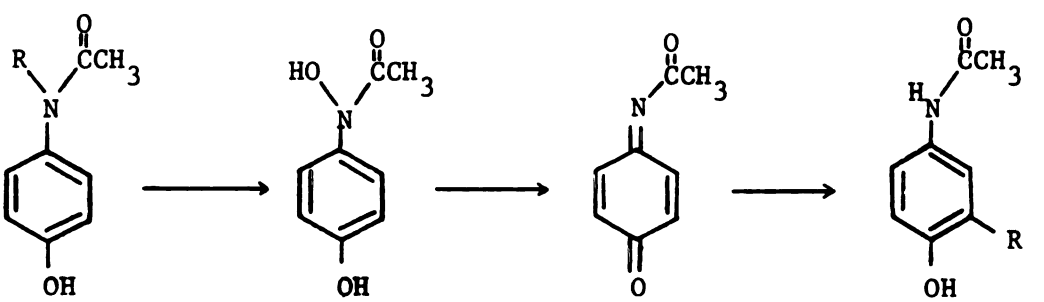
13 14 15 16

Further evidence for the diol epoxide 15 being the key reactive metabolite comes from the identification of diol epoxide-macromolecule adducts. The major reaction product 16 from the incubation of the diol epoxide with DNA results from the nucleophilic attack of the C-2 amino group of deoxyguanosine residues at C-10 of the diol epoxide.[5,30] The diol epoxide reacts with RNA and poly G in cultured bronchial mucosa to give the same product 16.[31] To a lesser extent, the diol epoxide also binds covalently to deoxyadenosine and deoxycytidine residues of polynucleotides.[32]

The metabolic activation of toxic agents other than chemical carcinogens has also been extensively studied. One example is the metabolism of the commonly used analgesic acetaminophen (17). Although safe in therapeutic doses, large doses of this drug can cause severe hepatic and renal necrosis in man and animals.[33,34] Animals pretreated with the inducers of drug metabolizing enzymes, phenobarbital and 3-methylcholanthrene, showed increased incidence and heightened severity of the hepatic necrosis. Similar pretreatment with inhibitors of cytochrome P-450 systems, cobaltous chloride and piperonyl butoxide, prevented necrosis. This evidence, along with the lack of correlation between tissue levels of acetaminophen and the severity of hepatic necrosis, implicates a cytochrome P-450 generated metabolite as the toxic species in acetaminophen-induced liver necrosis.[35] When radiolabeled acetaminophen was given to mice, a direct correlation was found between the severity of hepatic necrosis and the extent of covalent hepatic binding of radiolabeled material. In addition, the effect of inducers or inhibitors of oxidative metabolism on the severity of the hepatic necrosis correlated directly with the degree of covalent binding.[36]

Results from in vitro binding experiments provide additional evidence for the conclusion that the covalent binding and the consequent hepatotoxicity of acetaminophen is mediated by hepatic microsomal cytochrome P-450 enzyme systems.[37]

Further study has shown that glutathione protects the liver against electrophilic acetaminophen metabolites. Pretreatment with agents that alter the level of hepatic glutathione demonstrate a reciprocal relationship between the level of glutathione and the severity of hepatic necrosis. As expected, the level of covalent binding is also inversely related to the glutathione level. Accordingly, covalent binding and hepatic necrosis result only after liver glutatione has been sufficiently depleted (greater than 70%) by the electrophilic metabolites generated from large doses of acetaminophen. The reactive metabolite of acetaminophen is detoxified via preferential reaction with glutathione to form the polar mercapturic acid product 18. Depletion of glutathione leads to alkylation of macromolecules and cell toxicity.[38,39]



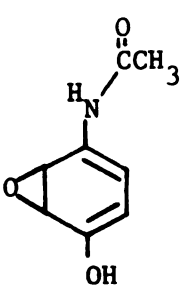
17 R = H

22 R = CH_3

20

19

18 R = $SCH_2CH(COOH)NHCOCH_3$



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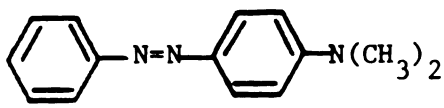
The electrophilic metabolite which is detoxified by glutathione and reacts adversely with biomacromolecules is believed to be the imidoquinone species 19. This reactive intermediate results from initial cytochrome P-450 catalyzed oxidation of acetaminophen to N-hydroxy-acetaminophen (20) followed by subsequent dehydration.[1] The evidence for the intermediary of the N-hydroxylated metabolite 20 versus an arene oxide 21 is growing. The N-methyl derivative of acetaminophen, N-methyl-4-hydroxyacetanilide (22), is not hepatotoxic;[1] additionally, N-hydro-acetaminophen has been shown to be five times more hepatotoxic than acetaminophen in mice.[40] It is believed that unlike the bioactivation of 2-AAF (3), further conjugation of the N-hydroxy derivative 20 is not required to generate the reactive, toxic intermediate 19; it is proposed that under physiological conditions, 20 decomposes readily to a reactive intermediate, presumably the imidoquinone 19, which is responsible for the observed toxicity.[40]

The cases cited above are illustrations of a large number of toxic agents which have been studied mechanistically. Great efforts are being made in attempts to elucidate the mechanisms of metabolic activation of a variety of structurally different chemical carcinogens such as 4-dimethylaminoazobenzene (23),[5] aflatoxin B_1 (24),[5,15,41] safrole (25)[18,42,43] 2,4-diaminoanisole (26),[44] vinyl chloride (27),[45,46] estrogens,[17,47] and nitrosamines.[48] The metabolic activation of a number of chemical agents which cause various forms of tissue lesion is also the subject of intensive study. Included among these agents are drugs like furosemide (28),[1,49] isoniazid (29),[50,51] and chloramphenicol (30).[52] Despite the structural diversity of all the compounds studied, results to date substantiate the theory that the actual toxic species in all cases is a metabolically-generated electrophilic intermediate.



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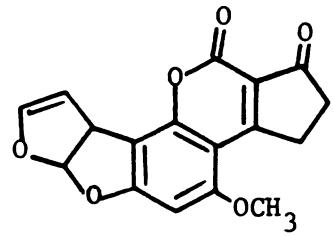

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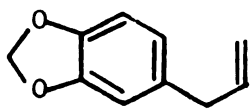

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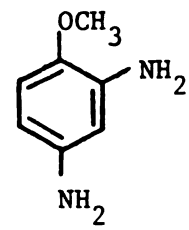

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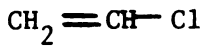

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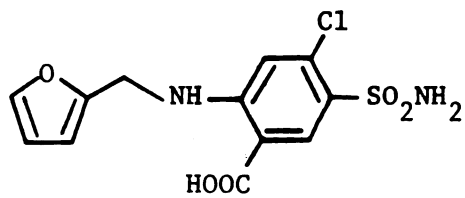

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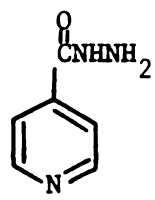

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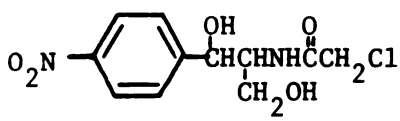
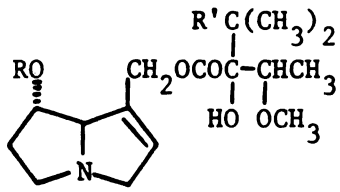


30

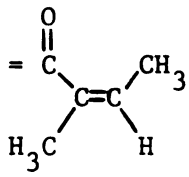
B. REACTIVE INTERMEDIATES DERIVED FROM NITROGENOUS COMPOUNDS

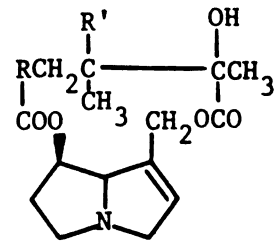
The common occurrence of nitrogen in drugs and other exogenous chemicals has made the metabolism of nitrogenous xenobiotics and particularly aliphatic amines a subject of considerable interest in recent years. Studies concerning the metabolism of aliphatic amines have not considered their potency for metabolic conversion to reactive intermediates. There is evidence in the literature, however, which strongly supports this possibility.

The hepatotoxic pyrrolizidine alkaloids are believed to be activated by a cytochrome P-450 related system.[53] The most thoroughly studied alkaloids, heliotrine (31), lasiocarpine (32), monocrotaline (33), and retrorsine (34), have structures typical to this class of toxins. The minimal structural requirement for hepatotoxicity is the dehydro-pyrrolizidine ring with the esterified hydroxyl group as shown in 35.[54] Similar to cytotoxic compounds discussed above, these alkaloids are chemically unreactive and regardless of the route of administration, the liver, the site of metabolism, is also the site of tissue damage.[55] These alkaloids are known to undergo various metabolic transformations. Hydrolysis of the carboxylic agent ester functionalities gives non-toxic water soluble acids and amino alcohols.[53] Rat liver microsomes can oxidize these alkaloids to the corresponding water-soluble, relatively non-toxic N-oxides.[56] It has been shown that these toxic alkaloids are metabolized *in vivo* by the liver to pyrrole-like (dihydropyrrolizine) derivatives 36.[57] These pyrrole metabolites, which have been isolated and identified, are believed to be formed by a hepatic microsomal mixed function oxidase system.[56]



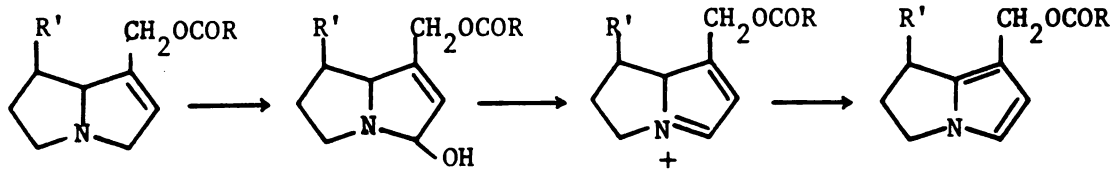
31 R = H; R' = H

32 R =  ; R' = OH



33 R = $CHCH_3$; R' = OH

34 R = $C{=}CCH_3$; R' = H



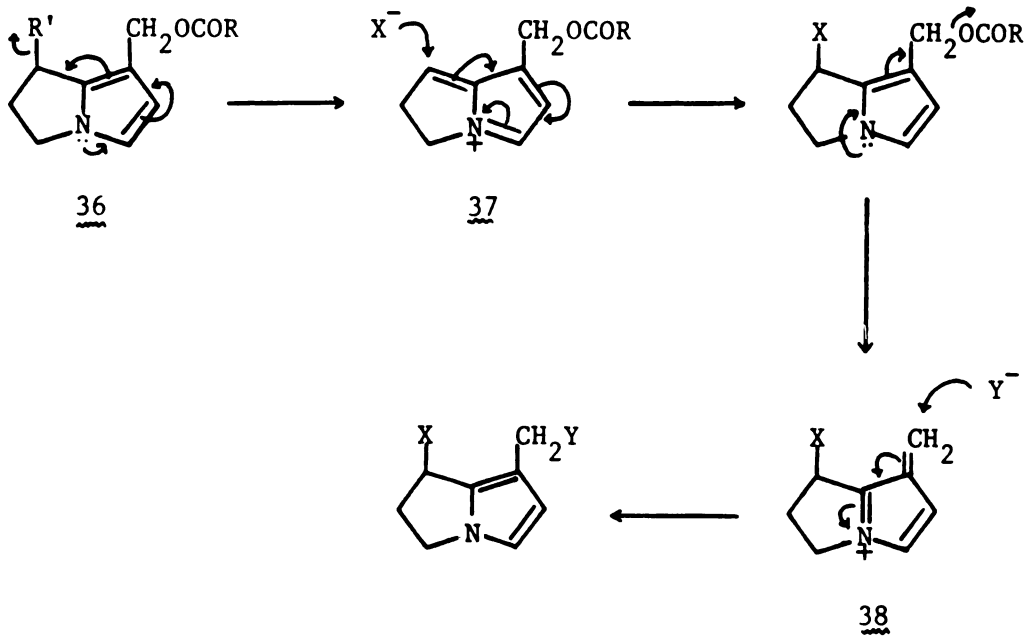
35 R = branched chain alkyl

R' = H, OH, or O-acyl

36

The formation of pyrrolic derivative does not necessarily mean the direct metabolic dehydrogenation of this system. Microsomal α-carbon hydroxylation of the pyrrolizidine ring generates a carbinolamine intermediate which immediately dehydrates and the resulting product then rearranges to the isolated pyrrole metabolite 36. There are several pieces of evidence implicating these pyrrole derivatives in the cytotoxicity of the pyrrolizide alkaloids. Whereas the parent alkaloids are relatively unreactive chemically, the dihydropyrrolizine esters are highly reactive towards nucleophiles such as amines, thiols, and OH^- under mild conditions.[58,59] The pyrrolic derivative of mono-

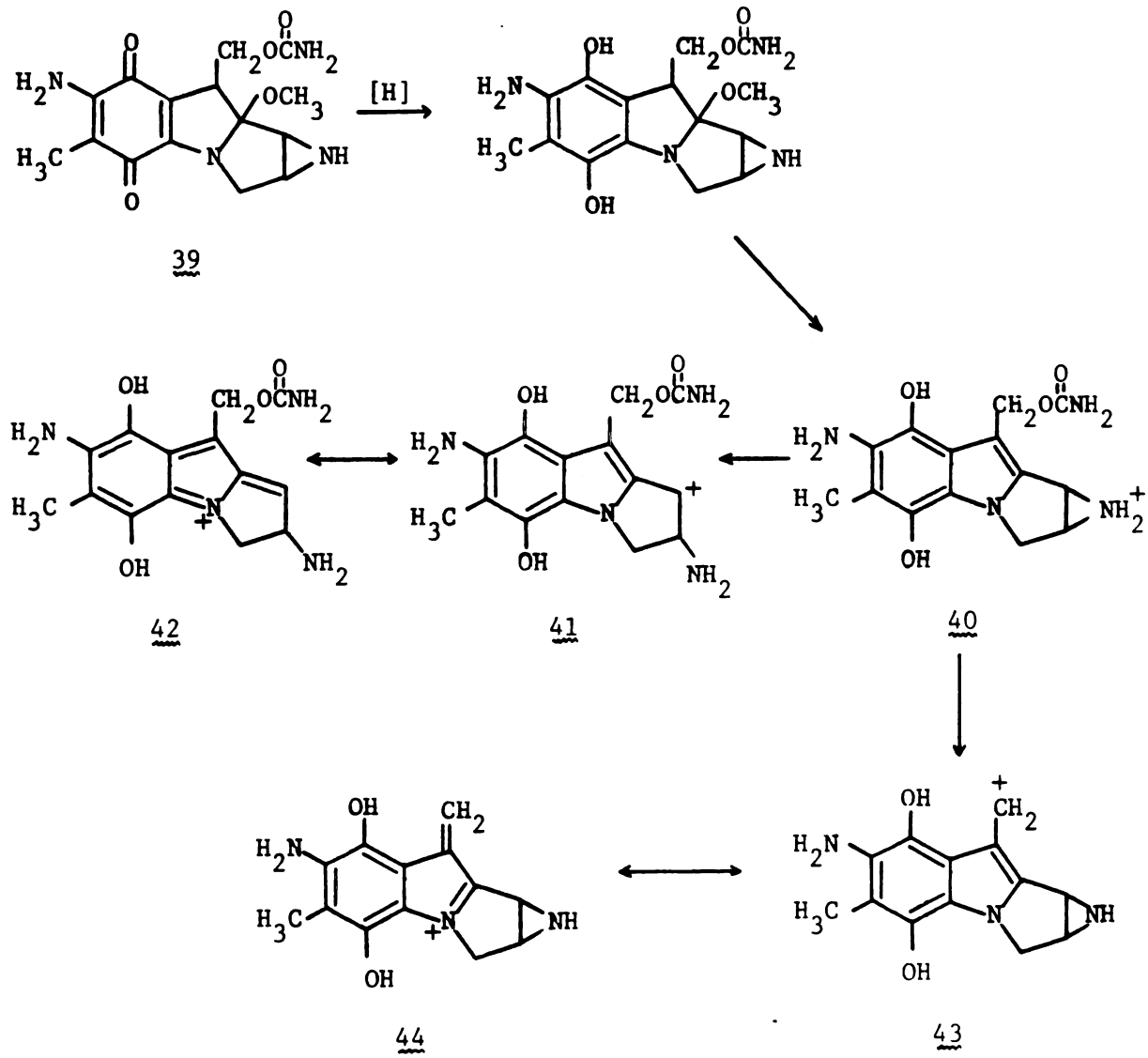
crotaline was shown to be ten times more toxic than the parent alkaloid.[60] There is also a correlation between the amounts of pyrrolic metabolites found in liver tissue *in vivo* and *in vitro* and the acute hepatotoxicity to the rat *in vivo*.[56] In addition, the pyrrolic derivatives of monocrotaline and retrorsine have been shown to be capable of cross-linking DNA *in vitro*.[61] Finally, in a manner similar to that seen for other metabolically activated toxic agents such as acetaminophen, alteration of the level of glutathione has been shown to be inversely related to the acute toxicity of retrorsine in rats.[62]



36 37 38

It is believed that the toxicity of the pyrrolic metabolite lies in its ability or tendency to rearrange to the highly reactive conjugated iminium ions 37 and 38 which are capable of alkylating cellular macromolecules.[58] Thus, initial α-carbon hydroxylation of the pyrrolizidine alkaloids produces a pyrrolic metabolite which ultimately forms a toxic electrophilic iminium species.

The electrophilic iminium moiety has been suggested as the functional group responsible for the binding or cross-linking of other compounds with DNA. The antibiotic mitomycin C (39) is capable of cross-linking DNA. Generally non-reactive in its natural oxidized state, mitomycin C acts as an alkylating agent upon chemical or enzymatic reduction. Although the exact mechanism of the cross-linking reaction is not known, structure-activity studies have shown the requirement of the aziridine ring for cross-linking activity.[63] A possible mechanism for the activation of mitomycin C involves initial reduction of the quinone fol-

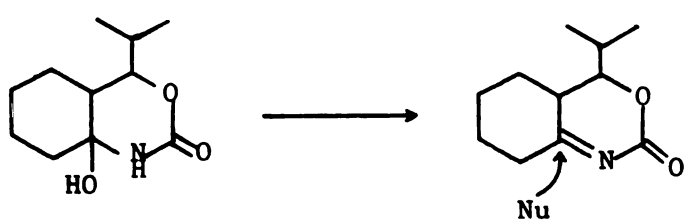


39 42 41 40 44 43

lowed by spontaneous loss of the methoxy group, a reaction which is driven by the formation of the fully aromatic indole system.[40]

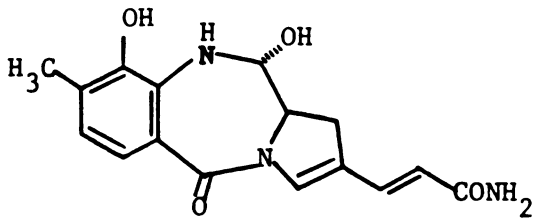
The cleavage of the aziridine ring to the carbonium species 41 is facilitated by the indole system which can act to delocalize the positive charge. The stabilization of charge by the iminium species 42 makes these electrophilic species more susceptible to nucleophilic attack at C-1. Another possible alkylating species 43, generated by the fission of the C-10-oxygen bond, can be stabilized by the indole nitrogen to give rise to 44. Delocalization of charge by the iminium species 44 would make C-10 reactive to nucleophilic attack.[64]

The carbinolamide moiety is believed to be responsible for the antitumor activity of the antileukemic agent maytansine. Studies with carbinolamide analogs such as 45 have shown that these analogs and maytansine can alkylate DNA under slightly acidic conditions. In addition, ether derivatives of these analogs fail to alkylate DNA or show antileukemic activity. These and other observations have led investigators to suggest that an alkylating species is formed by the dehydration of the carbinolamide. It is believed that the resulting azomethine lactone 46 is the reactive electrophilic metabolite which can react with biological nucleophiles.[65]



45 46

Anthramycin (47) and tomaymycin (48) are antitumor agents which form covalent complexes with DNA. These and related antibiotics or antitumor agents have in common the pyrrolo(1,4)benzodiazepine nucleus with a carbinolamine or imine function at the 10- and 11-positions. Studies with these and model compounds indicate the alkylation of DNA by these compounds is not by bivalent cross-linking or intercalation but through covalent interaction with one acid-sensitive functional group on the molecule. Structure-activity studies demonstrate that the carbinolamine function is necessary for reaction with DNA. A postulated mechanism for the formation of the pyrrolo(1,4)benzodiazepine antibiotic-DNA complexes requires the initial dehydration of the carbinolamine 49 to form the imine species 50 which can react with cellular nucleophiles. It is proposed that the electrophilic imine 50 is the species responsible for the alkylation of DNA.[66]



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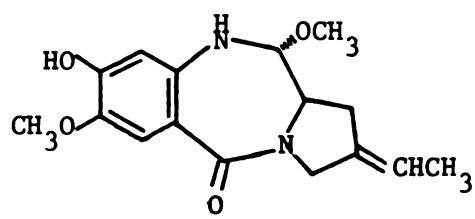

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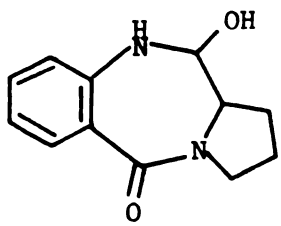

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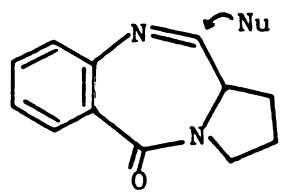
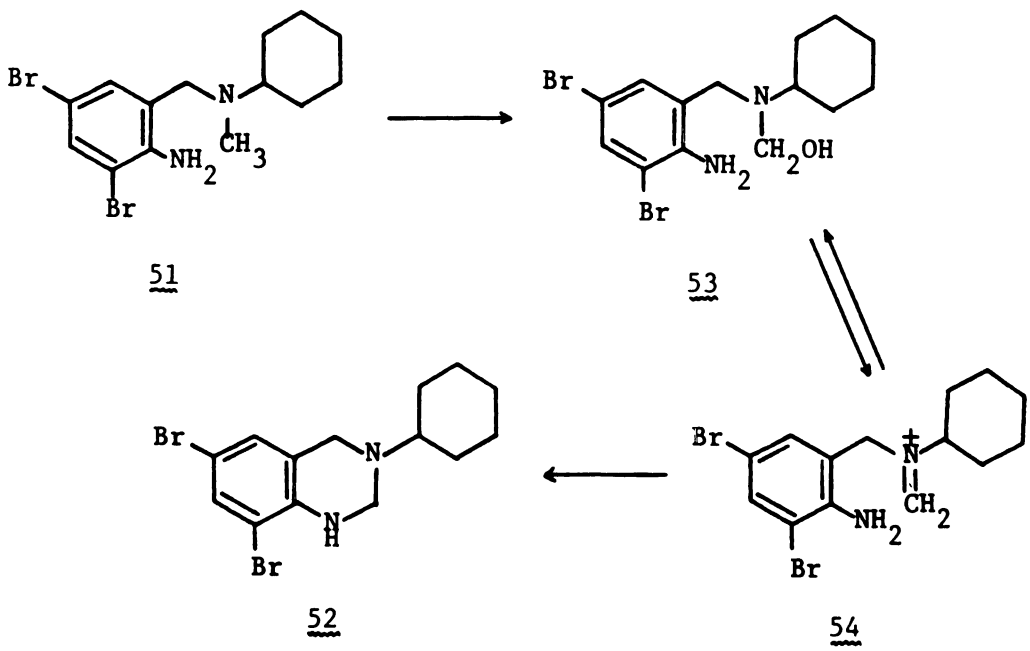


50

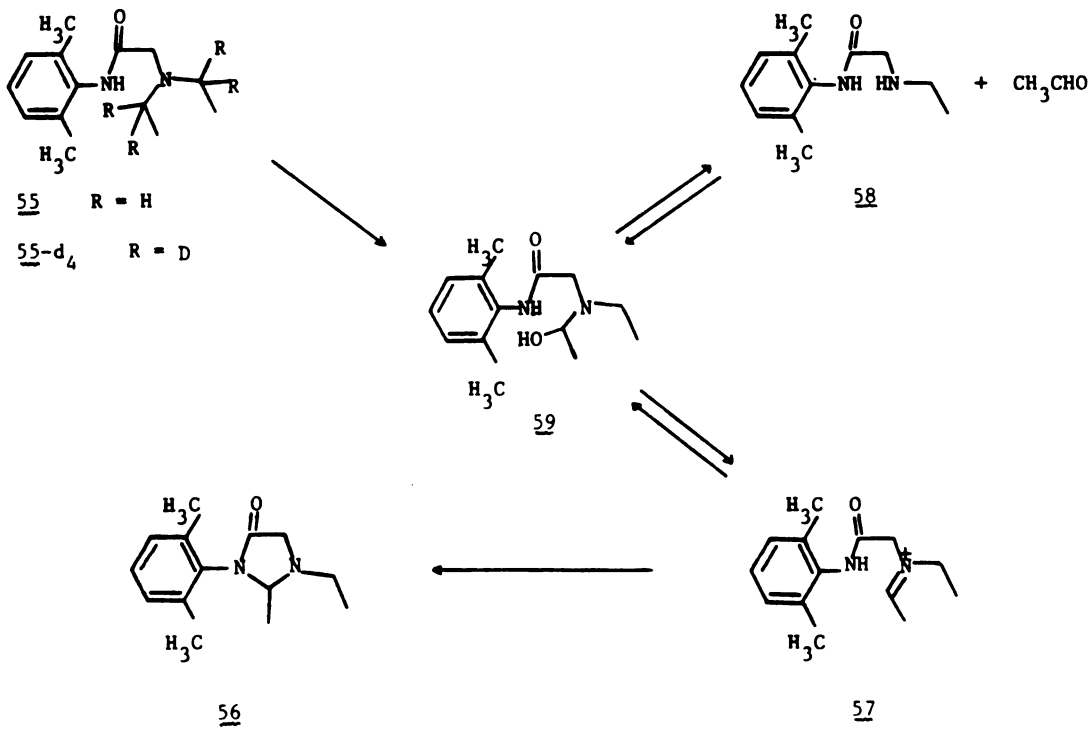
These examples clearly demonstrate the potential formation of reactive metabolites from aliphatic amines. In all cases, the actual reactive electrophilic intermediate is believed to be a carbinolamine-imine type species. Products of the interaction between reactive imines and biological macromolecules have not yet been characterized. However, reaction products resulting from intramolecular attack by nucleophiles on metabolically generated iminium species have been characterized.

The secretolytic drug Bisolvon (51) was found to be extensively metabolized (70%) by rabbits. The cyclic tetrahydroquinazoline 52 was found to be a minor metabolite in rabbit urine. A plausible mechanism for the formation of this cyclic metabolite invokes initial α-carbon hydroxylation of the N-methyl group to give the N-hydroxymethyl intermediate 53 which would spontaneously dehydrate reversibly to the iminium species 54. Nucleophilic attack by the aromatic amino group on the electrophilic species 54 would then give rise to the observed cyclic product 52.[67]



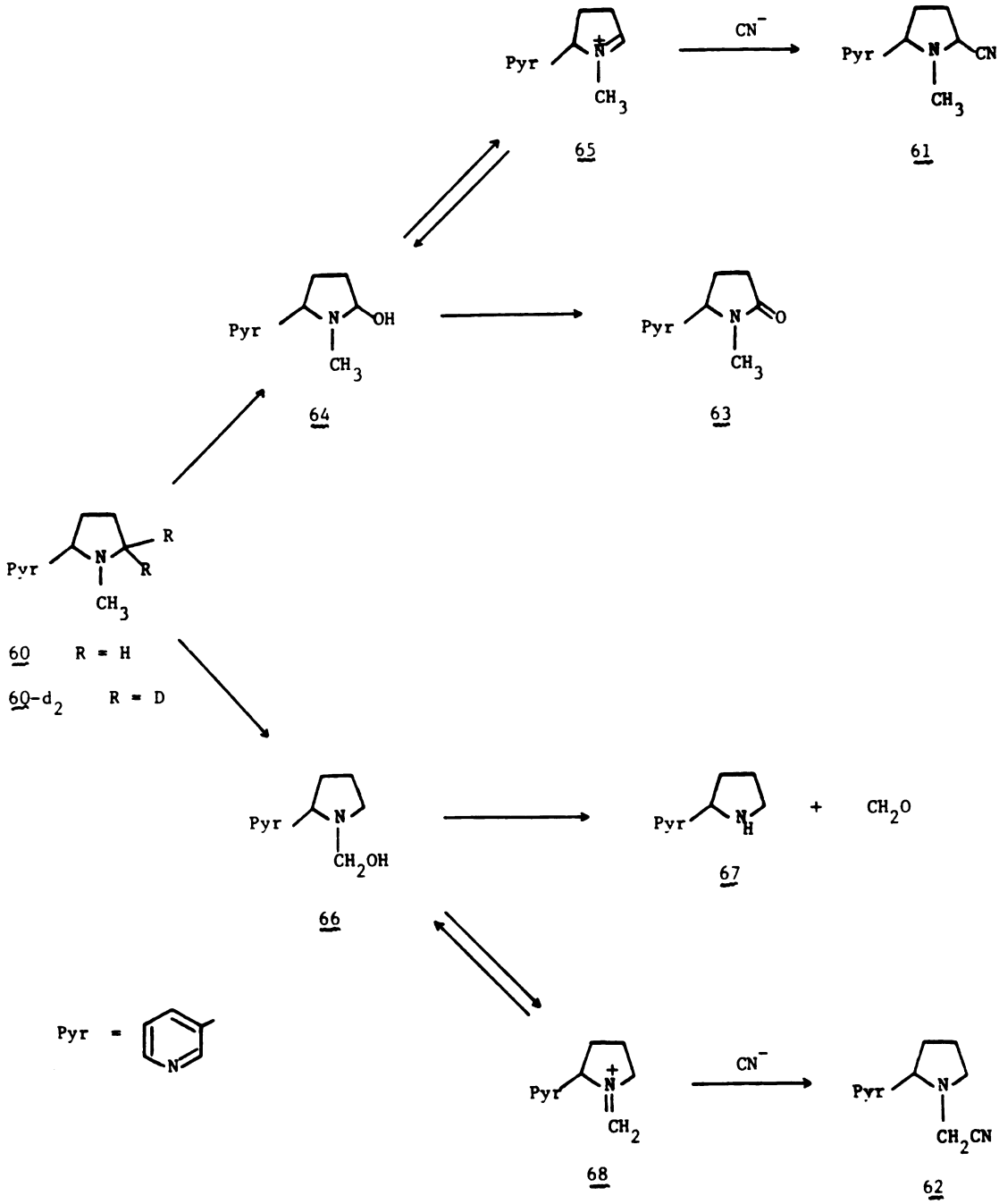
51 53 52 54

Similar evidence for metabolic iminium intermediates have been obtained with lidocaine (55), a widely used local anesthetic and anti-arrhythmic agent. The cyclic imidazolidinone product 56 has been isolated from the urine and plasma of humans.[68,69] Similar to the case of Bisolvon, 56 is presumed to arise from the intramolecular nucleophilic attack of the amide nitrogen on the electrophilic iminium carbon of 57. There are two possible pathways for the metabolic formation of the electrophilic iminium species 57. One involves formation of the Mannich type intermediate from the condensation of the N-dealkylated product 58 with endogenous acetaldehyde or trace aldehyde impurities in solvent. This condensation has been shown to be facile under physiological conditions suggesting that at least a portion of the observed cyclic product is an artifact resulting from this reaction.[70] The other possible route to 57 is *via* initial microsomal oxidation to the carbinolamine intermediate 59. This carbinolamine species is also most probably an intermediate in



the N-dealkylation of lidocaine to 58. This α-carbon hydroxylation product can either rearrange to the dealkylated amine 58 and acetaldehyde or it can dehydrate reversibly to 57 which can subsequently give rise to 56.

Additional evidence for metabolic iminium intermediates has been obtained in studies on the metabolism of the tobacco alkaloid nicotine



(60). In vitro studies on the metabolism of nicotine by rabbit liver microsomes in the presence of sodium cyanide have led to the characterization of two isomeric cyanonicotine compounds. The two cyano adducts which are believed to result from cyanide attack on iminium intermediates are 5'-cyanonicotine (61) and N-(cyanomethyl)nornicotine (62).[71,72]

The major mammalian metabolite of nicotine, cotinine (63), is believed to result from the further metabolism of the initial carbinolamine 64 by a soluble aldehyde oxidase. In turn, the carbinolamine, 5'-hydroxynicotine (64), results from the mixed function oxidation of nicotine.[73] Ionization of this initial metabolic hydroxylation product would produce the electrophilic species, nicotine $\Delta^{1'(5')}$ iminium ion (65), which is trapped by the cyanide ion to form the isolable 5'-cyanonicotine (61).[71] The structure of the cyano adduct has been confirmed by independent synthesis.[74] Metabolic formaton of the iminium ion and the resulting 5'-cyanonicotine is catalyzed by a mixed function oxidase system.

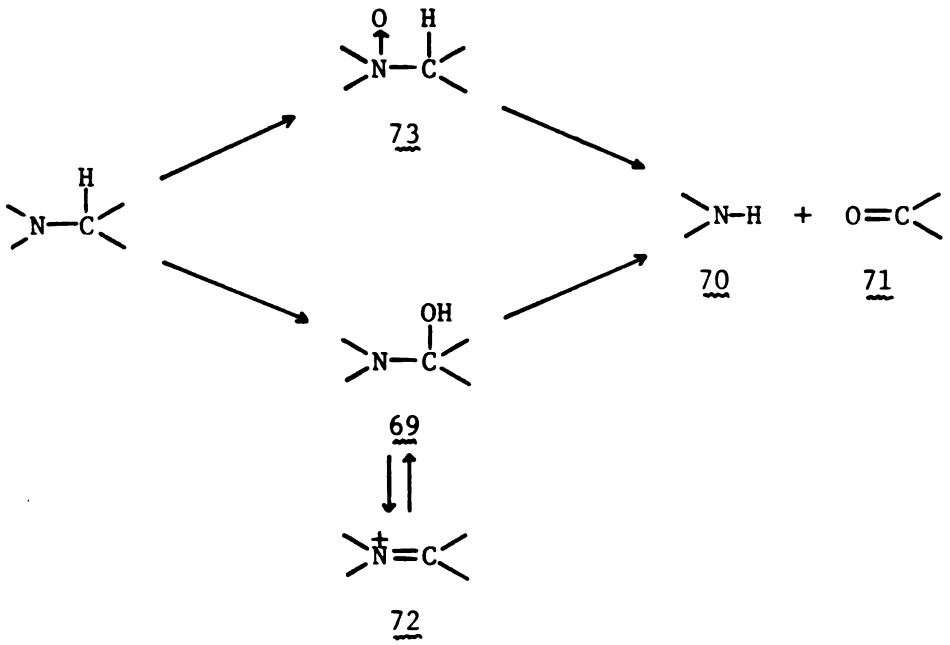
A similar mechanism has been postulated to rationalize the formation of N-(cyanomethyl)nornicotine (62). The carbinolamine, N-(hydroxymethyl)nornicotine (66), is formed from the mixed function oxidase catalyzed oxidation of nicotine. This carbinolamine species can cleave to yield nornicotine (67) and formaldehyde or ionize to the nicotine-N-methyleniminium ion 68. This iminium ion can then undergo nucleophilic attack by cyanide ion to form the observed N-(cyanomethyl)nornicotine.[72] The structure of this cyano product has been confirmed by synthesis.[75] However, N-(cyanomethyl)nornicotine can be formed from the facile condensation of metabolically generated nornicotine, formaldehyde, and sodium cyanide. Undoubtedly, part of the observed cyano product results from this indirect pathway, but isotope dilution studies have shown that, to a significant extent, the N-(cyanomethyl)nornicotine is formed without

prior C-N bond cleavage.[72] This suggests, in turn, that the reactive methyleniminium ion 68 is generated directly in the course of microsomal metabolism of nicotine. The characterization of these two cyano adducts implies the formation of the corresponding reactive iminium ions. It is most likely that the immediate precursors to the iminium ions are the carbinolamine species which result from α-carbon hydroxylation by the mixed function oxidase system. It is believed that α-carbon hydroxylation is the initial step in the oxidative N-dealkylation of amines.

C. α-CARBON HYDROXYLATION

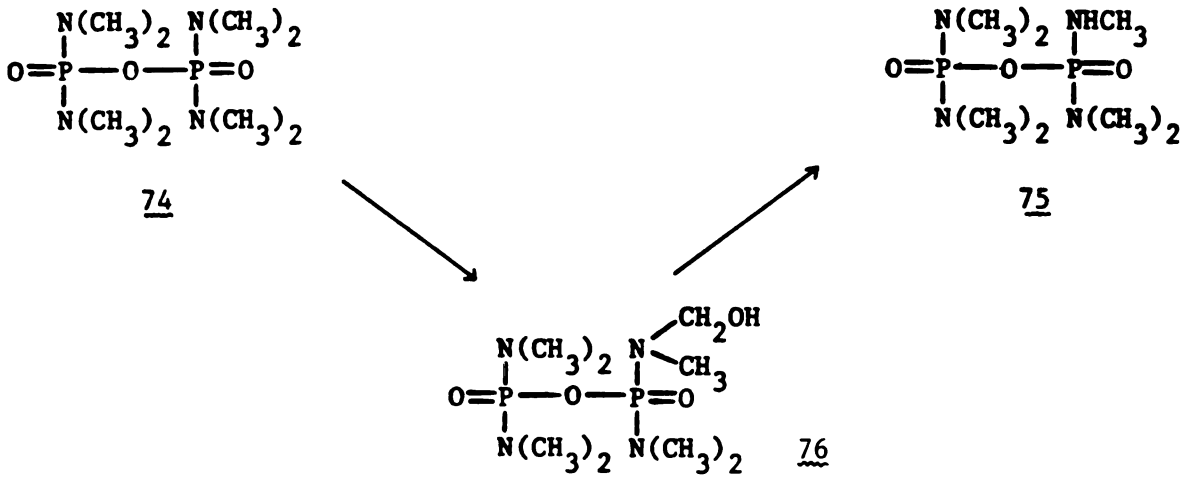
Two mechanisms have been proposed to describe the oxidative N-dealkylation of an amine. The first mechanism invokes the direct hydroxylation of the carbon alpha to the nitrogen heteroatom as the initial step.[76,77] The resulting unstable carbinolamine 69 then fragments to the observed product, a dealkylated amine 70 and a carbonyl compound 71. As mentioned before, an alternative pathway for the carbinolamine is reversible dehydration to an iminium species 72. The second postulated mechanism (which is usually discussed in terms of tertiary amines) involves initial oxidative attack on the nitrogen atom.[78] The resulting tertiary amine N-oxide 73 then undergoes further alteration by undefined paths which eventually lead to the observed products 70 and 71.

There is considerable evidence in support of the first mechanism. The proposed highly-reactive carbinolamines have been difficult to isolate. Nevertheless, there have been instances, mostly involving



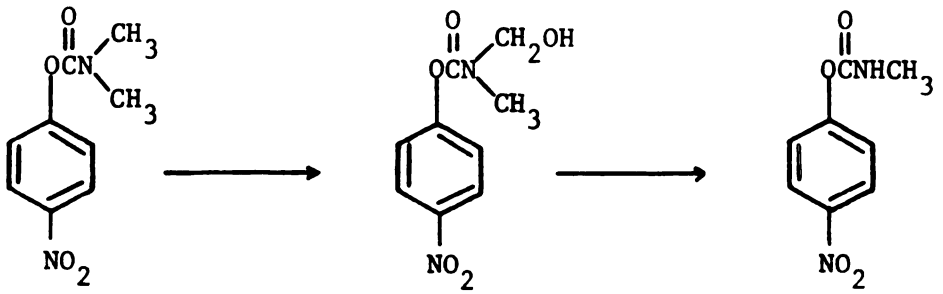
N-demethylation, in which the stable C-oxidized intermediates of amides (carbinolamides) and sp^2 nitrogen heterocyclic systems have been characterized. Additionally, some conjugates of α-hydroxy nitrogen systems have been isolated.

Octamethylpyrophosphoramide (74) is metabolized *in vivo* and *in vitro* to its heptamethylderivative (75). It has been proposed that the N-hydroxymethyl compound 76 is an unstable intermediate in this metabolic pathway.[79]



74 75 76

Studies on the metabolism of the N,N-dimethyl-p-nitrophenyl carbamate (77) have produced evidence for the presence of a carbinol intermediate. The results of these studies indicate 77 is metabolized by a microsomal enzyme system to an organic product which is stable under

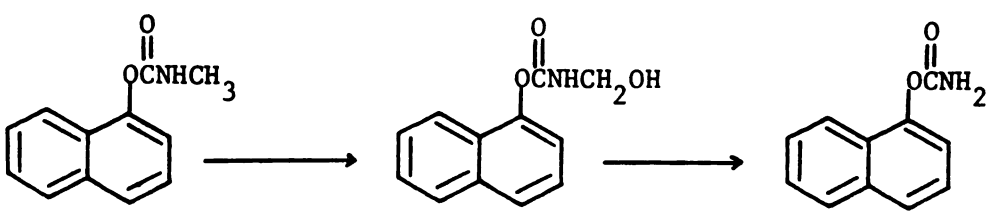


77 78 79

the incubation conditions (pH 7-8) and liberates formaldehyde only under the acidic conditions used in the formaldehyde assay. This product is proposed to be N-(hydroxymethyl)-N-methyl-p-nitrophenylcarbamate (78), an intermediate in the N-demethylation of 77 to 79.[80]

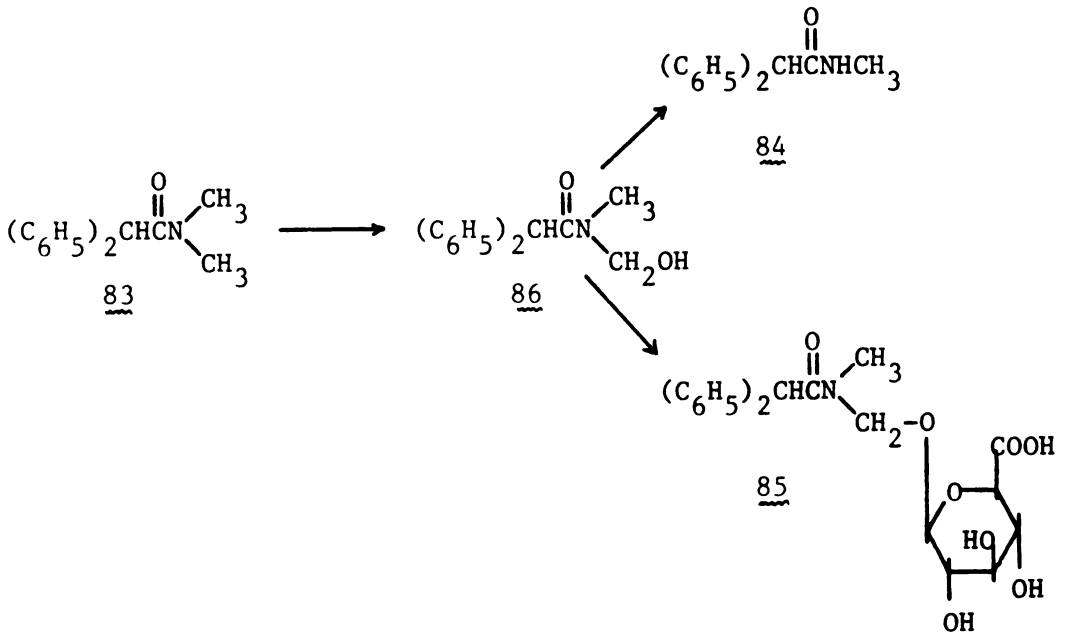
More direct proof of α-carbon hydroxylation was reported by Dorough and Casida who isolated 1-napthyl-N-(hydroxymethyl)carbamate (80) from the hepatic microsomal incubation of 1-napthyl-N-methylcarbamate (81).[81] Additional evidence implicating the hydroxymethylcarbamate 80 as an intermediate in the demethylation of 81 comes from studies on the oxidation of 81 by a chemical hydroxylation system. The N-demethylation product, 1-napthylcarbamate (82), and 1-napthyl-N-(hydroxymethyl)carbamate (80) were isolated and characterized from the incubation of 81 in the Udenfriend chemical hydroxylation system which consists of ascorbic acid, ferrous salt, EDTA, phosphate buffer, and molecular oxygen.[82]

Further evidence for the carbinol intermediate arises from the isolation of N-hydroxymethyl intermediates as their glucuronides. The major *in vivo* metabolite of the herbicide diphenamid (83) is the N-demethylated product nordiphenamid (84). The O-glucuronide of N-methyl-N-hydroxymethyldiphenylacetamide (85) has been isolated from the urine of diphenamid-dosed rats. Apparently, the unstable N-hydroxymethyl compound

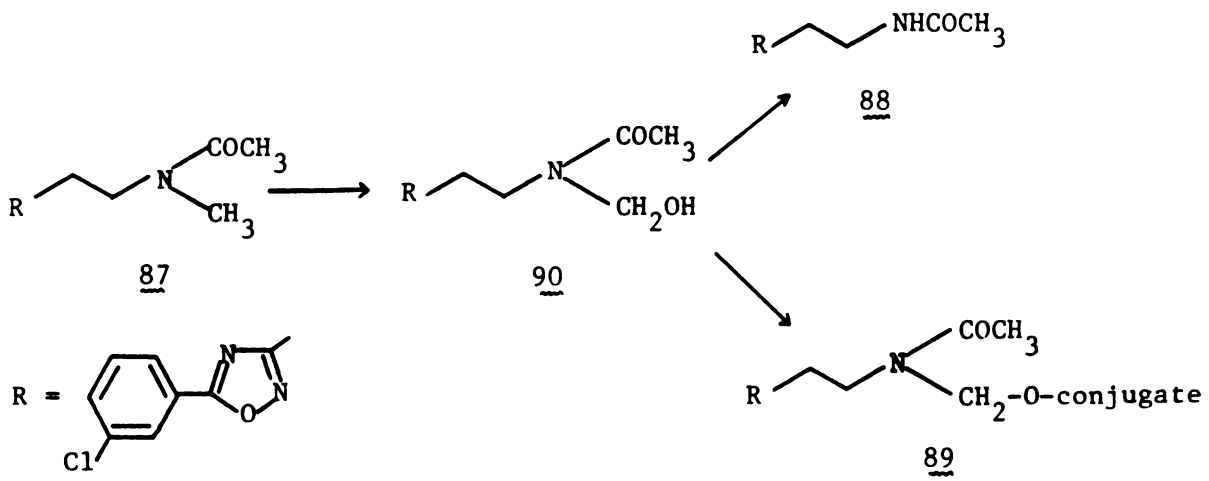


81 80 82

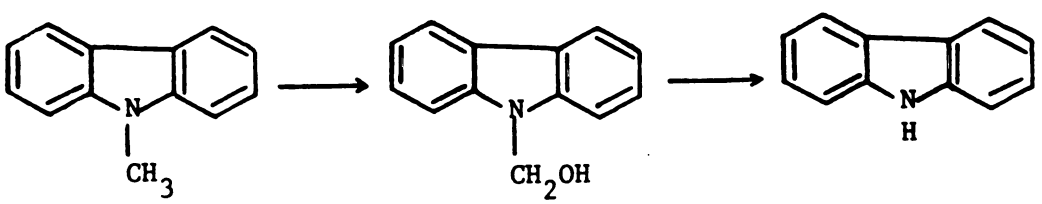
86, a possible intermediate in the N-demethylation reaction, is stabilized by glucuronide formation.[83]



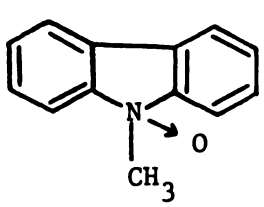
Investigations into the metabolism of the anticonvulsant oxadiazole (87) have shown that the N-demethylated secondary amide 88 is a major urinary metabolite in humans. The isolation of the glucuronide or sulfate conjugate of the N-hydroxymethyl metabolite 89 from human urine is evidence for a carbinol intermediate 90 in the N-dealkylation reaction.[84]



Not only is the evidence for carbinol intermediates documented in the metabolism of N-methylamide type compounds but also in the metabolism of aromatic N-methylamines. N-Hydroxymethylcarbazole (91) has been isolated as a metabolic intermediate in the in vivo N-demethylation of N-methylcarbazole (92). The N-demethylated product, carbazole (93), has been well characterized in vivo and in vitro. The hydroxymethyl product has been isolated and identified in vitro and is found as the glucuronide conjugate in vivo. It appears that the delocalization of the nitrogen lone pair by the two aryl moieties enhances the stability of 91. The finding that more formaldehyde is produced from the incubation of N-(hydroxymethyl)carbazole (91) than from N-methylcarbazole (92) indicates that the formation of the former may be rate-limiting in N-demethylation.[85] Furthermore, the N-oxide 94 was not detected as a metabolite in these experiments;[85] but then, one would not expect N-oxidation to occur to any great extent due to the delocalization of the nitrogen lone pair into the aromatic rings. So, with systems containing sp^2 hybridized nitrogen, reasonable evidence supports the α-carbon hydroxylation hypothesis for N-dealkylation.

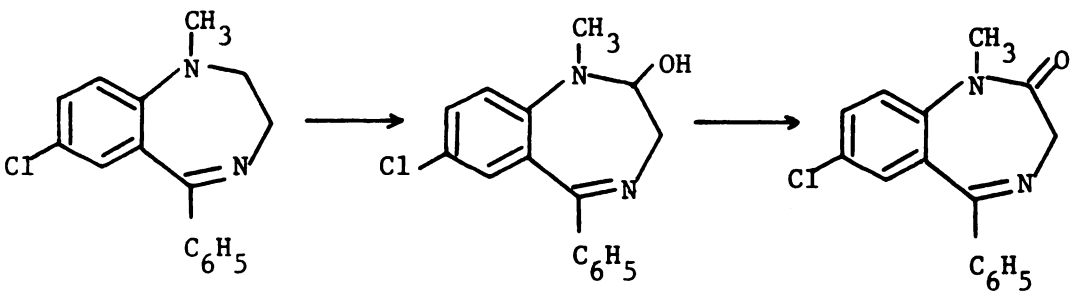


92 91 93



94

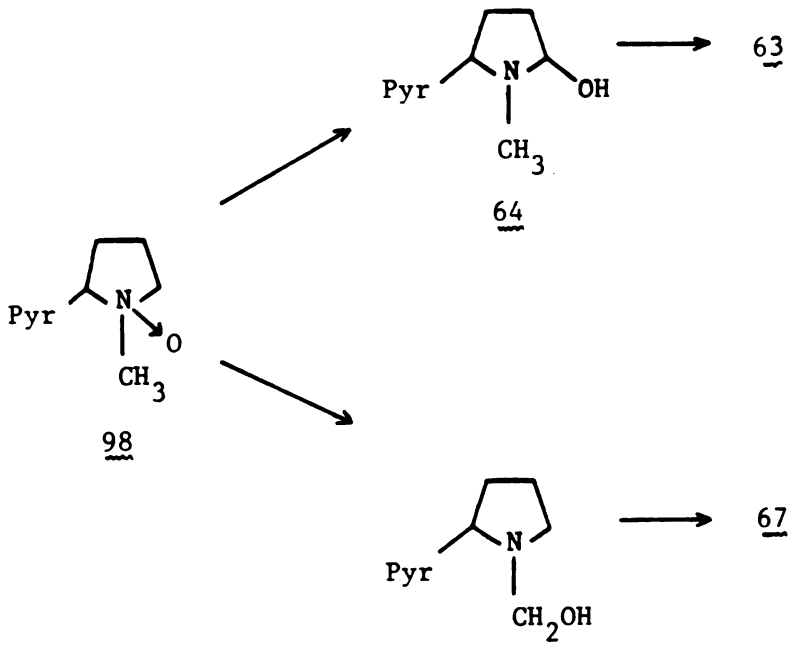
A carbinolamine has been isolated from the in vitro metabolism of the cyclic amine, medazepam (95). The oxidation of cyclic amines to lactams can be classified as a biotransformation reaction which involves initial α-carbon hydroxylation. As will be discussed later concerning pyrrolidine ring metabolism, a second enzyme system may be responsible for the conversion of the carbinol intermediate to the carbonyl product. Apparently, a rat liver microsomal enzyme can oxidize the initial 2-hydroxy medazepam metabolite 96 to the corresponding lactam, diazepam (97). Dog liver lacks this enzyme and 96 is metabolized via alternate pathways. This inability to metabolize 96 to diazepam has led to the isolation of the carbinolamine 96 as a metabolite from dog liver preparations.[86]



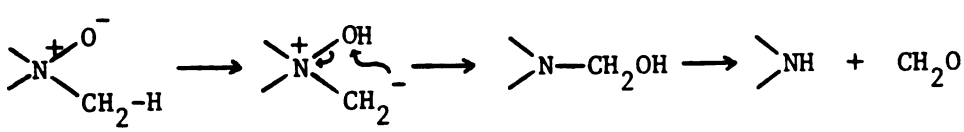
95 96 97

As discussed earlier, nicotine (60) is another example of a cyclic amine which is postulated to undergo a two step four-electron oxidation to the lactam cotinine (63).[73] Nicotine is also known to under oxidation to nicotine N'-oxide (98). Incubation of nicotine N'-oxide in a metal complex system [iron (III)-tartaric acid] yields nornicotine and formaldehyde as the major products. In order to rationalize the generation of nornicotine, cotinine, and other known nicotine metabolites by this chemical system, rearrangements of the N-oxide involving available protons on the carbon(s) α to the N-oxide nitrogen have been

proposed. It is postulated nicotine N'-oxide (98) rearranges to the N-(hydroxymethyl)nornicotine (66) and the 5'-hydroxynicotine (64) intermediates to give respectively, nornicotine (67) and cotinine (63).[87]

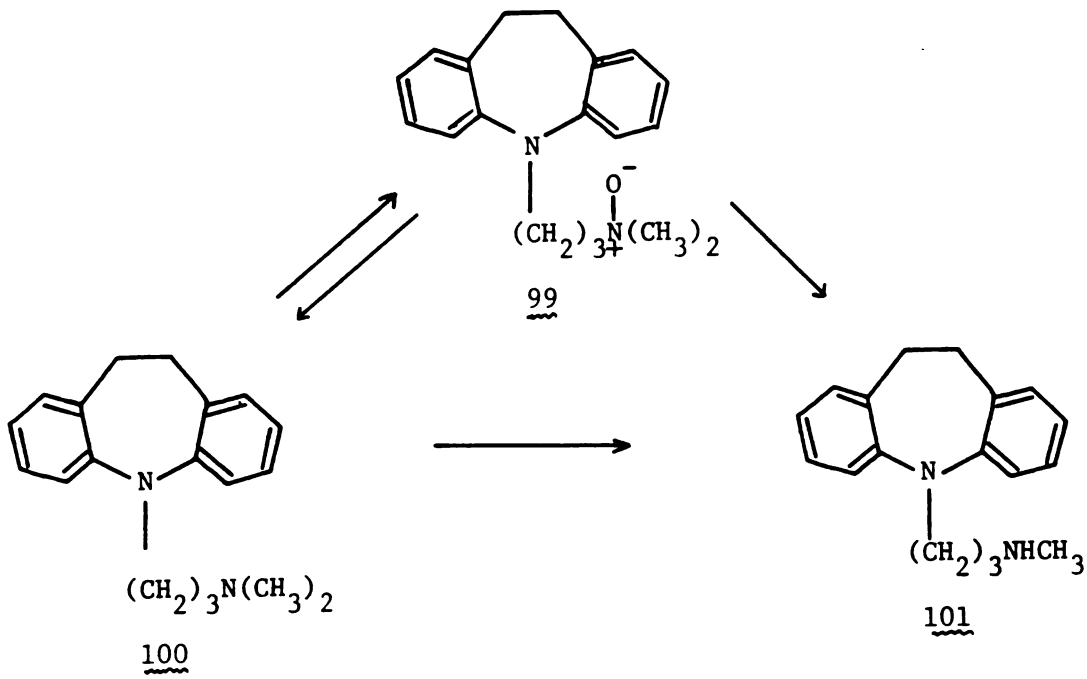


The involvement of the N-oxide in dealkylation might be described in the following manner. The tertiary amine is oxidized to an N-oxide which can rearrange to a carbinolamine; the carbinolamine in turn fragments to the dealkylated amine. The rearrangement of the N-oxide to the carbinolamine might proceed via an intramolecular migration of the oxygen from the nitrogen to the adjacent α-carbon.[88]



Another possible role for the N-oxide in N-dealkylation involves its reduction back to the tertiary amine. The tertiary amine can then undergo carbon hydroxylation and dealkylation. Imipramine N-oxide (99) is known to undergo in vitro metabolic reduction to imipramine (100) and

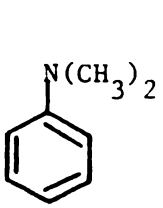
demethylation to desmethylimipramine (101). Both reactions are mediated by an extramicrosomal enzyme system.[89] However, because N-oxidation of imipramine is small compared to direct N-dealkylation of 100 and because the rate of N-oxide reduction is rapid, the demethylation of imipramine N-oxide is considered negligible in the metabolism of imipramine.[90]



99 100 101

A number of other N-oxides have been detected as metabolites of tertiary amines which would be expected to readily N-demethylate. An example is the metabolism of the extensively-studied N,N-dimethylaniline (102). N,N-Dimethylaniline-N-oxide (103) has been detected in a microsomal incubation of 102. This conversion is dependent on NADPH and O_2.[91] It is postulated that the N-oxide is an intermediate which is acted upon by N-oxide demethylase to give N-methylaniline (104) and formaldehyde. This conjecture is compatible with the observation that the N-oxide is demethylated at a rate greater than the overall oxidative demethylation rate of 102.[92] Further studies on the metabolism of N,N-dimethylaniline in pig liver microsomes indicate the involvement of two reactions. Ini-

tially, a NADPH or NADH and O_2 dependent N-oxide synthetase is involved in the generation of the N-oxide; then, an N-oxide dealkylase completes the demethylation reaction. The observation that only the N-oxide is found in the presence of SKF-525A or carbon monoxide is consistent with the proposed two-step mechanism.[93]



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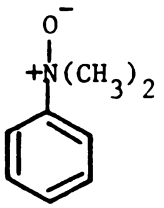
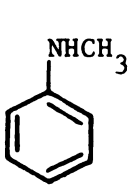


103



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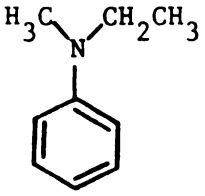
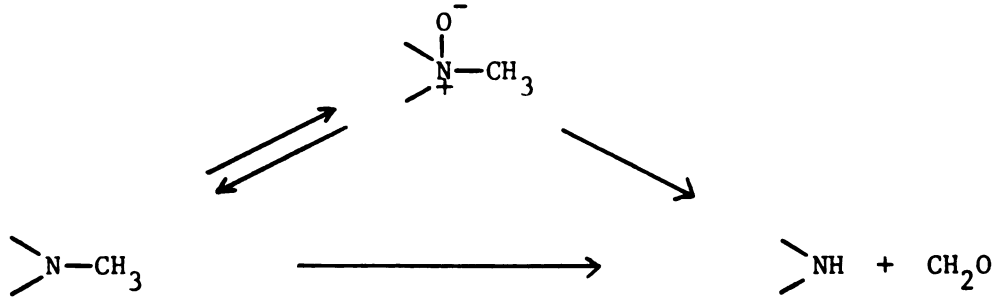


105

The participation of the N-oxide in metabolic dealkylations is summarized in the following scheme:[94]



Although the involvement of the N-oxide intermediate in N-dealkylation of tertiary amines is feasible, evidence is lacking for N-oxidized intermediates in the general scheme of N-dealkylation. Many compounds which readily undergo N-dealkylation do not appear to form N-oxide products. For example, as noted above, amides, which are unlikely to form N-oxidation products do undergo oxidative N-dealkylation.[95] Other examples include imides and carbamates.[96]

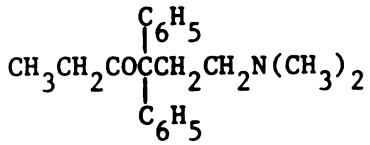
Beckett[97] and Bickel[94] have suggested that N-oxidation and α-carbon hydroxylation are distinct, nonrelated metabolic reactions. They maintain that oxidation at nitrogen may or may not lead to dealkylation

whereas oxidation at the α carbon leads to N-dealkylation *via* the carbinolamine. The two reactions are alternate pathways available for the oxidative metabolism of a tertiary amine. The existence of two alternate paths would explain why, despite the NADPH dependence of both paths, α-carbon hydroxylation (dealkylation) is mediated by a microsomal cytochrome P-450 enzyme system whereas N-oxidation is usually catalyzed by a microsomal flavoprotein enzyme system.[89]

The co-existence of the α-carbon oxidation and nitrogen oxidation pathways was demonstrated in studies on the effects of various modifications of the incubation media on the *in vitro* metabolism of N,N-dimethylaniline (102)[98] and N-ethyl-N-methylaniline (105).[99] The formation of formaldehyde from N,N-dimethylaniline was affected by various chemical agents in a manner dissimilar to the influence of the same agents on the demethylation of N,N-dimethylaniline N-oxide, thus supporting the postulated theory.[98] The effects of various agents on the N-dealkylation and N-oxidation of 105 led to the same conclusion.[99] Furthermore, many N-oxides apparently do not undergo direct dealkylation and tertiary amine dealkylation rates are generally greater than the combined rate of N-oxide formation and rate of N-oxide dealkylation.[89] An exception to these generalizations is the case of N,N-dimethylaniline-N-oxide (103). Studies on the rate of formation and the rate of demethylation of 103 have clearly demonstrated that this N-oxide may act as an intermediate in the oxidative demethylation of N,N-dimethylaniline.[91,92]

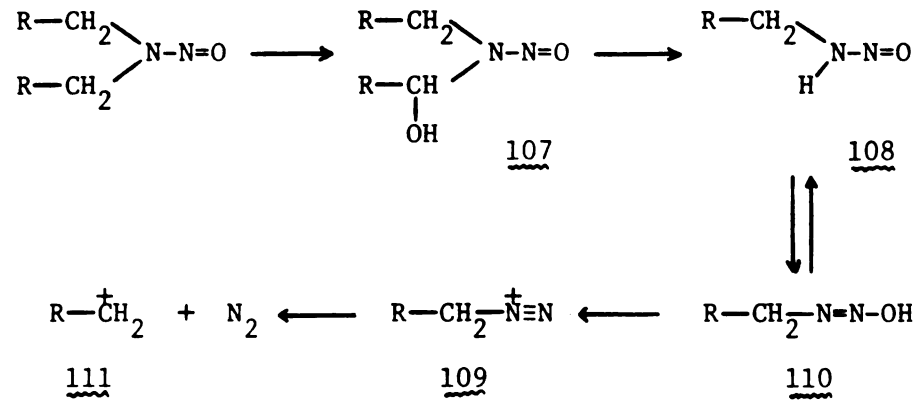
The use of enzyme inhibitors which selectively inhibit one of the two enzymatic paths seems to demonstrate the coexistence of the two metabolic pathways most convincingly. The effect of various metabolic inhibitors on the N-demethylation and N-oxidation of normethadone (106)

in liver microsomal preparations was examined. In particular, the data showed that SKF 525A inhibited N-dealkylation (14% of control) but did not affect N-oxide formation whereas dithiothreitol blocked N-oxidation (16% of control) without affecting N-dealkylation.[100] The selective inhibition of N-oxide and lactam (via α-carbon hydroxylation) formation from nicotine has also been observed. Cysteamine and dithiothreitol effectively (11% and 58% of control, respectively) block formation of nicotine N'-oxide without affecting cotinine formation.[101] Thus, it is generally accepted that N-oxidation and α-C-hydroxylation are two independent metabolic pathways.

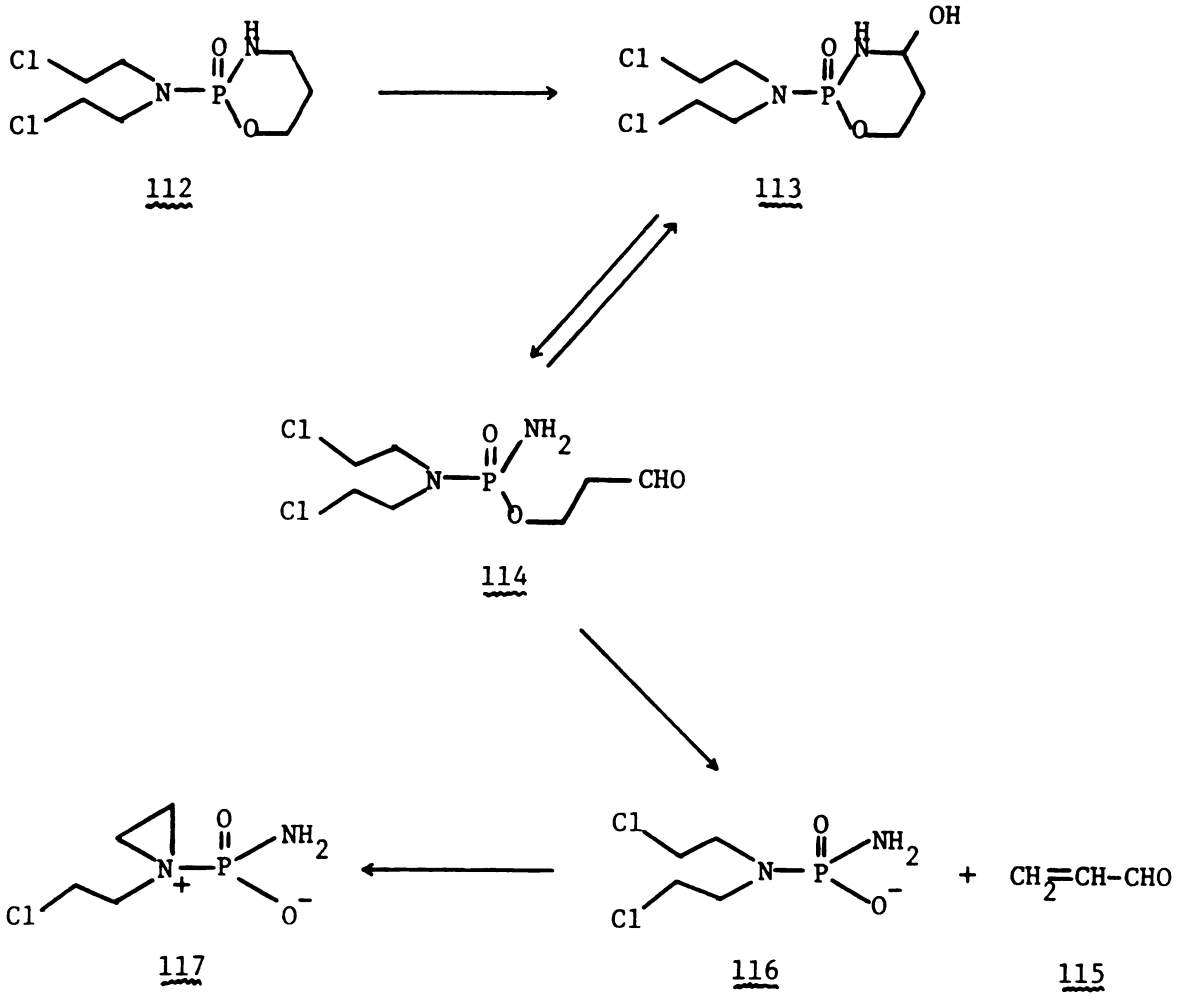


106

As discussed in Section II.B., the α-carbon hydroxylation process produces a carbinol intermediate which may be converted directly to a reactive electrophilic metabolite. In addition, α-carbon hydroxylation is known to be the premiere step in the bioactivation sequence of other xenobiotics. It is known that the carcinogenic N-nitrosamines are activated by microsomal mixed function oxidases. The critical step mediated by cytochrome P-450 is a α-carbon hydroxylation reaction to produce the unstable carbinolamine 107 which subsequently dealkylates to yield a carbonyl compound and the labile monoalkylnitrosamine 108.[102] The dealkylated product rearranges to the unstable alkyldiazo intermediate 109 possibly via the diazohydroxide species 110. Ultimately, the diazo compound 109 decomposes to gaseous nitrogen and the carbonium ion 111 that serves as the active alkylating species.[103]



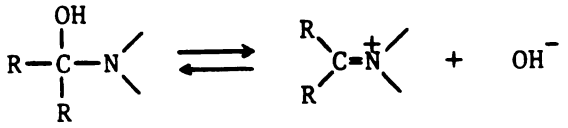
One last example illustrating the importance of α-carbon hydroxylation is the metabolic activation of the antitumorigenic agent cyclophosphamide (112). Metabolic hydroxylation is the key transformation which initiates a series of reactions leading to the ultimate active species. In this case, mixed function oxidase mediated α-carbon hydroxylation produces a carbinolamide 113 which undergoes N-dealkylation to the open chain amino-aldehyde 114. This unstable intermediate eliminates acrolein (115) and forms N,N-bis(2-chloroethyl)-phosphorodiamidic acid (116).[104] It is believed that the phosphorodiamidic acid is responsible for the antitumor properties of cyclophosphamide. Presumably 116 alkylates macromolecules via the electrophilic aziridinium species 117.[105]

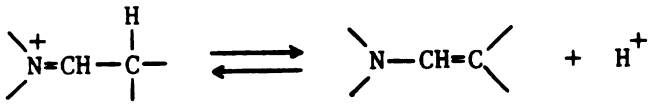

Cl
Cl
N
P
O
H
N
O
112
OH
113
NH2
CHO
114
117
116
+
CH2=CH-CHO
115

CHAPTER III

THE CHEMISTRY OF THE IMINIUM INTERMEDIATE

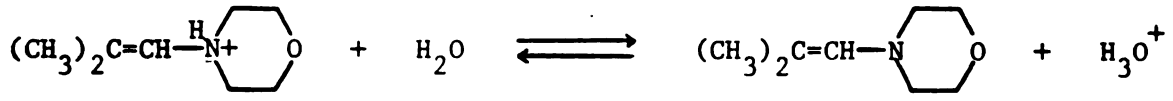
The evidence presented thus far strongly suggests that the electrophilic intermediate generated from the metabolism of tertiary aliphatic amines is an iminium species. The iminium species exists in a reversible equilibrium with the carbinolamine. In addition, the iminium species is actually the conjugate acid of the corresponding enamine. Several reports in the literature describe the hydrolysis of enamines. Primarily, these are kinetic studies concerned with the rates of hydrolysis of various enamines. Since iminium and carbinolamine species are believed to be involved, the results of these studies will be briefly reviewed.



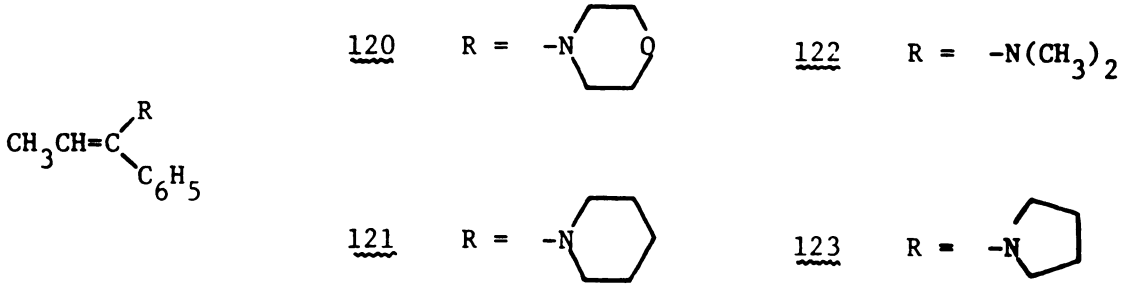


The hydrolysis of enamines has been reviewed.[106] In neutral or basic solution the hydrolysis of enamines has been found to be pH dependent. Results obtained from rate measurements for the hydrolysis of 4-(2-methylpropenyl)morpholine (118) are explained in the terms of an equilibrium between the free enamine 118 and the nitrogen protonated species 119.[107] Protonation of the enamine leads to the formation of the enammonium species which then rearranges rapidly to the more stable, β-carbon protonated iminium ion. The formation of the iminium ion by β-carbon protonation (Eq. B) is believed to be the rate-limiting step in the hydrolysis of

propiophenone enamines (120, 121, 122, and 123) in basic (pH > 10) medium.[108]



119 118



120 R = -N O 122 R = $-N(CH_3)_2$

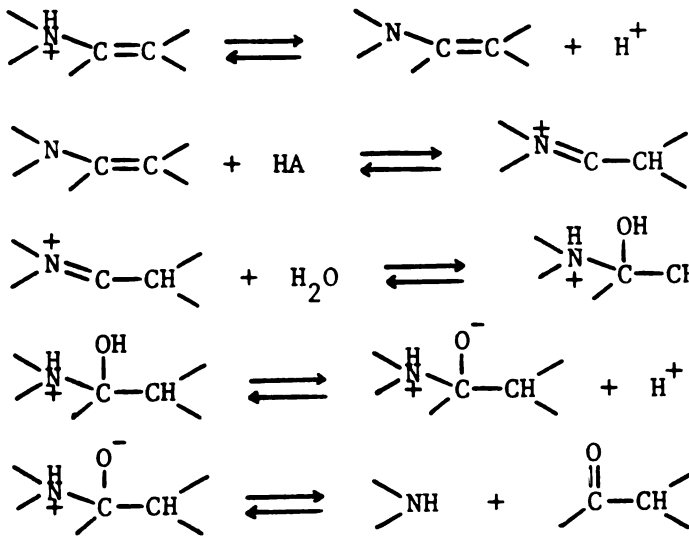
121 R = -N 123 R = -N

In neutral (pH 6-10) medium, the formation of the iminium species is the rate determining step (Eq. B) for the hydrolysis of 118 which is general acid-catalyzed from pH 4 to 9.[106] Whereas, in the case of the propiophenone enamines, the rate limiting step of hydrolysis below pH 8 is now the uncatalyzed attack of water on the iminium ion (Eq. C). In this pH range (6-10) a buildup of the iminium ion is observed; obviously, this iminium species exists in rapid equilibrium with the enamine at this stage.[108]

In the weakly acidic pH range (1-6), the hydrolysis of 118 has changed so that the rate limiting step is the general base-catalyzed hydration of the predominant iminium ions to the carbinolamine (Eq. C).[106] Similarly, the rate of hydrolysis of the propiophenone enamines is determined by water attack on the iminium species in this pH range. As will be discussed, a similar phenomenon is encountered in the hydrolysis of Schiff's bases.[108]

At strongly acidic pH's (pH < 1), the hydrolysis of both compounds

behaves similarly; transition in the rate-limiting step from hydration of the iminium ion (Eq. C) to decomposition of the resulting carbinolamine (Eq. E) occurs. The N-protonated carbinolamine apparently loses a proton to become a zwitterionic species (Eq. D) before rate-limiting decomposition to carbonyl compound and amine.[108,109]



Equation (A)

(B)

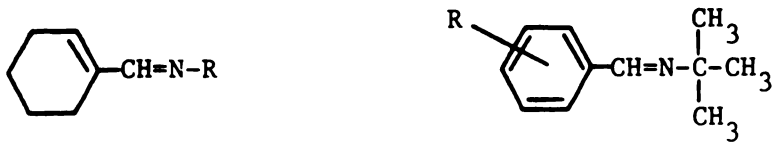
(C)

(D)

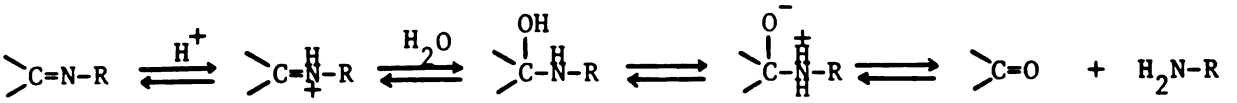
(E)

The hydrolysis of a series of Schiff bases derived from cyclohexene-1-carboxaldehyde 124[110] and a series of para- and meta- substituted benzylidene-1, 1-dimethylethylamines 125[111] has been investigated over a wide pH range. At neutral or basic pH's, the rate-limiting step for the hydrolysis of these Schiff bases is the formation of the carbinolamine 126. Above pH 9, the formation of the carbinolamine occurs via the rate-limiting attack of OH^- on the iminium species 127. In the pH range from 4 to 9, it is primarily the attack of H_2O on the protonated Schiff base which is the rate-limiting step. At pH 4, a transition occurs; the rate-limiting step is no longer the formation of the carbinolamine from H_2O attack on the now fully protonated Schiff base but the decomposition of the carbinolamine. The iminium species 127 and the carbinolamine 126 are in rapid equilibrium; a proton transfer occurs to form the zwitterion 128 and the breakdown of

128 becomes the rate determining step.



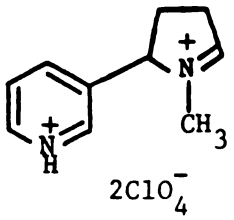
124 125



127 126 128

These results seem to indicate that in the reverse reaction for the formation of the iminium species, the dehydration of the carbinolamine is the rate-limiting step. In the physiological pH range, the carbinolamine will most likely disproportionate to the carbonyl compound and the amine. But dehydration to the iminium species does occur; the resulting iminium species exists in a rapid equilibrium with its proton tautomer, enamine, and has been shown to build up rapidly in the physiological pH range.

The behavior of the diperchlorate iminium salt of nicotine 129 has been recently examined.[112] Only the iminium form 65 was observed in acidic or neutral solutions and only the carbinolamine 64 was observed in strongly basic solutions. Only at pH < 1 was the iminium ion found to be stable; in less acidic solutions, dimerization to form 130 occurs. The formation of 130 probably results from the nucleophilic attack of the enamine species 131 on the corresponding iminium ion.[113]



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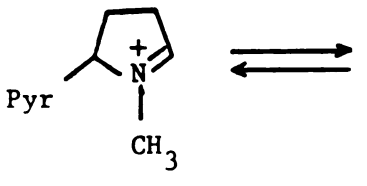
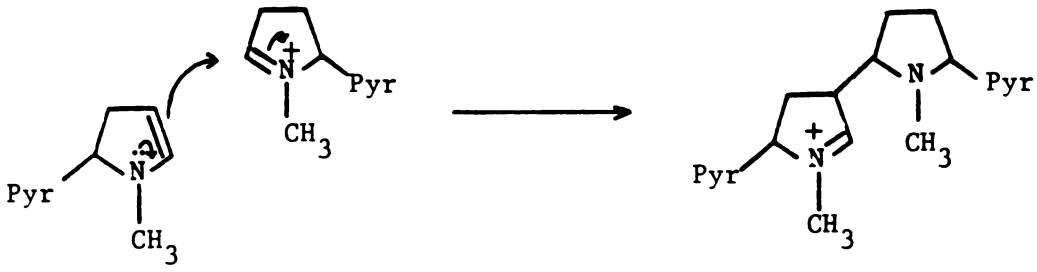
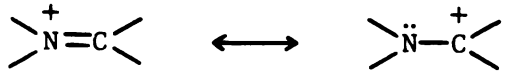


65 64



131 130

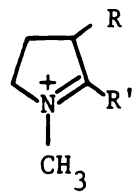
The reactivity of iminium ions towards nucleophilic agents has been reviewed;[114] the reactivity of methyleniminium salts in particular has also been recently reviewed.[115] The electrophilicity of iminium ions is mostly due to the positive charge residing on the α-carbon; this can be most clearly seen in the resonance form of the iminium ion.



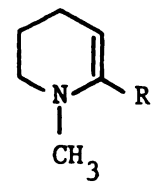
The reactivity of iminium ions is such that almost all nucleophiles will react and only the stability of the product determines whether such adducts can be isolated.[116]

The reduction of iminium salts by hydride ion can be achieved by a number of reducing agents. Lithium aluminum hydride, sodium borohydride, Zn-HOAc, and diborane are a few of the reagents reported to reduce iminium

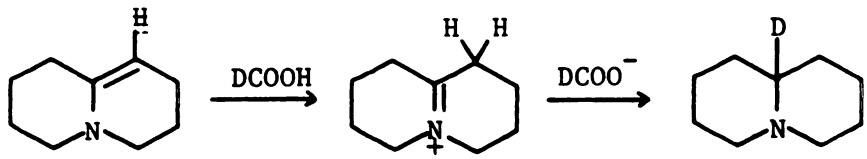
salts to the corresponding amines.[117] One interesting reaction is the reduction of a series of iminium salts 132 and enamines 133 by formic acid. By examining the action of various deuterium-labeled formic acids (DCOOH, DCOOD, HCOOD) on $\Delta^{1(10)}$-dehydroquinolizidine (134), Leonard and Sauers determined that the enamine is initially and reversibly protonated at the β-carbon to give an iminium species 135 which is subsequently reduced by hydride ion.[118]



132 R, R' = H or Alkyl

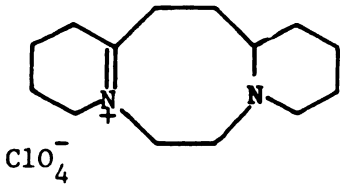


133 R = Alkyl



134 135

The reaction of methyleniminium salts with C-H acidic methine or methylene compounds has been reviewed.[115] Similar to other simple iminium compounds, the methyleniminium salts also react with organometallic reagents to form C-alkylated products. The reactivity of iminium salts with Grignard reagents is well known; alkyl-lithium reagents react in the same way. Example of this C-alkylation are found in the reaction of the iminium salts, $\Delta^{1(6)}$-dehydrosparteinium perchlorate (136), $\Delta^{5(10)}$-dehydroquinolizidinium perchlorate (137), and $\Delta^{4(9)}$-hexahydropyrrocolinium perchlorate (138), with Grignard reagents to give 139, 140, and 141, respectively.[119,120]



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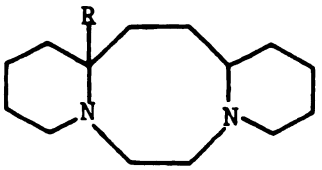
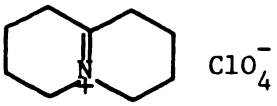


139 R = CH_3, C_2H_5, n-C_3H_7, n-C_4H_9, $CH_2C_6H_5$

149 R = CN



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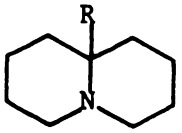
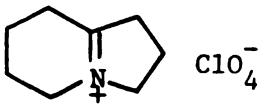


140 R = CH_3, C_2H_5, n-C_3H_7, $CH_2C_6H_5$

150 R = CN



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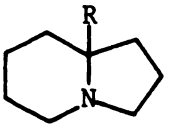


141 R = CH_3, C_2H_5, n-C_3H_7, $CH_2C_6H_5$

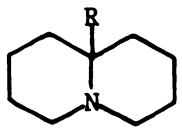
151 R = CN

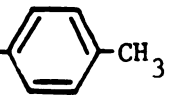
Methyleniminium salts are reported to undergo reaction with mercaptans or thiophenols to yield dialkylaminomethyl thioethers 142.[115] $\Delta^{5(10)}$-Dehydroquinolizidinium perchlorate is also reported to react with the potassium salts of p-thiocresol and α-napthylmethylmercaptan to give 10-(p-thiocresyl)-quinolizidine (143) and 10-(α-napthylmethylmercapto)-quinolizidine (144) respectively. Both mercapto products are unstable and tend to decompose to $\Delta^{1(10)}$-dehydroquinolizidine (145). Efforts to isolate analogous quinolizidine products with potassium alkoxides and phenoxides failed due to the intability of the products.[120] But methylenium

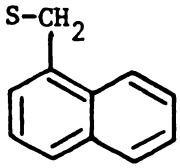
salts do react with alcohols and phenols to form the corresponding dialkylaminomethyl ethers 146.[115]

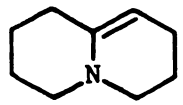
$$R_2\overset{+}{N}{=}CH_2 + R'SH \longrightarrow R_2N\text{-}CH_2\text{-}SR'$$

142



143 R = 

144 R = 

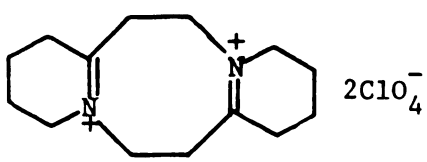


145

$$R_2\overset{+}{N}{=}CH_2 + R'OH \longrightarrow R_2N\text{-}CH_2\text{-}OR'$$

146

The nucleophile cyanide ion reacts with iminium salts to give isolable adducts. Leonard has shown that iminium salts such as 136, 137, 138, $\Delta^{1(6),11(16)}$-didehydrosparteinium diperchlorate (147), and 1,2-dimethyl-Δ^{1}-pyrrolinium perchlorate (148) react with cyanide ion to give the respective cyano addition products 149, 150, 151, 152, and 153.[119,120,121]



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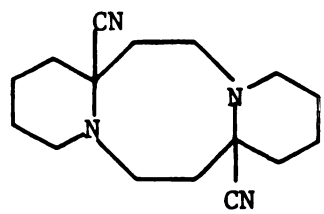

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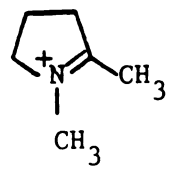

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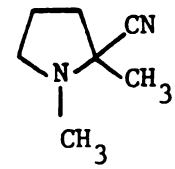
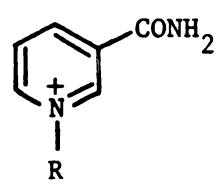
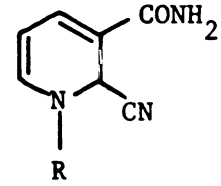
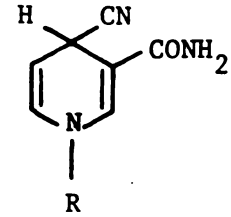


153

In a reaction that will be of interest in our proposed studies, cyanide is reported to form a complex with a special iminium system found in N-substituted nicotinamides. Colowick et al. have observed that nicotinamide adenine dinucleotide (154, NAD^+) and N-methylnicotinamide (155) react with KCN to form a detectable complex.[122] They propose that the addition of cyanide occurs at the 2-position of the pyridinium ring to give the cyano complex 156. All of the NAD^+ is converted to the complex by 1.0 M KCN in less than 1 minute at pH 11 (25°); whereas under the same conditions at 0.01 M KCN, it takes 20 minutes for equilibrium to reach 50% complex formation. At a lower pH of 8.2, 100% conversion to the complex requires 10 minutes at a KCN concentration of 1.0 M and 50% conversion requires 50 minutes at 0.1 M KCN. It was also observed that the cyano-complex is unstable and dissociates rapidly to NAD^+ and cyanide when the cyanide concentration is lowered 1000-fold from 1.0 M. Later studies involving deuterium-exchange experiments argue for cyanide attack at the 4-position of NAD^+ to give 157.[123]







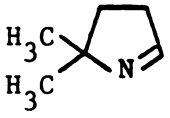
154 R = Adenine dinucleotide

155 R = CH_3

156

157

Other studies demonstrated that addition of HCN to the Schiff bases, 5,5-dimethyl-Δ^1-pyrroline (158) or 4,5,5-trimethyl-Δ^1-pyrroline (159), afforded the corresponding cyano adducts 160 and 161.[124]



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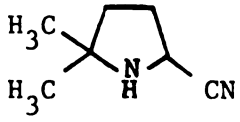

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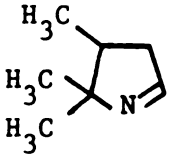

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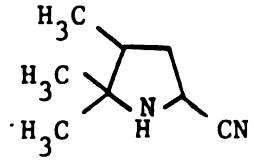
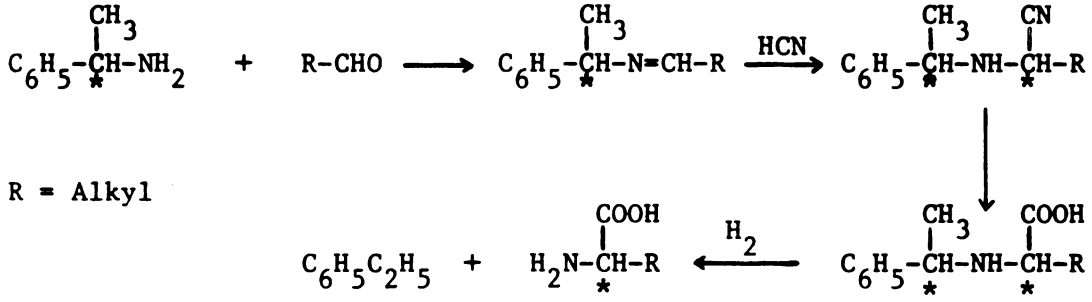


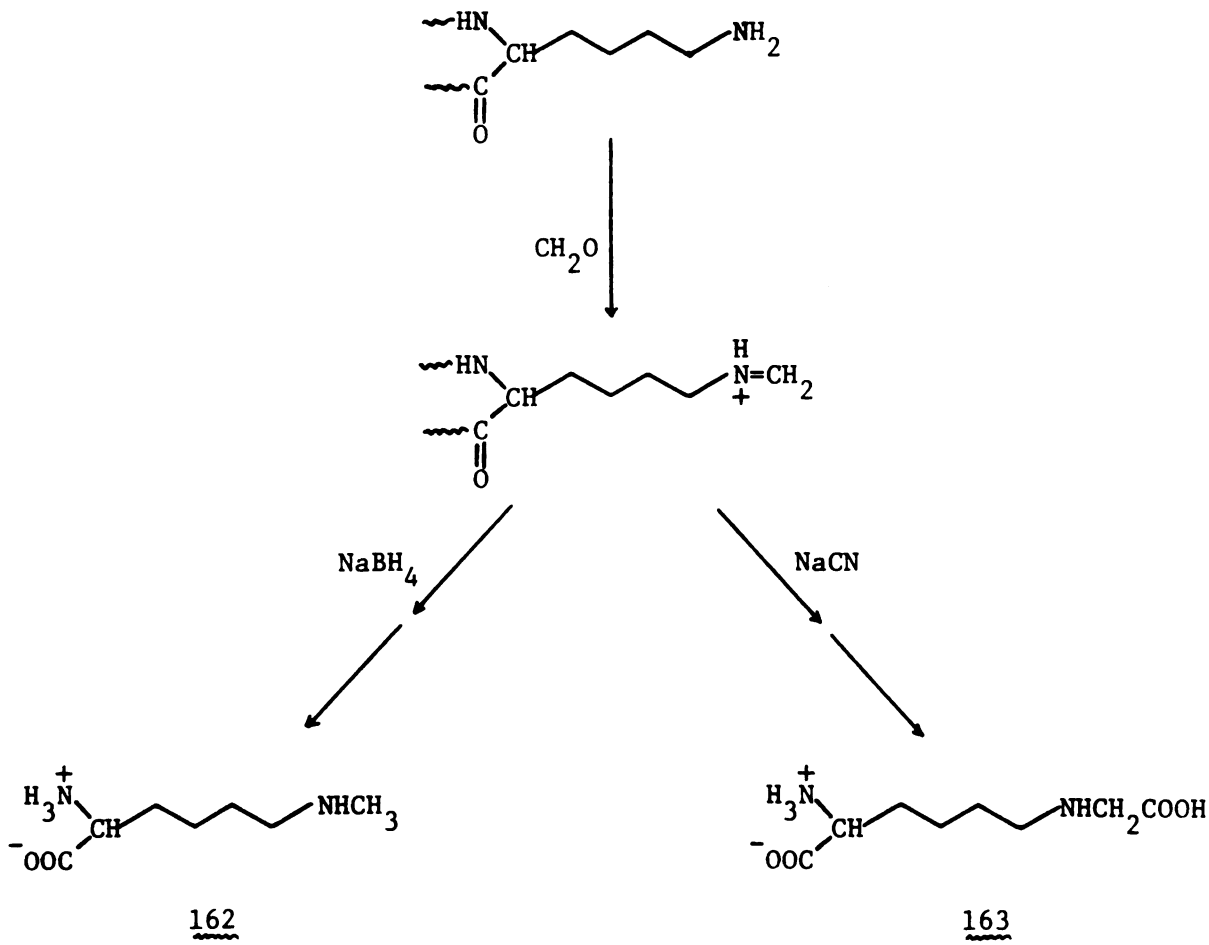
161

The reactivity of cyanide with Schiff bases was utilized by Patel and Worsley in a modified Strecker synthesis to generate optically active α-amino acids in good yield.[125] The synthetic scheme is shown below; the addition of HCN to the Schiff base has been observed to be stereoselective but a satisfactory mechanism has not been proposed.



Cyanide has also been shown to form adducts with Schiff bases involved in enzyme mediated reactions. It has been clearly demonstrated that 2-keto-4-hydroxyglutarate aldolase is inactivated in the presence

of stoichiometric amounts of formaldehyde and $NaBH_4$ or NaCN. Apparently, formaldehyde forms a Schiff base with a reactive amine moiety of the enzyme; subsequently, addition of $NaBH_4$ reduces the Schiff base to an isolable adduct which has been identified as N^6-methyllysine (162). Analogously in the presence of cyanide, it is most likely that a cyanomethylamine is formed. Evidence for this cyano complex is the 1:1:1 stoichiometry of the complex (enzyme:formaldehyde:cyanide) and the oxidation and identification of N^6-carboxymethyllysine (163) from the acid hydrolysis of the enzyme/formaldehyde/cyanide complex.[126] A postulated scheme to explain these results is presented below:



162 163

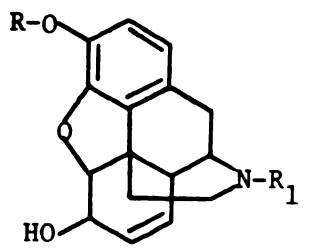
In addition to inhibiting enzymes which function *via* a Schiff base mechanism, cyanide is known to inhibit other enzyme-catalyzed reactions. Cyanide can cause toxic effects by complexing the metal ion in cytochrome oxidase and methemoglobin and it can inhibit other enzymes by cyanohydrin formation.[126]

The effect of cyanide ion on cytochrome P-450 mediated mixed function oxidase systems is unclear and has not been extensively studied. It is known that cyanide interacts with cytochrome P-450 to give a modified type II difference spectrum.[127,128] More recent work has demonstrated the existence of three forms of cytochrome P-450 in rat liver microsomes which have different binding affinities for cyanide.[129]

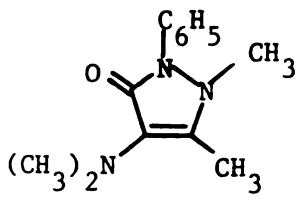
The literature contains numerous reports of the inhibitory or non-inhibitory effects of cyanide on the *in vitro* metabolism of various compounds. Cyanide (1 mM) has little or no effect on ethylmorphine (164) N-demethylation.[130] It does not inhibit the oxidative demethylation of aminopyrine (165) at 0.5 mM concentrations[131] nor does cyanide affect the formation of nicotine N-oxide (98) from nicotine. Cyanide (1 mM) does not affect the N-oxidation of normethadone (106) but it does inhibit (20-25%) the demethylation of this compound.[100] Para-hydroxylation of *o*-chloroaniline (166) is 69% inhibited by 2 mM KCN.[132] Cyanide (10 mM) causes 65% inhibition of the formation of cotinine (63) from nicotine;[101] cyanide is proposed to act in this case as an aldehyde oxidase inhibitor preventing the oxidation of the 5'-hydroxynicotine intermediate (64).[73] But the formation of 5'-cyanonicotine (61) as a competitive reaction to cotinine formation was not considered.

More recent studies have shown that at a concentration of 1.0 mM, cyanide inhibits the hydroxylation of aniline (167) by 64% and the N-

demethylation of aminopyrine (165), codeine (168), and ethylmorphine (164) by 39%, 37%, and 38%, respectively.[133] At 10 mM cyanide concentration, the N-demethylation of aminopyrine, ethylmorphine, and methamphetamine (169) is inhibited by 78%, 79%, and 90%, respectively.[134]



164	R = CH_2CH_3; R_1 = CH_3
164-d_3	R = CH_2CH_3; R_1 = CD_3
168	R = CH_3; R_1 = CH_3
244	R = H; R_1 = CH_3
244-d_3	R = H; R_1 = CD_3



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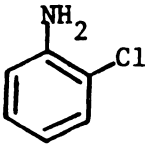

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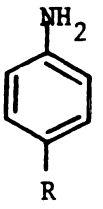
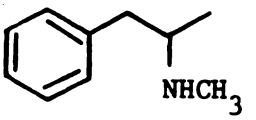


167 R = H

170 R = OH



169

Evidence has been presented for a "cyanide sensitive factor" in the desaturation of fatty acids by liver microsomal systems. It is believed that this cyanide sensitive factor is the terminal component, an oxygen activating enzyme, in the desaturase system. This fatty acid desaturation system consists of a NADH-cytochrome b_5 reductase, NADPH-cytochrome c reductase, and cytochrome b_5 in addition to the cyanide sensitive factor. However, the system is not believed to involve cytochrome P-450.[135,136]

The hydroxylation of aniline (167) to p-aminophenol (170) by liver microsomes was found to be inhibited noncompetitively by cyanide at low

aniline concentrations and competitively at high concentrations of aniline.[137] These workers have also demonstrated that cyanide inhibits the binding of aniline to cytochrome P-450 (type II) non-competitively at low aniline concentrations and competitively at high substrate concentrations. These results suggest that the primary inhibitory action by cyanide is modification of substrate-cytochrome P-450 binding accompanied by inhibition of aniline hydroxylase activity. Noncompetitive inhibition of hydroxylation and binding by cyanide suggest separate cytochrome P-450 binding sites for aniline and cyanide, perhaps a specific binding site for aniline and the sixth ligand of the heme iron for cyanide. At high substrate concentrations, aniline binds not only at its specific binding site but also competes directly with cyanide for binding at the sixth ligand of the heme iron, thus explaining the observed competitive inhibition of binding and hydroxylation.[138]

Other studies dispute these claims and suggest that cyanide inhibition is due to a reduced affinity of cytochrome P-450 to oxygen in the presence of cyanide. This lowered affinity for oxygen is believed to be a result of modification of the cytochrome P-450 molecules by cyanide which binds to the reduced form of cytochrome P-450. Inhibition of NADPH-cytochrome P-450 reductase by cyanide was also observed.[133]

More recent studies have shown that the N-demethylation of aminopyrine is 78% inhibited by 10 mM cyanide. These investigators found that cyanide inhibited the N-demethylation of aminopyrine (measured by H_2CO formation) in a noncompetitive manner and modified the binding of aminopyrine to cytochrome P-450. Formation of a cyanide-cytochrome P-450 complex is presumed to be responsible for the modification of the binding and, as a consequence, the inhibition of the N-demethylation reaction.[134] However,

these investigators did not consider the possible interaction of cyanide with reactive metabolites of aminopyrine and its effects on the dealkylation and binding of aminopyrine.

The characterization of cyano adducts from the metabolism of nicotine is significant because of the possible toxicological implications of the metabolic iminium intermediate. In order to gain further insight into the mechanism of metabolism of aliphatic amines and the nature of the metabolically generated electrophilic intermediate, we have examined the in vitro metabolism of aliphatic amines in the presence of an added nucleophile, cyanide. Cyanide ion has proven to be an effective nucleophile which reacts to form stable adducts with iminium ions in chemical systems and, as shown in the case of nicotine, in biological systems.

CHAPTER IV

1-BENZYLPYRROLIDINE

A. BACKGROUND

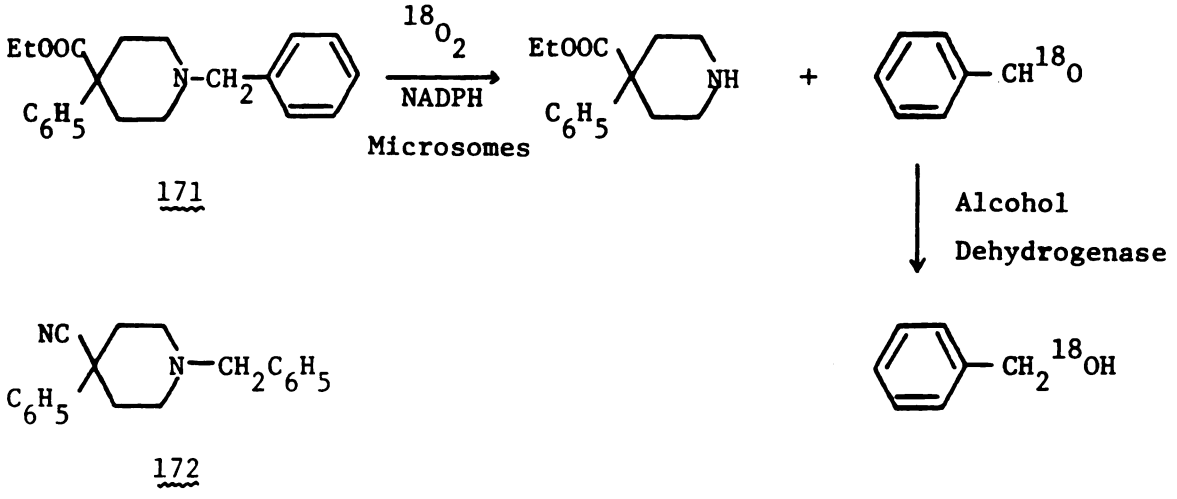
To further characterize the metabolic pathway leading to the formation of electrophilic intermediates, we examined the rabbit liver microsomal metabolism of the model tertiary amine 1-benzylpyrrolidine (1) in the presence and absence of cyanide ion. It was expected that the structural simplicity of this molecule would facilitate chemical manipulations and metabolic profile analysis. With the aid of specifically deuterium labeled analogs and gc-eims, we hoped to gain further information on the nature of the putative iminium ion intermediate.

1-Benzypyrrolidine (1) has two unique carbon atoms alpha to nitrogen which are prime targets for metabolic hydroxylation. Metabolic α-carbon hydroxylation can occur on the benzylic carbon or at C-2 of the pyrrolidine ring. The metabolism of 1 has not been previously studied but there are several examples in the literature concerning studies on the metabolism of compounds having a 1-substituted pyrrolidine ring or an N-benzyl moiety.

1. METABOLISM OF THE N-BENZYL MOIETY

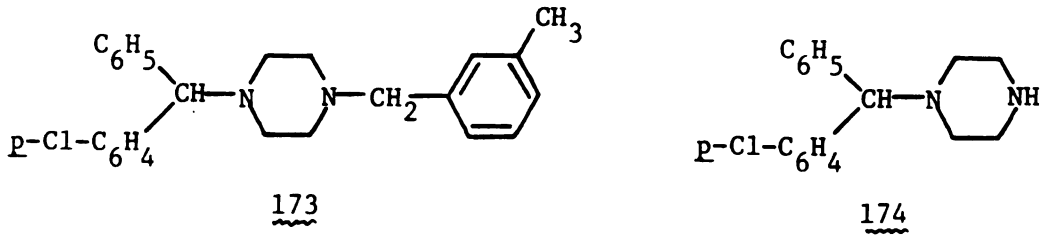
Examples in the literature of N-debenzylation are relatively few which may be a reflection of the infrequent occurence of N-benzyl groups in drugs. The debenzylation of N-benzyl-4-phenyl-4-carbethoxypiperidine (171) to yield benzaldehyde was studied to determine the source of the carbonyl oxygen in enzymatic dealkylation reactions.[76] Incubation of

171 with an NADPH generating system, rat liver microsomes, and alcohol dehydrogenase in the presence of $^{18}O_2$ yielded benzyl alcohol. The benzyl alcohol, which is the enzymatically coupled reduction product of the initially formed benzaldehyde, was found to contain 25-29% ^{18}O; the low incorporation of ^{18}O label into the product was rationalized in terms of the exchange of the benzaldehyde oxygen atom with water. McMahon interpreted these results to support direct hydroxylation of the α-carbon in the N-dealkylation process.[76] Similar experiments conducted with 1-benzyl-4-cyano-4-phenylpiperidine (172) as substrate found 78% ^{18}O incorporation in the recovered benzyl alcohol.[77]



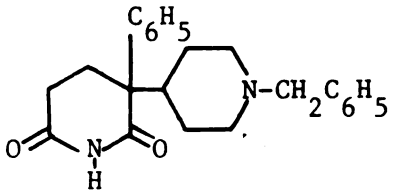
171 172

In rats treated with the teratogenic agent, meclizine (173), the apparent debenzylation product norchlorcyclizine (174), was detected in a wide variety of body tissues.[139]



173 174

When the anticholinergic agent benzetimide (175) was administered to rats, the N-debenzylated product norbenzetimide (176) was the major metabolite found in the urine (66% of total metabolites) and the feces (48%).[140]



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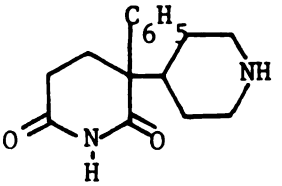
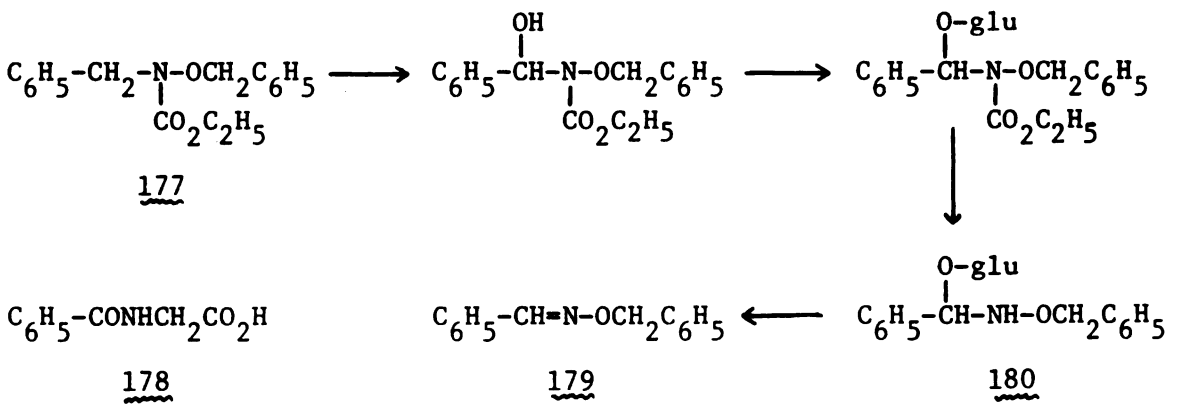


176

In vivo studies with ^{14}C-labeled ethyl-N-benzyl-N-benzyloxycarbamate (177) showed that the N-benzyl group is cleaved and oxidized to benzoic acid which is then conjugated to yield the isolated hippuric acid (178).[141] Hydrolysis of another major urinary metabolite of 177 with β-glucuronidase gave rise to O-benzyl-benzylaldoxime (179). Characterization of this hydrolysis product allowed the authors to propose the O-glucuronide structure 180 for this urinary metabolite.[142] A proposed metabolic pathway to account for the formation of 179 invokes initial α-carbon hydroxylation at the N-benzyl position.[142]

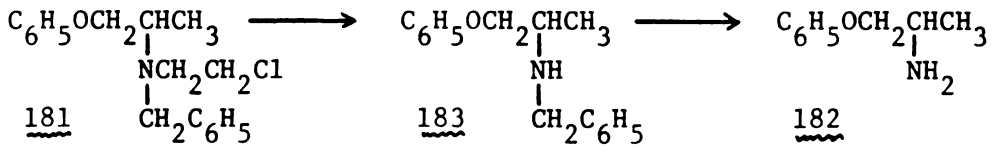


177

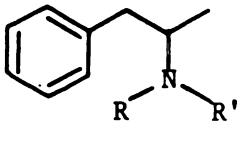
178 179 180

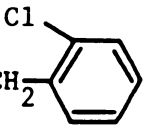
The α-adrenergic blocking agent phenoxybenzamine (181) undergoes extensive N-debenzylation in the dog. In a study on the metabolic fate of 181 in the rat, dog, and man, the N-debenzylated product 182 was

found to be a metabolite unique to the dog.[143] It was postulated that 182 results from the further metabolism of the initially formed intermediate 183.



N-benzylamphetamine derivatives are known to undergo metabolic debenzylation. For example, N-(o-chlorobenzyl)-α-methylphenylethylamine (184) is debenzylated to a slight extent (5%) to amphetamine (185).[144] The metabolism of N-benzylamphetamine (186) has been thoroughly studied; one of the major metabolic paths is that of debenzylation to amphetamine and benzaldehyde or benzyl alcohol.[97,145] However, benzphetamine (187) prefers to undergo demethylation as opposed to debenzylation. The rapid rate of formaldehyde liberation and NADPH oxidation is evidence that the hydroxylation and dealkylation of 187 proceeds swiftly.[146] The liberation of formaldehyde from benzphetamine is used as an assay for N-demethylation but the possible formation of benzaldehyde has not been examined in these cases.[147,148]





185 R = R' = H

186 R = H; R' = $CH_2C_6H_5$

187 R = CH_3; R' = $CH_2C_6H_5$

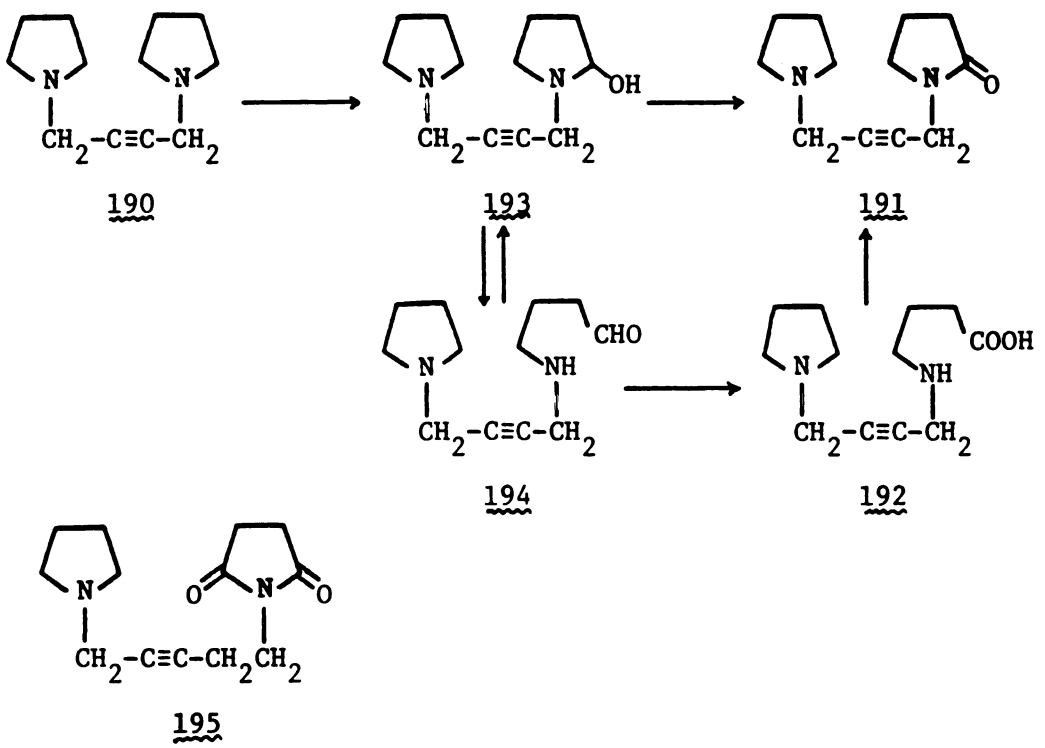
Thus, it seems that 1-benzylpyrrolidine should, on the basis of reports in the literature, undergo N-debenzylation to yield benzaldehyde and pyrrolidine. Before considering this possibility in detail, we wish

to first explore the consequences of metabolic attack on the pyrrolidine ring.

2. METABOLISM OF THE PYRROLIDINE RING

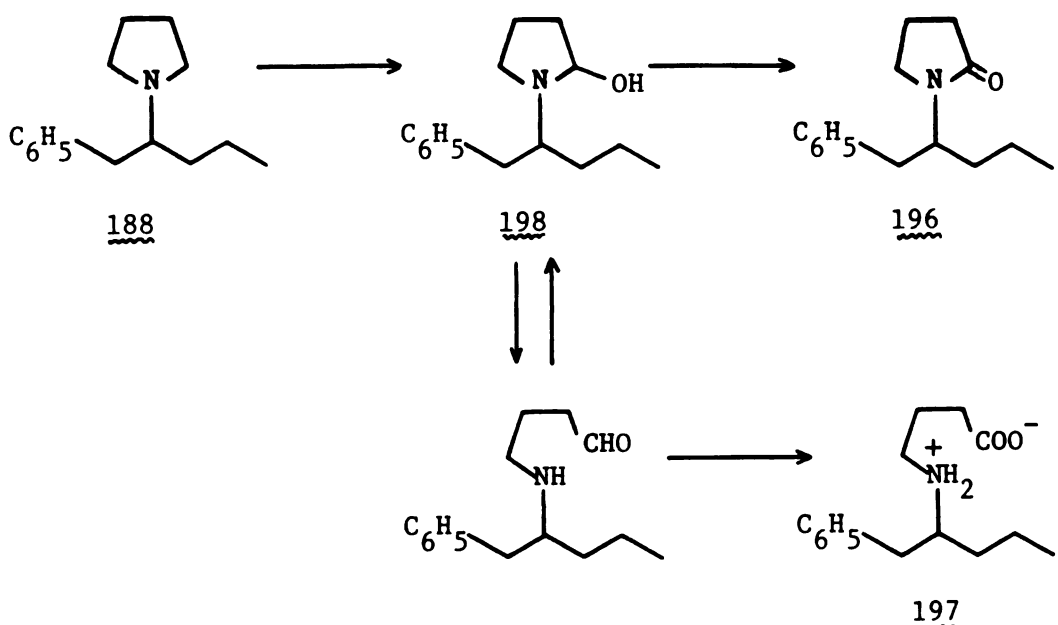
A search of the literature has revealed four cases in which the metabolism of compounds containing a 1-substituted pyrrolidine ring has been studied. The compounds of interest are the CNS stimulant prolintane (188), the antibacterial agent piromidic acid (189), the tobacco alkaloid nicotine (60), and the first compound to be discussed, tremorine (190).

Tremorine (190) is metabolized at C-2 of the pyrrolidine ring to yield the lactam, oxotremorine (191).[149] The characterization of another tremorine metabolite, N-(4-pyrrolidino-2-butynyl)-γ-amino-butyric acid (192), from the urine of rat has led to the following proposed metabolic scheme:[150]



This scheme involves initial metabolic hydroxylation at the ring α-position to give the α-hydroxytremorine intermediate 193 which can exist in equilibrium with the open chain aminoaldehyde 194 or undergo oxidation directly to 191. Further oxidation of the aminoaldehyde 194 can give rise to the isolated amino acid 192; the postulated ring closure of 192 to the lactam 191 has been demonstrated to occur *in vivo* but not *in vitro*.[150] The metabolic profile of the structurally similar N-(5-pyrrolidinopent-3-ynyl)-succinimide (195) has been demonstrated to be similar to that of tremorine.[151]

In *in vitro* rabbit liver preparations, the lactam oxoprolintane (196) has been found to be the major metabolite (50-60%) of prolintane (188);[152] this lactam is also the major metabolite isolated from the urine of rabbits. The amino acid 197 has also been found as a metabolite *in vivo*.[153] It has been postulated that 197 is an intermediate in the formation of the lactam 196. But attempts to detect the formation of 196 from incubations of 197 with rabbit liver preparations have failed. Furthermore, prolintane was found to be metabolized by rabbit liver



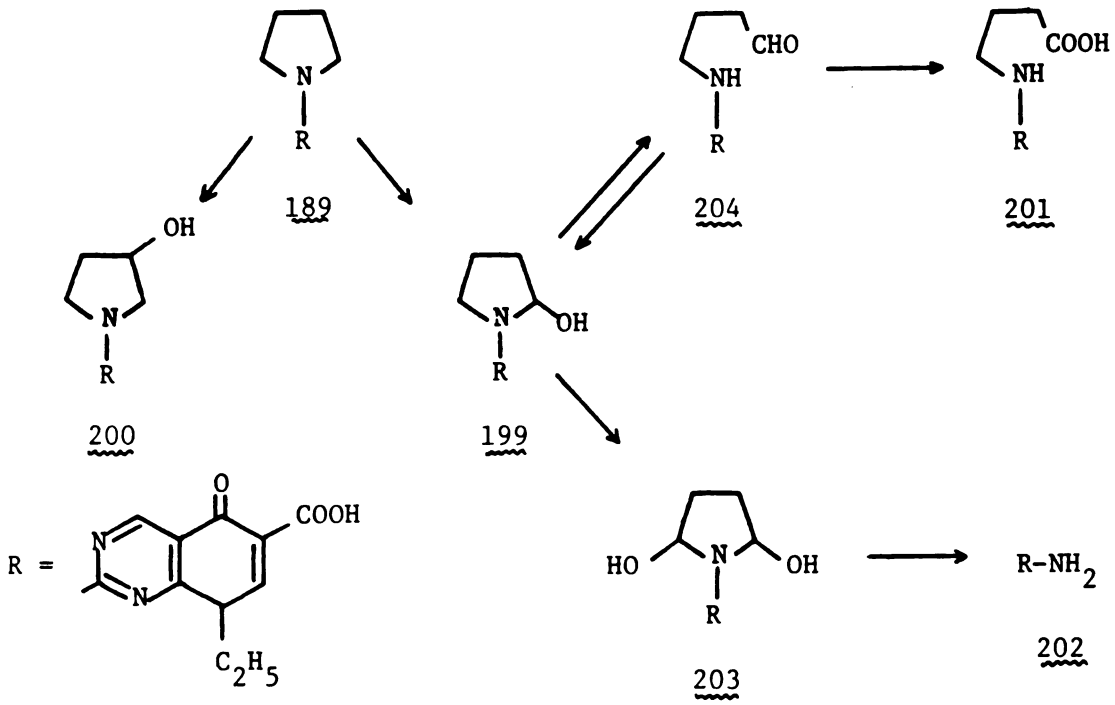
188 198 196

197

microsomes in the presence of NADPH and O_2; the microsomal metabolites were not identified but oxoprolintane was not detected in these studies.[154] The soluble fraction is required for the *in vitro* formation of the lactam from prolintane. These results suggest the amino acid 197 and the lactam 196 are metabolites which arise separately from a common α-hydroxy intermediate 198.

In more recent experiments, the conversion of 197 to the lactam 196 under continuous extraction conditions has been demonstrated. Apparently, the conditions for the extraction of metabolites leads to the artifactual formation of 196 from 197.[155] These results have led to the conclusion that in rabbits and in rats the major urinary metabolite is the amino acid 197.[156]

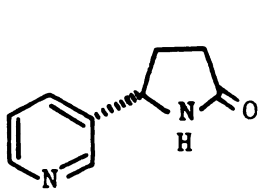
The pyrrolidine moiety of piromidic acid (189) has been found to be extensively metabolized in rats and humans. The major metabolites found in the blood, urine, and bile of rats and humans as the free metabolite or glucuronide conjugate are the α-hydroxypyrrolidine (199), the 3-hydroxypyrrolidine (200), the γ-aminobutyric acid (201), and the 2-amino piromidic acid derivatives (202).[157] The metabolism of piromidic acid by rat liver preparations results in a similar metabolic profile.[158] The first step in the metabolism of 189 is a mixed function oxidase catalyzed hydroxylation reaction at the 2- or 3-position of the pyrrolidine ring to yield 199 or 200 respectively. The *in vitro* rate of formation of the 2-hydroxypyrrolidine derivative was found to be ten times faster than the rate of formation of 200. The 3-hydroxypyrrolidine is not further metabolized; whereas 199 is extensively metabolized to 201 and 202. It is known that α-carbinolamines, including 2-hydroxypyrrolidine derivatives, are chemically unstable and not generally iso-



lable. In this case, the 2-hydroxypyrrolidine metabolite is stable enough to be isolated and fully characterized. It is believed 199 is stabilized by hydrogen bonding between the pyrrolidinyl hydroxyl group and one of the nitrogen atons of the pyrimidine ring; the basicity of the pyrrolidine nitrogen is also decreased by delocalization of the lone pair electrons into the pyrimidine ring. The 2-amino product 202 is formed upon further metabolism of 199 presumably *via* the 2,5-dihydroxypyrrolidine intermediate 203.[159] The carbinolamine probably exists in equilibrium with the open chain amino aldehyde 204 in physiological medium; 199 can be converted to the amino acid 201 by aldehyde dehydrogenase. Contrary to the postulated pathways for lactam formation in other pyrrolidine ring systems, no evidence for lactam formation from the α-hydroxypyrrolidine or α-aminobutyric acid derivatives of prolintane were found.[158]

Most of the known metabolites of nicotine result from enzymic

oxidation of the pyrrolidine ring. Nicotine N'-oxide (98) is a product of metabolic N-oxidation of the pyrrolidine nitrogen. Nicotine can also be N-demethylated to nornicotine (67). The major *in vitro* and *in vivo* metabolite of nicotine is the lactam cotinine (63); cotinine itself is further metabolized to demethylcotinine (205), *trans*-3-hydroxycotinine (206), and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide (207).[160]



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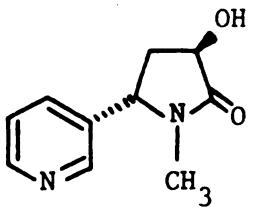

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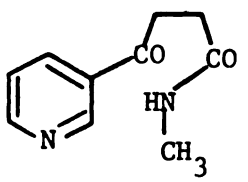

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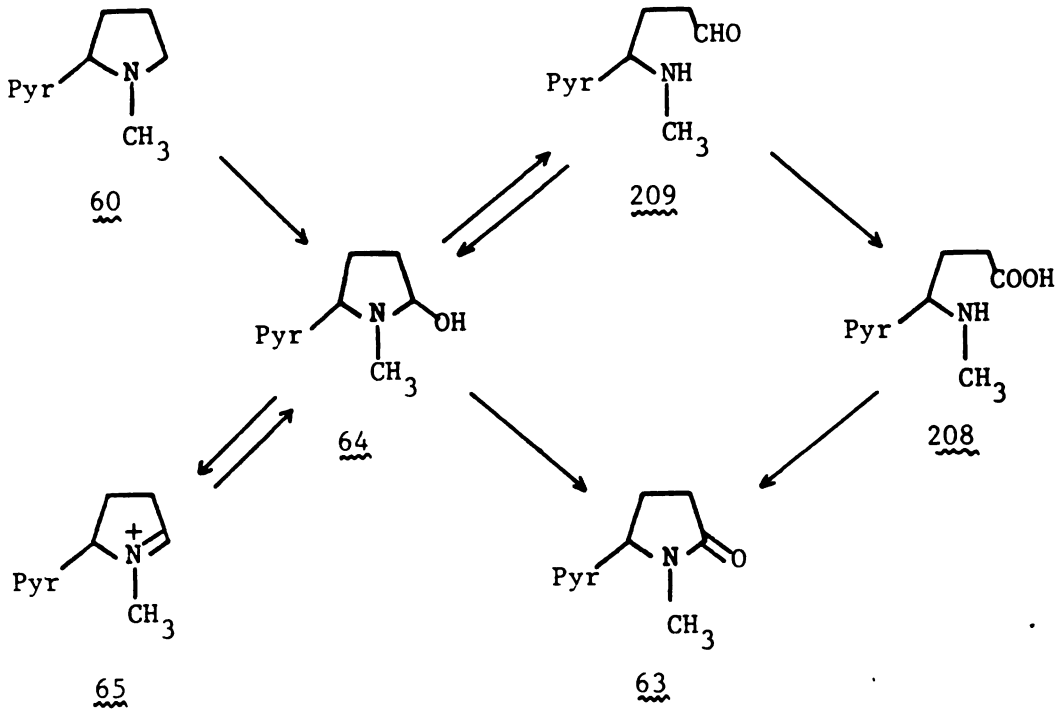
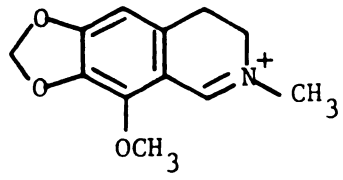
As mentioned before, the formation of cotinine is believed to proceed *via* the 5'-hydroxynicotine intermediate 64. The isolation of γ-(3-pyridyl)-γ-methylaminobutyric acid (208) from the urine of dogs treated with nicotine brought forth the suggestion that the cotinine metabolite results from the ring closure of this amino acid.[161] Incubations of 208 with rabbit liver preparations have resulted in the formation of only trace amounts of the lactam.[73] It is now generally accepted that the soluble enzyme aldehyde oxidase is responsible for the oxidation of the carbinolamine 64 to cotinine.

5'-Hydroxynicotine can exist in equilibrium with its open chain aldehyde form 209; oxidation of 209 would account for the observed amino acid product 208. However, 64 can also exist in equilibrium with nicotine $\Delta^{1'(5')}$ iminium ion (65).[71] Five or six-member ring systems containing an imine or iminium moiety are fairly stable and prefer to exist in the cyclic form.[162] There is a great deal of evidence that indicates



cotarnine exists only as the iminium salt 210 or as the carbinolamine 211 or in a equilibrium of these two forms; no evidence could be obtained for the existence of the postulated open chain amino aldehyde 212.[163] By analogy with the nicotine system, this evidence argues against the existence of the amino aldehyde tautomer 209 and supports the equilibrium between 64 and 65. The incubation of nicotine with rabbit liver preparations under an $^{18}O_2$ atmosphere did not lead to ^{18}O incorporation in the resulting cotinine. This result can be easily rationalized in view of the carbinolamine-iminium equilibrium. Evidently, the oxygen of the initially formed 5'-hydroxynicotine is exchanged in this equilibrium prior to aldehyde oxidase catalyzed oxidation of 64 to cotinine.[71] In recent studies, Brandange and Lindblom have demonstrated that the diperchlorate salt of nicotine $\Delta^{1'(5')}$ iminium ion (129) exists primarily as the iminium ion at pH 7.4[112] and that 65 can act as a substrate for purified rabbit liver aldehyde oxidase.[164]



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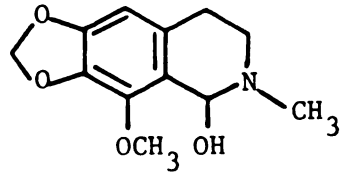

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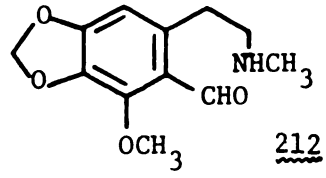
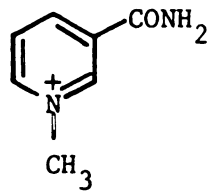


212

The soluble hepatic enzyme aldehyde oxidase is believed to be responsible for the formation of the lactam cotinine from its precursors. Aldehyde oxidase is similar to the enzyme xanthine oxidase in that both contain iron, flavin adenine dinucleotide, and molybdenum in the ratio of 4:1:1.[165] Additionally, both enzymes can reduce oxygen and many other electron acceptors, oxidize aldehydes to acids, catalyze the oxidation of carbon adjacent to nitrogen in a number of aromatic heterocyclic systems, and act as nitroreductases.[165,166] It has been shown that these two enzymes have the ability to oxidize a wide variety of substituted pyrimidines, purines, and other N-heterocycles. Although the specificities of the enzymes display some general common features, it appears that the substrate specificities are complementary rather than identical.[167]



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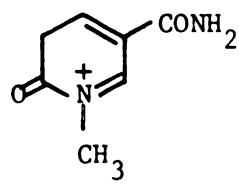
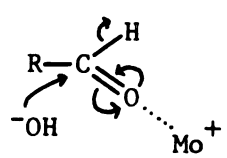
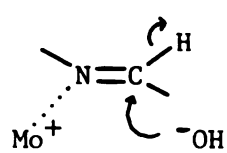


214

The oxidation of N'-methylnicotinamide (213) to N'-methyl-2-pyridone-5-carboxamide (214) or of acetaldehyde to acetic acid are typical examples of the oxidative reactions catalyzed by aldehyde dehydrogenase. It is proposed that molybdenum acts as a Lewis acid at the active site

of these enzymes, ligation of the carbonyl oxygen of the aldehyde or the ring nitrogen adjacent to the susceptible carbon in aromatic heterocycles would facilitate the attack of a hydroxide ion from the medium and the removal of the hydride ion resulting in a net oxidative reaction.[168] The molybdenum atom might also act as the initial acceptor of electrons from the substrate.[168] In iminium compounds the attack of the hydroxide ion on the α-carbon would be facilitated by the nature of the polarized C-N bond. The ability of nicotine $\Delta^{1'(5')}$ iminium ion to serve as a substrate for aldehyde oxidase has prompted Brandange and Lindblom to suggest that this enzyme would be more appropriately named an iminium oxidase.[164] The irreversible inactivation of both xanthine oxidase and aldehyde oxidase by cyanide is proposed to result from the binding of cyanide at the substrate binding site. Additional results suggest the presence of a sulfhydryl group at the active site.[168]



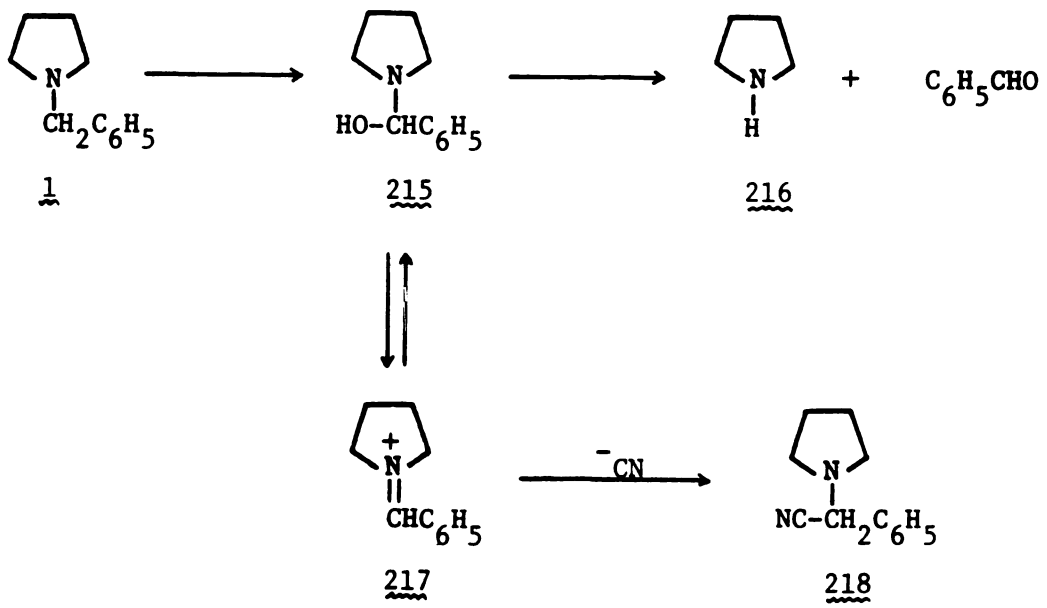


B. STUDIES ON THE METABOLISM OF 1-BENZYLPYRROLIDINE

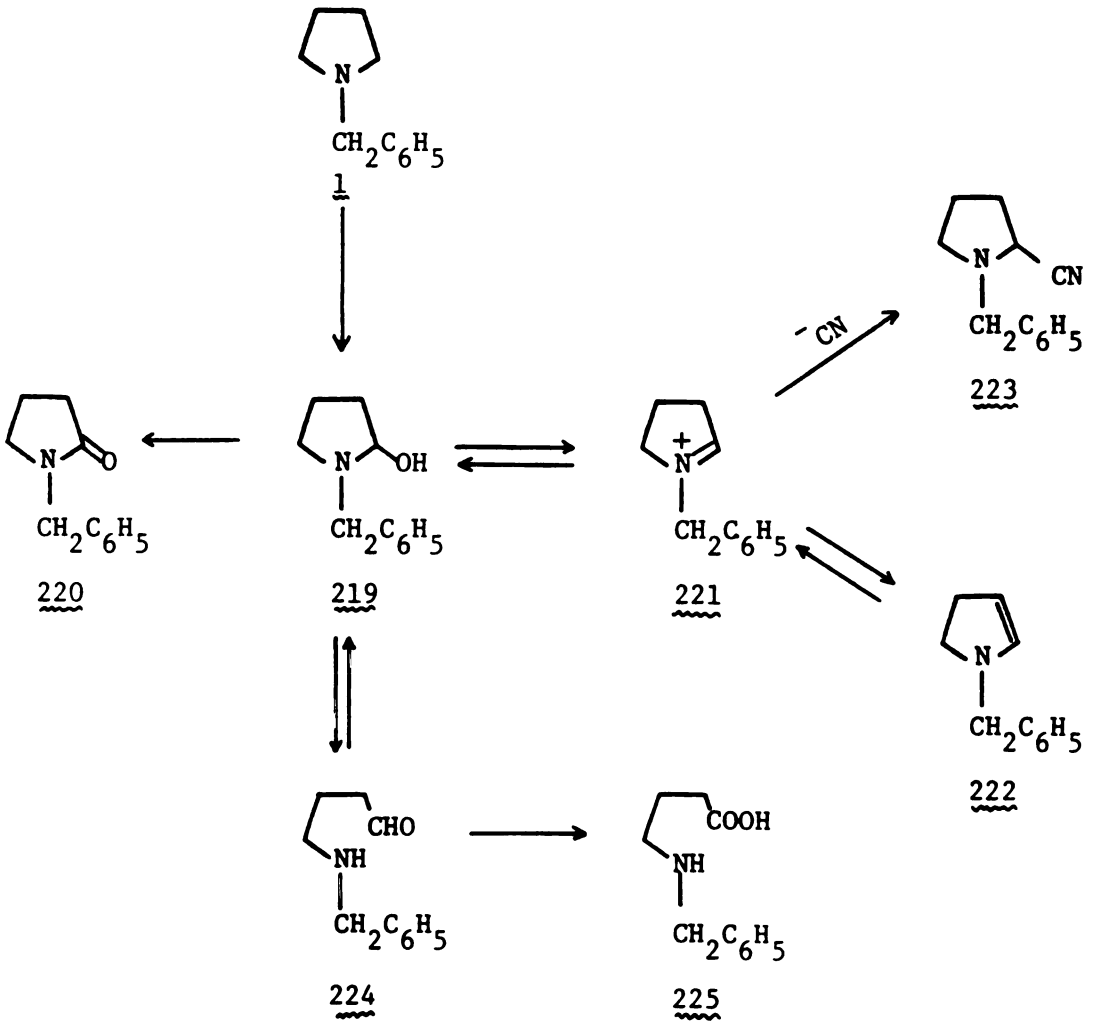
1. CHARACTERIZATION OF THE IN VITRO METABOLITES OF 1-BENZYLPYRROLIDINE.

1-Benzylpyrrolidine (1) would be expected to undergo extensive metabolism at either the benzylic carbon or C-2 on the pyrrolidine ring. The possibility that other metabolic processes such as N-oxidation or aromatic oxidation can lead to the formation of electrophilic intermediates from 1 should not be overlooked and will be considered. The α-carbon hydroxylation process is most likely to generate reactive iminium intermediates; therefore, the possible products resulting from this metabolic transformation will be of primary consideration.

Metabolic oxidation at the benzylic carbon atom of 1 would give rise to the unstable carbinolamine 215 which would be expected to cleave to yield pyrrolidine (216) and benzaldehyde. The carbinolamine also could be in reversible equilibrium with the reactive iminium species 217 which could be trapped with a nucleophile such as cyanide to yield the stable adduct 218.



Metabolic hydroxylation at the 2-position of the pyrrolidine ring would generate the carbinolamine 219 which could undergo further oxidation to the lactam 220. The carbinolamine could be in equilibrium with the iminium species 221 which also could equilibrate with the enamine 222 or react with cyanide ion to give the cyano product 223. In addition, 219 could also be in equilibrium with the open-chain amino-aldehyde 224 which could undergo oxidation to the amino acid 225.



Before attempting to characterize possible products from the incubation of 1 with liver preparation and cyanide, the metabolism of 1 by rabbit liver 100,000 x g microsomal preparations was examined.

The gc analysis of the base fraction (diethyl ether extract) isolated from 1-benzylpyrrolidine postincubates showed the presence of two major

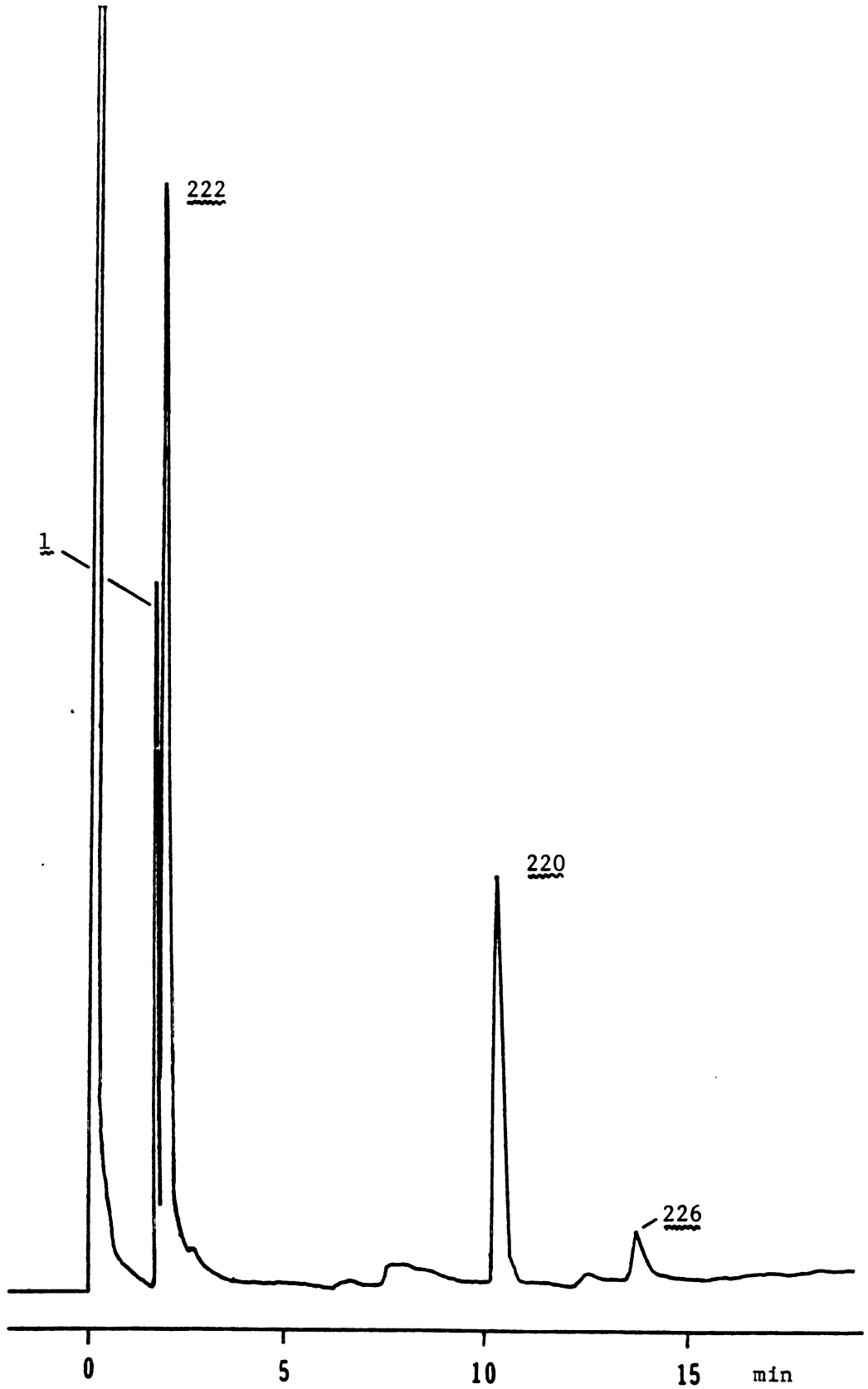


Figure 1. The gc tracing of the base extract from the postincubate of 1.

and one minor metabolite (Figure 1). Quantitation of the metabolism of 1 with the aid of gc internal standards (see below) indicated that 60% of the starting substrate was metabolized after 1 hour at 37° in air. In the absence of NADPH or O_2 or when boiled microsomes were used only substrate was observed in the gc tracing. The microsomal metabolism of 1 was also found to be inhibited in a 9:1 CO/O_2 atmosphere. The data describing the kinetics of the disappearance of 1 and the formation of the metabolites are shown in Table I. The data presented are percentages of total recovered products observed in gc tracing).

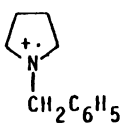
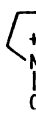
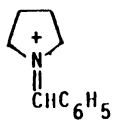
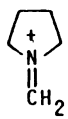
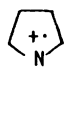
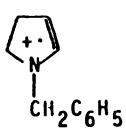
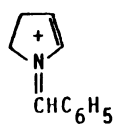
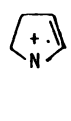
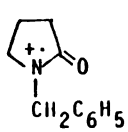
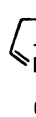
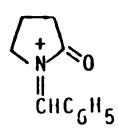
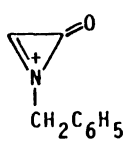
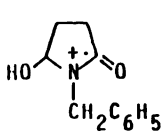
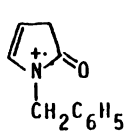
Table I. Kinetic study of the products obtained from the metabolism of 1-benzylpyrrolidine

		Time (mins)				
		0	10	20	30	40
Metabolic product (as % of total recovered products)	1	100	92	76	64	50
	222	--	--	16	24	36
	220	--	4	5	8	11
	226	--	--	--	--	0.8

The structure assignments (described below) and gc-eims characteristics of these compounds and unmetabolized 1 are summarized in Table II.

The tropylium ion ($C_6H_5CH_2^+$) at m/e 91 dominates the gc-eims spectra of all four compounds. The appearance of this fragment ion in these spectra as well as in the mass spectra of the cyano adducts discussed

Table II. GC-EIMS characterization of products obtained from the metabolism of 1-benzylpyrrolidine.

Metabolite M^+(%)	Retention Time (min)	% Formation (of total metabolites)	Major GC-EIMS Fragment Ions m/e (%)				
 161(26) $\underline{1}^+$	1.6	--	 160(31) $\underline{11}^+$	 $\underline{111}^+$	 84(68) $\underline{1111}^+$	 70(59) $\underline{11v}^+$	$C_6H_5CH_2^+$ 91(100)
 159(22) $\underline{222}^+$	2.0	71	 158(8) $\underline{2221}^+$	 $\underline{22211}^+$	 157(5) $\underline{222111}^+$	 68(17) $\underline{2221v}^+$	
 175(70) $\underline{220}^+$	10.2	26	 174(9) $\underline{2201}^+$	 $\underline{22011}^+$	 146(37) $\underline{220111}^+$		
 191(1) $\underline{226}^+$	13.5	3	 173(32) $\underline{2261}^+$				

below indicates that the benzylic carbon atom of the substrate is not altered in these metabolically generated products. The possibility that substituent rearrangements might accompany the fragmentation was ruled out since the corresponding metabolites derived from 1-benzylpyrrolidine-α,α-d_2[1-α,α-d_2, prepared by $LiAlD_4$ reduction of 1-benzoylpyrrolidine (227), 93% deuteration] displayed a strong tropylium ion ($C_6H_5CD_2^+$) at m/e 93. For example, this is clearly illustrated in a comparison (Figure 2) of the gc-eims spectra of the lactam 220 (M^+ 175) derived from 1 versus 220-α,α-d_2 (M^+ 177) from the metabolism of 1-α,α-d_2. Attempts to characterize benzylic carbon-nitrogen cleavage products (pyrrolidine, benzyl alcohol, benzaldehyde, and benzoic acid) by gc analysis against authentic compounds also proved unsuccessful.

The gc-eims of 1-benzylpyrrolidine (Figure 3a) displayed in addition to the tropylium ion a strong parent ion at M^+ 161 and the benzylic C-N cleavage fragment ion $1iv^+$ at m/e 70 (Table II). In this and other related compounds, loss of the proton α to nitrogen generated the fragment ions $1i^+$ and $1ii^+$ complicating deuterium content measurements in the parent ion mass region. A third ion ($1iii^+$) resulting from cleavage β to nitrogen appeared at m/e 84. The gc-eims of 1-α,α-d_2 (Figure 4) supports the assignments for fragment ions $1i^+$ - $1iv^+$; in particular, the ion corresponding to $1iv^+$ remains at m/e 70 as expected and the deuterated analog of $1iii^+$ shifts from m/e 84 to m/e 86. Further confirmation of these fragments ion assignments were obtained from the gc-eims of 1-benzylpyrrolidine-2,2-d_2 (1-2,2-d_2, Figure 3b) prepared by $LiAlD_4$ reduction (97% deuteration) of 1-benzyl-2-pyrrolidinone (220). The metabolism of 1-2,2-d_2 was examined to confirm the structure assignments of the metabolically derived compounds encountered in this study.

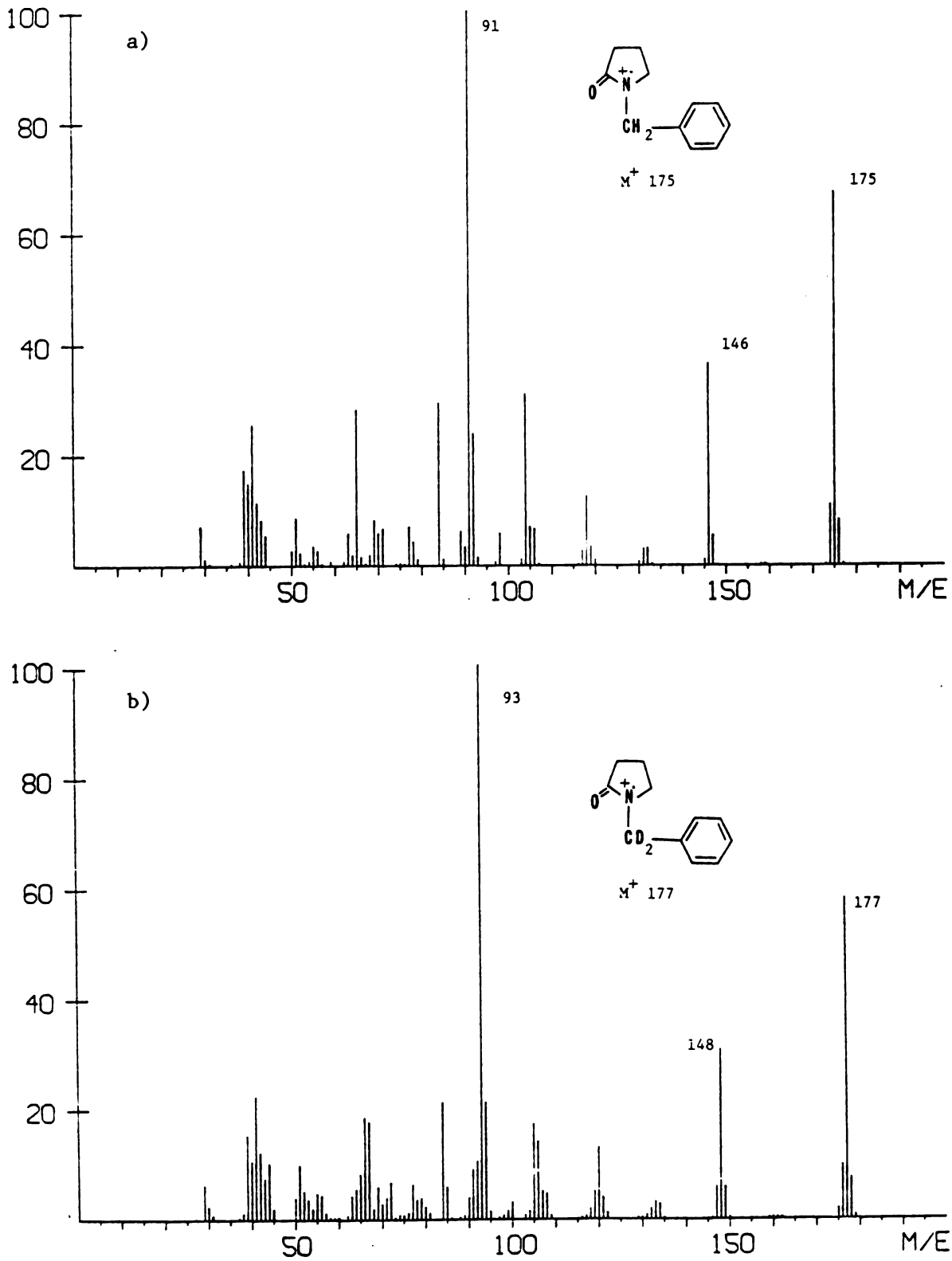


Figure 2. The gc-eims of a) 1-benzyl-2-pyrrolidinone (220) from the metabolism of 1 b) 220-α,α-d_2 from the metabolism of 1-α,α-d_2.

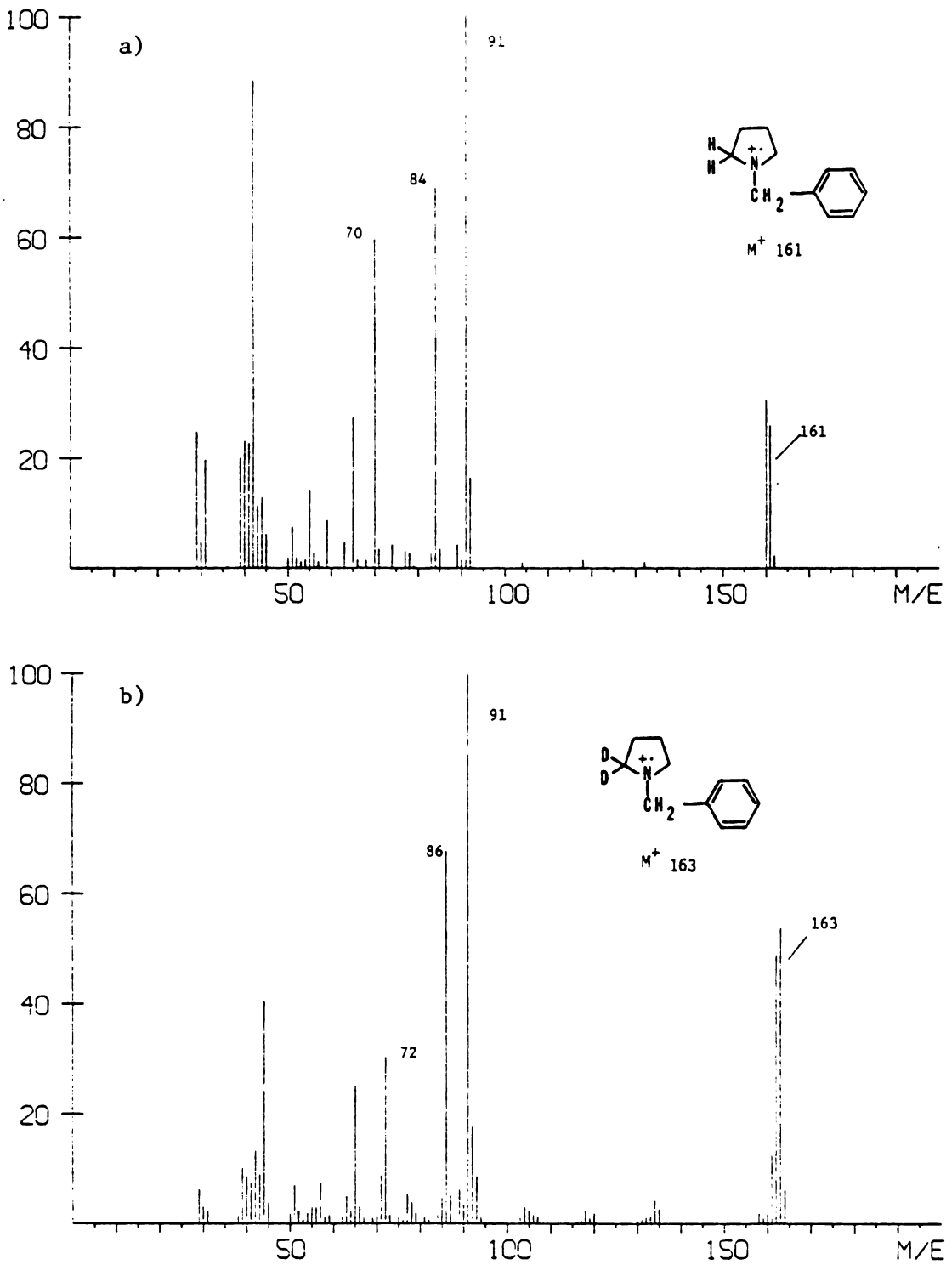


Figure 3. The gc-eims of a) 1-benzylpyrrolidine (1); b) 1-benzylpyrrolidine-2,2-d_2 (1-2,2-d_2).

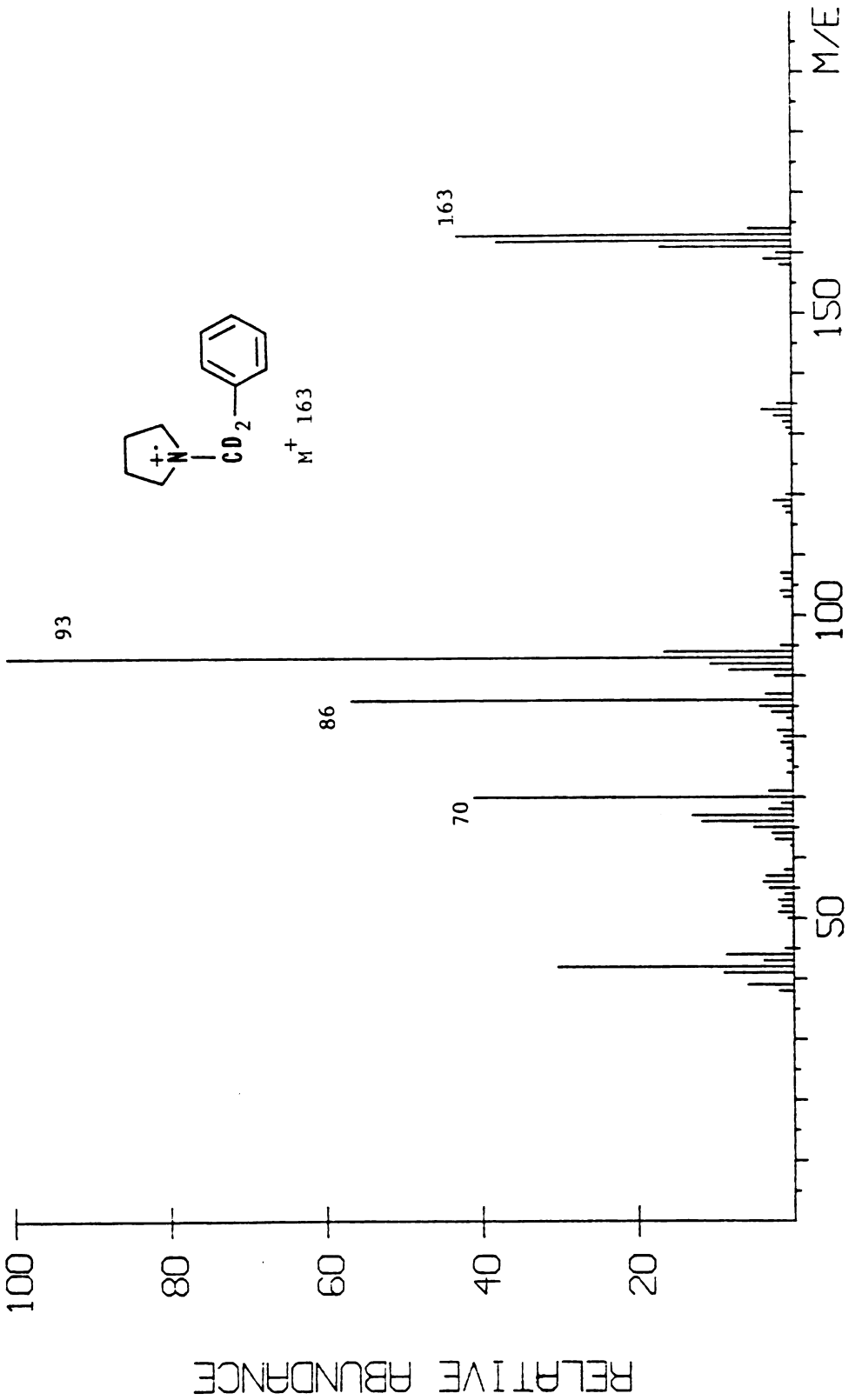
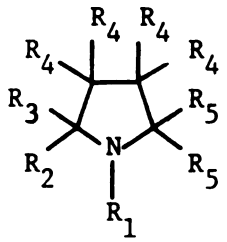


Figure 4. The gc-eims of 1-benzylpyrrolidine-α,α-d_2 (1-α,α-d_2).



	R_1	R_2	R_3	R_4	R_5
1	$CH_2C_6H_5$	H	H	H	H
1-α,α-d_2	$CD_2C_6H_5$	H	H	H	H
1-2,2-d_2	$CH_2C_6H_5$	D	D	H	H
1-d_4	$CH_2C_6H_5$	H	H	D	H
1-α,α-$^3H_2$	$C^3H_2C_6H_5$	H	H	H	H
227	$\overset{O}{\overset{\|}{C}}-C_6H_5$	H	H	H	H

The metabolite eluting just after 1-benzylpyrrolidine displayed a parent ion at M^+ 159 (Figure 5a). This information plus the fragmentation data listed in Table II led to the tentative structure assignment of 1-benzyl-2-pyrroline (222) for this compound. The mass spectrum of metabolite 222 derived from 1-2,2-d_2 (Figure 5b) showed the expected cluster of ions at masses 158, 159, 160, and 161, due to oxidative attack at C-2 (to yield 222-d_1, M^+ 160) and C-5 (to yield 222-d_2, M^+ 161). The fragment ions corresponding to the deuterated analogs of $222i^+$, $222ii^+$, and $222iii^+$ also were present. The deuterated fragment ions corresponding to $222iv^+$ shifted from m/e 68 to m/e 69 and 70. If one excludes the unlikely possibility of multiple migrations of the double bond, these data confirm the α,β-location of the double bond of 222 since the 3-pyrroline structure 228 would be expected to retain both deuterium atoms originally present in the substrate molecule.

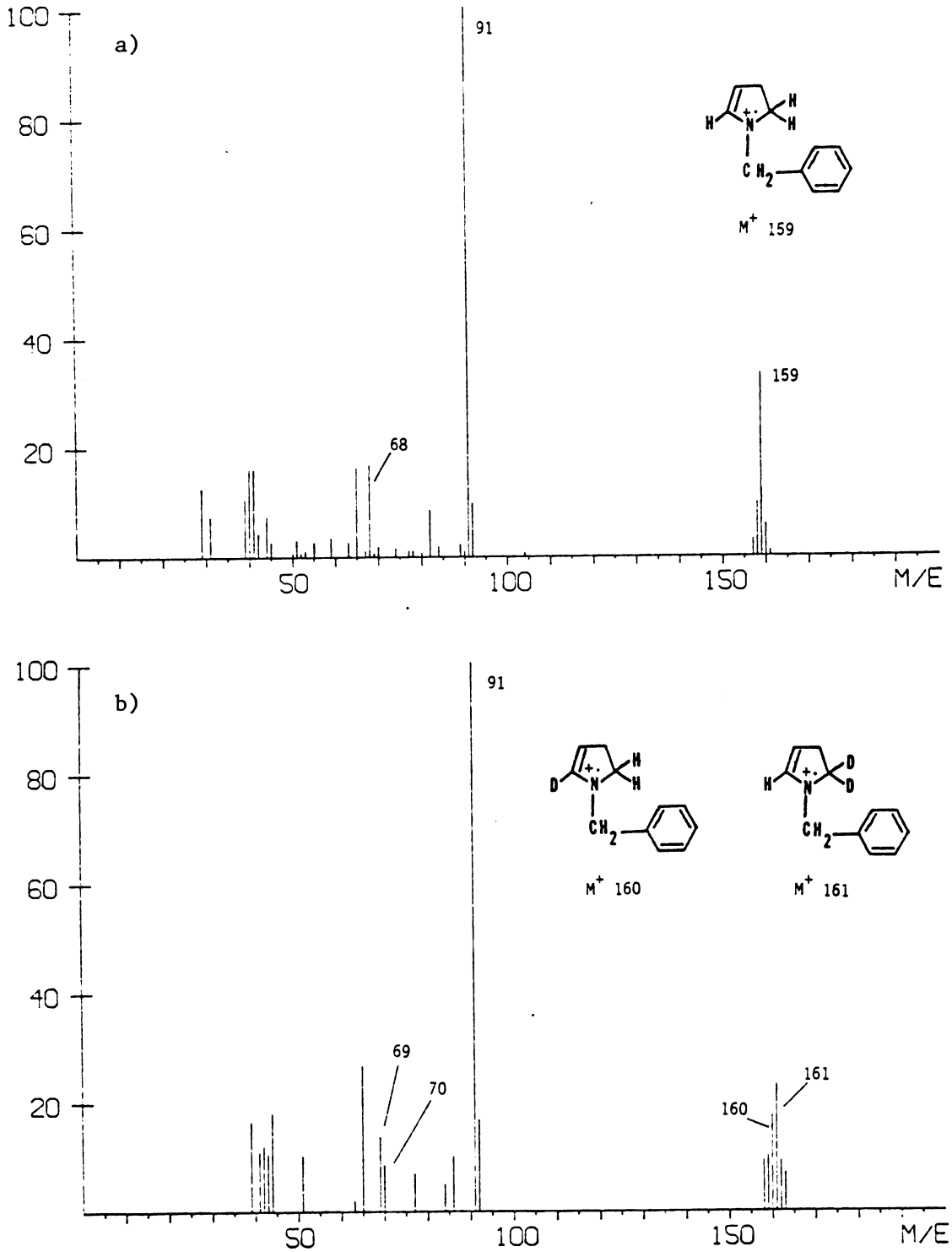
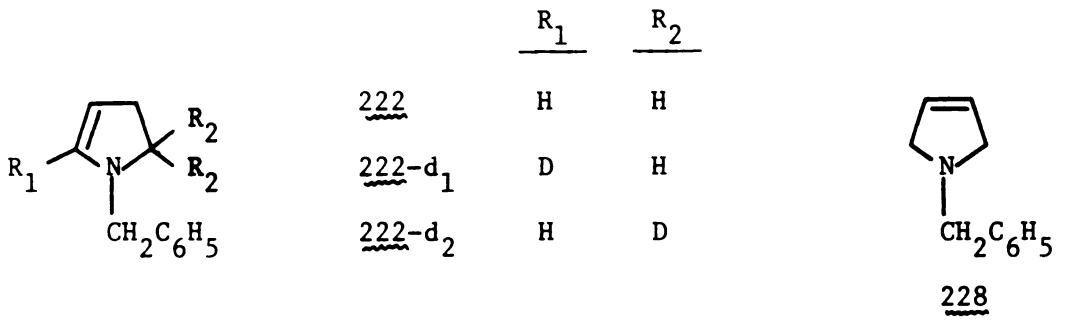
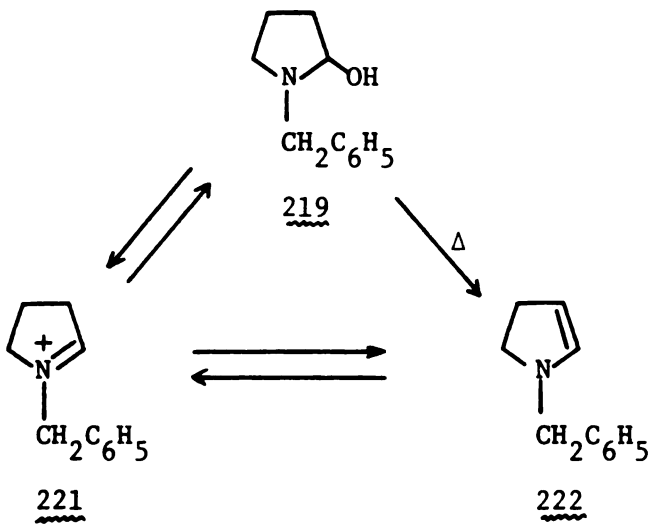


Figure 5. The gc-eims of a) 1-benzyl-2-pyrroline (222) from the metabolism of 1; b) 222-d_1 and 222-d_2 from the metabolism of 1-2,2-d_2.

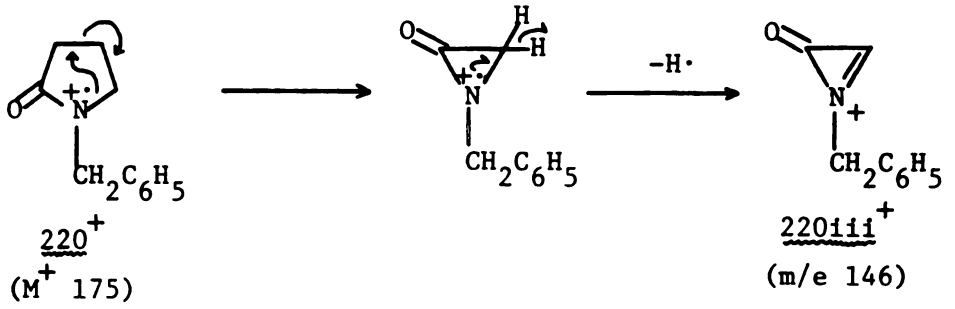


The characterization of 222 from microsomal postincubates does not necessarily mean the microsomal enzymes form this compound directly. As discussed above, the unstable carbinol intermediates of cyclic amines may undergo further oxidation by soluble aldehyde oxidase to lactams. In this case, the proposed carbinolamine 219, if extractable from the incubation mixture, would be expected to undergo thermal dehydration to 222 upon gc analysis. Alternatively, 219 could ionize to the pyrrolinium species 221 which is actually the conjugate acid of the enamine 222. Since the incubation mixtures are worked-up at pH 10, the iminium species 221 would be converted to the free base 222 under these conditions.



1-Benzyl-2-pyrrolidinone (220) was the second major metabolite characterized from microsomal incubations of 1. This metabolite had

gc and gc-eims characteristics (Figure 6a) identical to that of authentic 1. The strong fragment ion at m/e 146 has been assigned to structure 220iii$^+$ on the basis of its nominal mass. This fragment ion shifts to m/e 148 in the gc-eims of metabolite 220-α,α-d_2 (M^+ 177, Figure 2b) derived from 1-α,α-d_2. On the other hand, fragment ion 220iii$^+$ appears exclusively at m/e 146 in the gc-eims of the mixture of 220 and 1-benzyl-2-pyrrolidinone-5,5-d_2 (220-5,5-d_2, M^+ 177, Figure 6b) resulting from the metabolism of 1-2,2-d_2. These data indicate that the fragmentation to form 220iii$^+$ apparently involves the loss of the C4 and C5 methylene groups plus one additional proton. This unusual fragmentation process might be rationalized in the following scheme:



This question was further examined with the aid of 1-benzylpyrrolidine-3,3,4,4-d_4 (1-d_4), prepared by base-catalyzed deuterium exchange at C-3 and C-4 of 1-benzylsuccinimide (229) followed by AlH_3 reduction (90% deuteration). The gc-eims of metabolite 220-d_4 (M^+ 179) derived from the incubation of 1-d_4 is shown in Figure 7. According to our postulated fragmentation sequence, fragment 220iii$^+$ from 220-d_4 should appear at m/e 147 and should retain one of the two deuterium atoms from C-3; contrary to these expectations, fragment 220iii$^+$ from 220-d_4 appears at m/e 148.* This last result is not consistent with the postulated

*This figure m/e 148 was incorrectly reported as m/e 147 in pg 135 of B. Ho and N. Castagnoli, J. Med. Chem. 23 133 (1980).

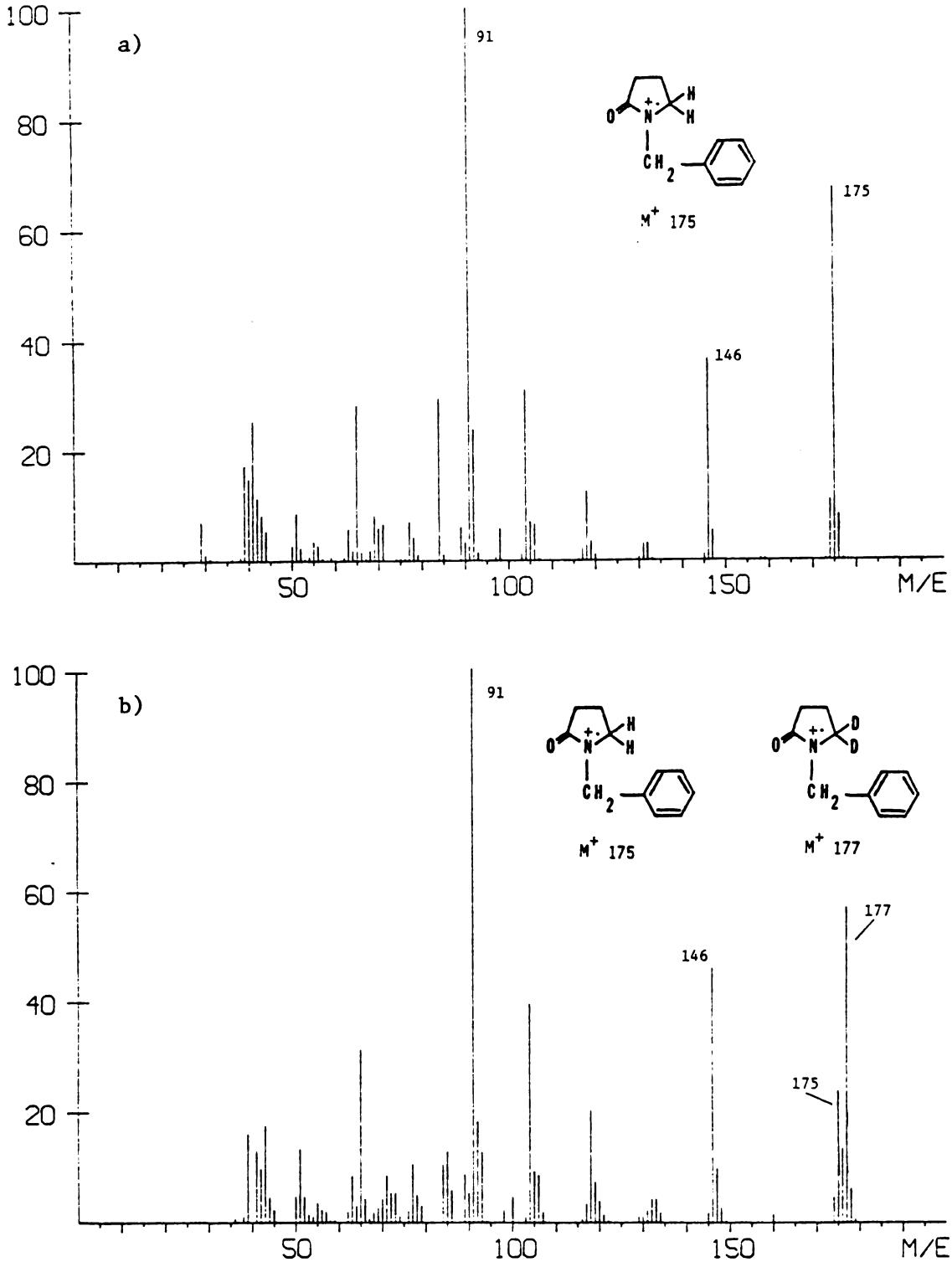


Figure 6. The gc-eims of a) 1-benzyl-2-pyrrolidinone (220) from the metabolism of 1; b) 220 and 220-d_2 from the metabolism of 1-2,2-d_2.

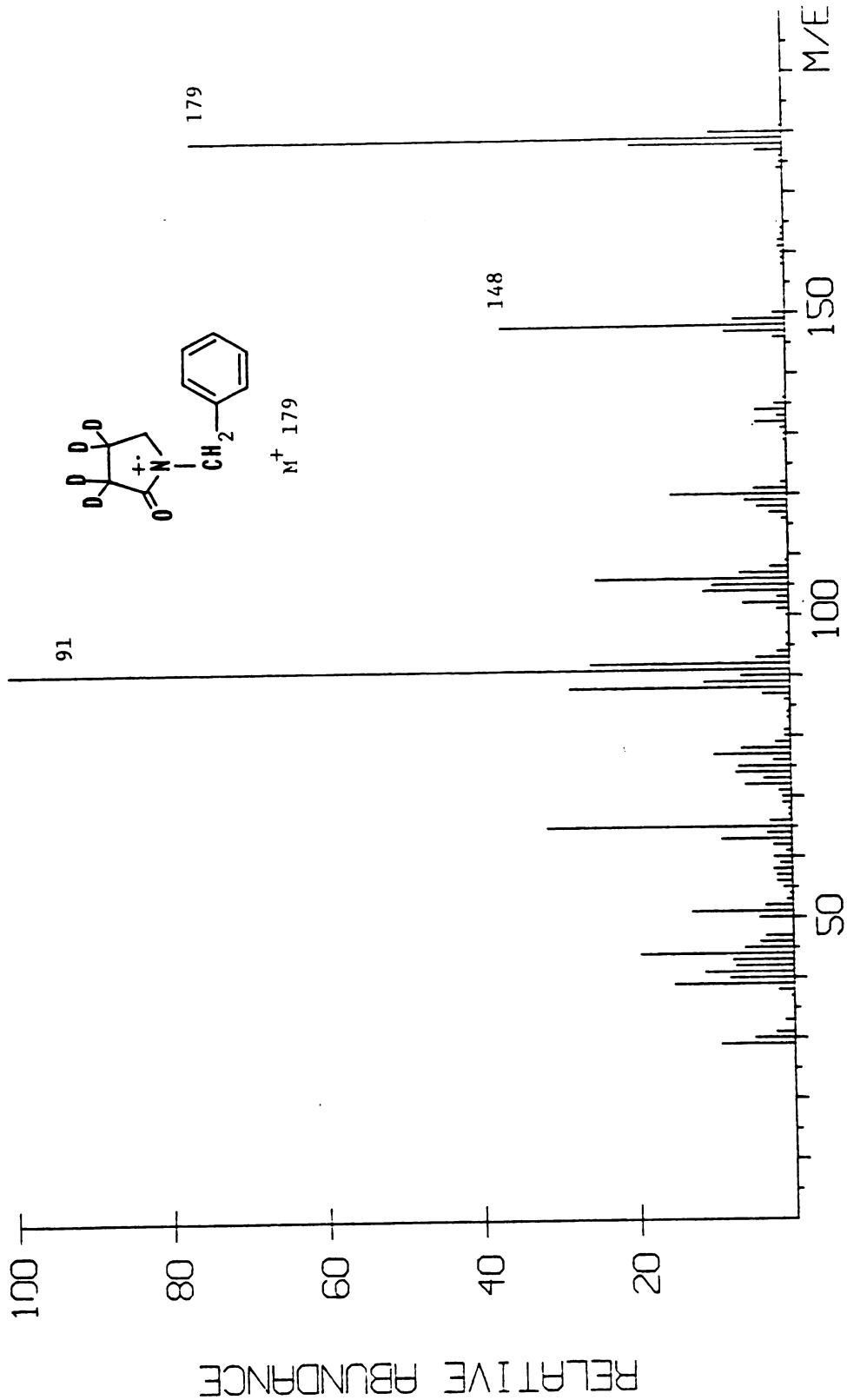
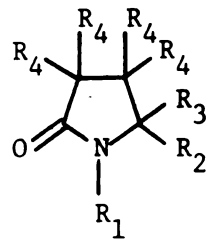


Figure 7. The gc-eims of 1-benzyl-2-pyrrolidinone-3,3,4,4-d_4 (220-3,3,4,4-d_4) from the metabolism of 1-3,3,4,4-d_4.

fragmentation process and is difficult to rationalize. The possibility of intramolecular proton transfer from the benzylic position can be ruled out because the tropylium ion from 220-α,α-d_2 (see Figure 2b) appears at m/e 93.



	R_1	R_2	R_3	R_4
220	$CH_2C_6H_5$	H	H	H
220-α,α-d_2	$CD_2C_6H_5$	H	H	H
220-5,5-d_2	$CH_2C_6H_5$	D	D	H
220-d_4	$CH_2C_6H_5$	H	H	D
226	$CH_2C_6H_5$	OH	H	H
226-d_4	$CH_2C_6H_5$	OH	D	H
229	$CH_2C_6H_5$	=O		H

A minor metabolite observed in the microsomal incubation of 1 was assigned the structure 1-benzyl-5-hydroxy-2-pyrrolidinone (226). The gc-eims (Figure 8a) displayed a weak parent ion at M^+ 191 (1-benzyl-2-pyrrolidinone + oxygen) and a fairly strong fragment ion at m/e 173 ($226i^+$) generated from the loss of water. The gc-eims of metabolite 226 derived from 1-2,2-d_2 as substrate (Figure 8b) established the location of the newly introduced oxygen atom. The appearance of two parent ions at M^+ 191 and 192 [resulting from 226 and 1-benzyl-5-hydroxy-2-pyrrolidinone-5-d_1 (226-d_1), respectively] and two corresponding fragment ions (from the loss of H_2O) at m/e 173 and 174 require the hydroxyl group to be located at C-5. If the two oxygen atoms were located at positions other than C-2 and C-5, one of the metabolites should retain both deuterium atoms originally present in 1-2,2-d_2.

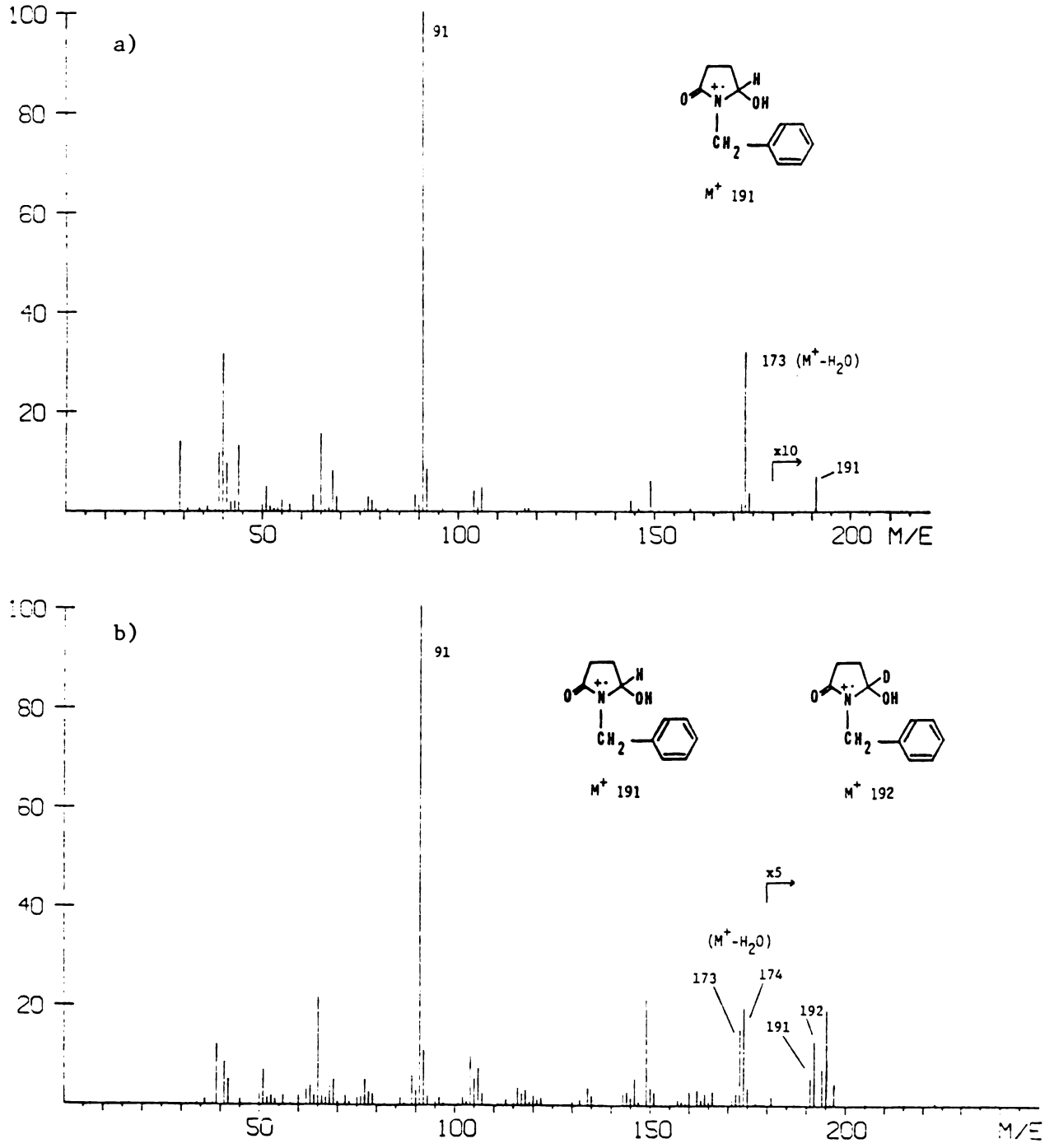
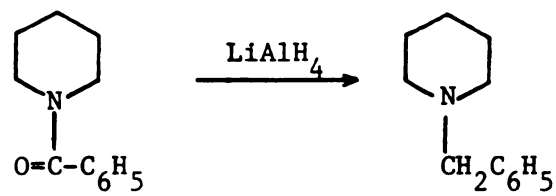


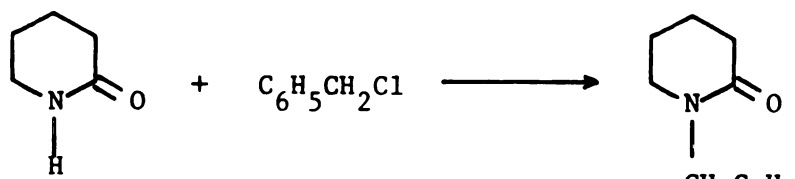
Figure 8. The gc-eims of a) 1-benzyl-5-hydroxy-2-pyrrolidinone (226) from the metabolism of 1; b) 226 and 226-d_1 from the metabolism of 1-2,2-d_2.

Since an appreciation of the quantitative aspects associated with the C-2 metabolite oxidation of 1 was important in the planned cyanide ion trapping experiments, a quantitative gc assay for 1-benzylpyrrolidine (retention time 1.6 min) and 1-benzyl-2-pyrrolidinone (retention time 10.2 min) was developed. The homologs 1-benzylpiperidine (230, retention time 2.4 min) and 1-benzyl-2-piperidone (231, retention time 12.1 min) were prepared as internal standards. 1-Benzylpiperidine was prepared by $LiAlH_4$ reduction of 1-benzoylpiperidine (232) and 231 was prepared by the condensation of 2-piperidone (233) with benzyl chloride. With the aid of these gc internal standards, we examined the influence of soluble enzyme systems on the metabolism of 1 by comparing the metabolic profiles obtained from 10,000 x g hepatic homogenate supernatant fractions with the washed 100,000 x g microsomal fraction. The amount of lactam 220 formed by the 10,000 x g supernatant (93% of total metabolites) is 4 to 5 times greater than that generated by the 100,000 x g microsomal preparation (21% of total metabolites). Yet, the level of substrate metabolism is virtually equal in both hepatic fractions. These findings are consistent with the proposal that the soluble aldehyde oxidase is at least in part responsible for the further oxidation of the initially formed 2-electron oxidation product, presumably the carbinolamine 219, to the lactam.[73] The absence of detectable amounts of enamine 222 in 10,000 x g postincubates of 1 also supports the role of soluble enzymes in the overall metabolism of cyclic amines to lactams. It is also possible that the lactam 220 might arise from the *in vitro* condensation of the amino acid 225 or is an artifact resulting from the work-up or analysis of 225. The presence of the amino acid 225 resulting from the metabolism of 1 has not been investigated and can not be ruled out. However, almost all of the

metabolized substrate can be accounted for as 220, 222 and 226 so that the amount of metabolized 1 that might exist as 225 would be negligible. The possible involvement of an amino acid intermediate in iminium ion formation would also be highly unlikely. Since our interests are focused on cyanide trapping of iminium ions, subsequent experiments were performed with 100,000 x g microsomal preparation to minimize loss of the postulated carbinolamine or iminium intermediate via oxidation to the lactam.



232 230



233 231

2. THE METABOLISM OF 1-BENZYLPYRROLIDINE IN THE PRESENCE OF SODIUM CYANIDE

Figure 9 shows the typical gc tracing obtained from the base extracts of 1-benzylpyrrolidine-sodium cyanide co-incubations. In addition to recovered substrate 1 and 1-benzyl-2-pyrrolidinone (220), four new components are present. The gc and gc-eims characteristics of these compounds are summarized in Table III. The gc assay showed that in the presence of cyanide, 35% of starting 1 is metabolized after 1 hour. The

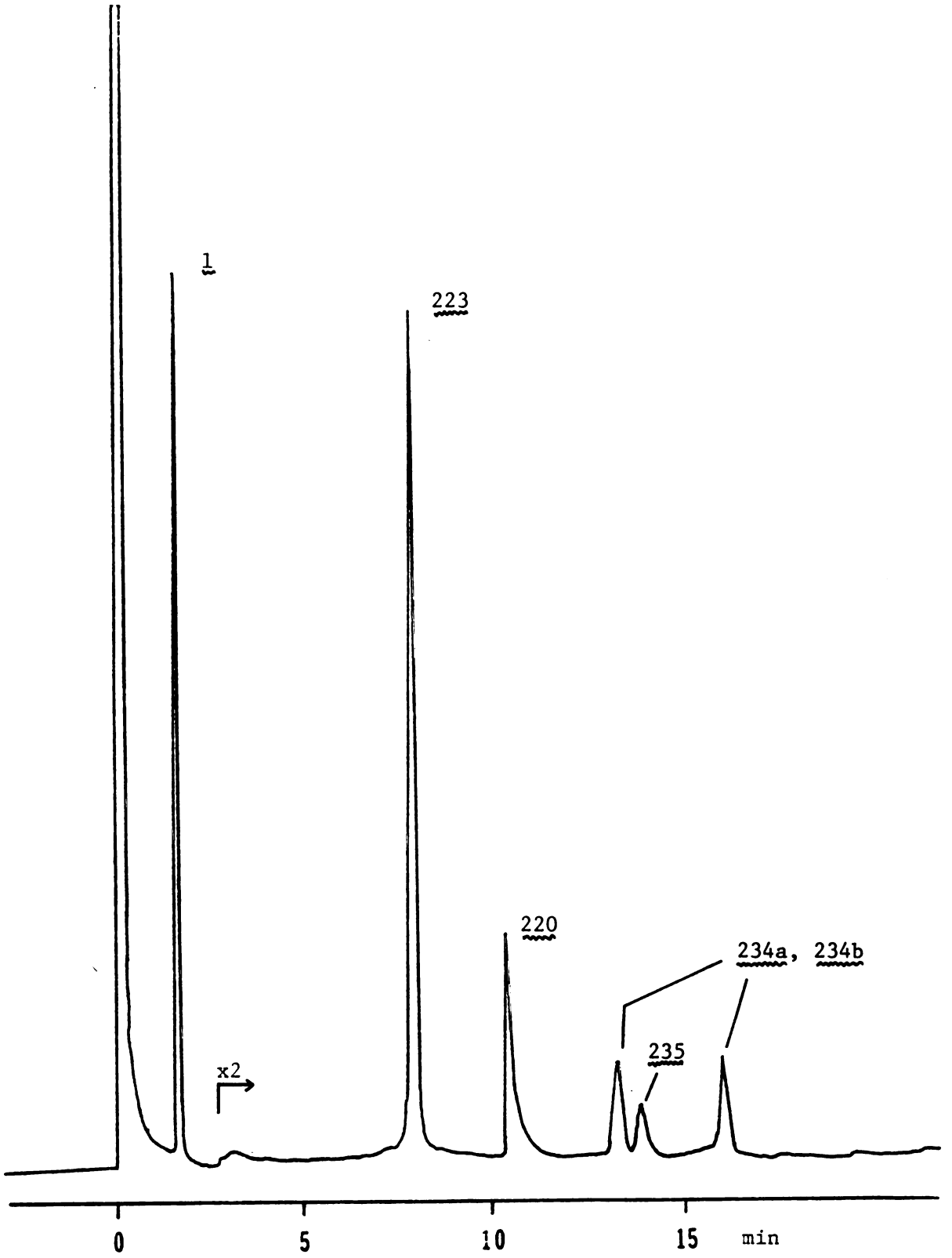
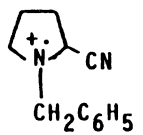
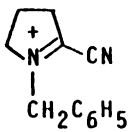
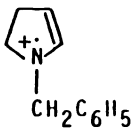
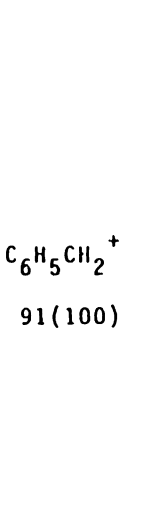
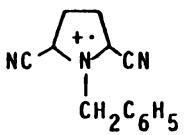
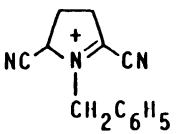
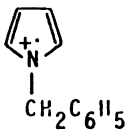
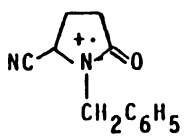
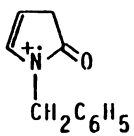
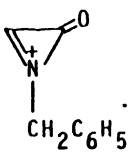


Figure 9. The gc tracing of the base extract from the postincubate of 1 in the presence of cyanide.

Table III. GC-EIMS characterization of products obtained from the metabolism of 1-benzylpyrrolidine in the presence of sodium cyanide.

Cyano Adduct M^+(%)	Retention Time (min)	% Formation (of total metabolites)	Major GC-EIMS Fragment Ions m/e (%)		
	7.6	65			
186(4)			185(4)	159(25)	
223^+			2231^+	22311^+	
	12.8,15.6	8,7			$C_6H_5CH_2^+$
211(2)			210(2)	157(13)	91(100)
$234a^+$; $234b^+$			2341^+	23411^+	
	13.4	4			$C_6H_5C{\equiv}NH^+$
200(47)			173(3)	146(62)	104(49)
235^+			2351^+	23511^+	

formation of the observed products requires NADPH and O_2 and is inhibited by a 9:1 CO/O_2 atmosphere. The rates of formation of these metabolic products are summarized in Table IV.

Table IV. Formation of the products obtained from the metabolism of 1 in the presence of sodium cyanide.

		TIME (mins)				
		0	10	20	30	40
Metabolic product	1	100	97	92	86	75
(As % of total recovered products)	223	--	3	5	9	14
	220	--	--	0.5	1.5	3.5
	234a	--	--	0.5	1	2
	235	--	--	--	0.1	1
	234b	--	--	0.5	1	2

Quantitatively the most important metabolite proved to be 1-benzyl-2-cyanopyrrolidine (223). The gc-eims of 223 (Figure 10a) displayed a parent ion at M^+ 186 which requires the substitution of one proton of the substrate 1 with a cyano group. The facile loss of HCN to form the fragment ion $223ii^+$ plus the presence of the tropylium ion (m/e 91) as the base peak eliminated on mass spectral grounds the isomeric α-cyano structure, α-1-pyrrolidinylphenylacetonitrile (218). The gc (retention time 7.4 min) and gc-eims characteristics of synthetic 218 (Figure 11; M^+ 186 (42), m/e 185 (43), 91 (35), 70 (100)) prepared from the reaction

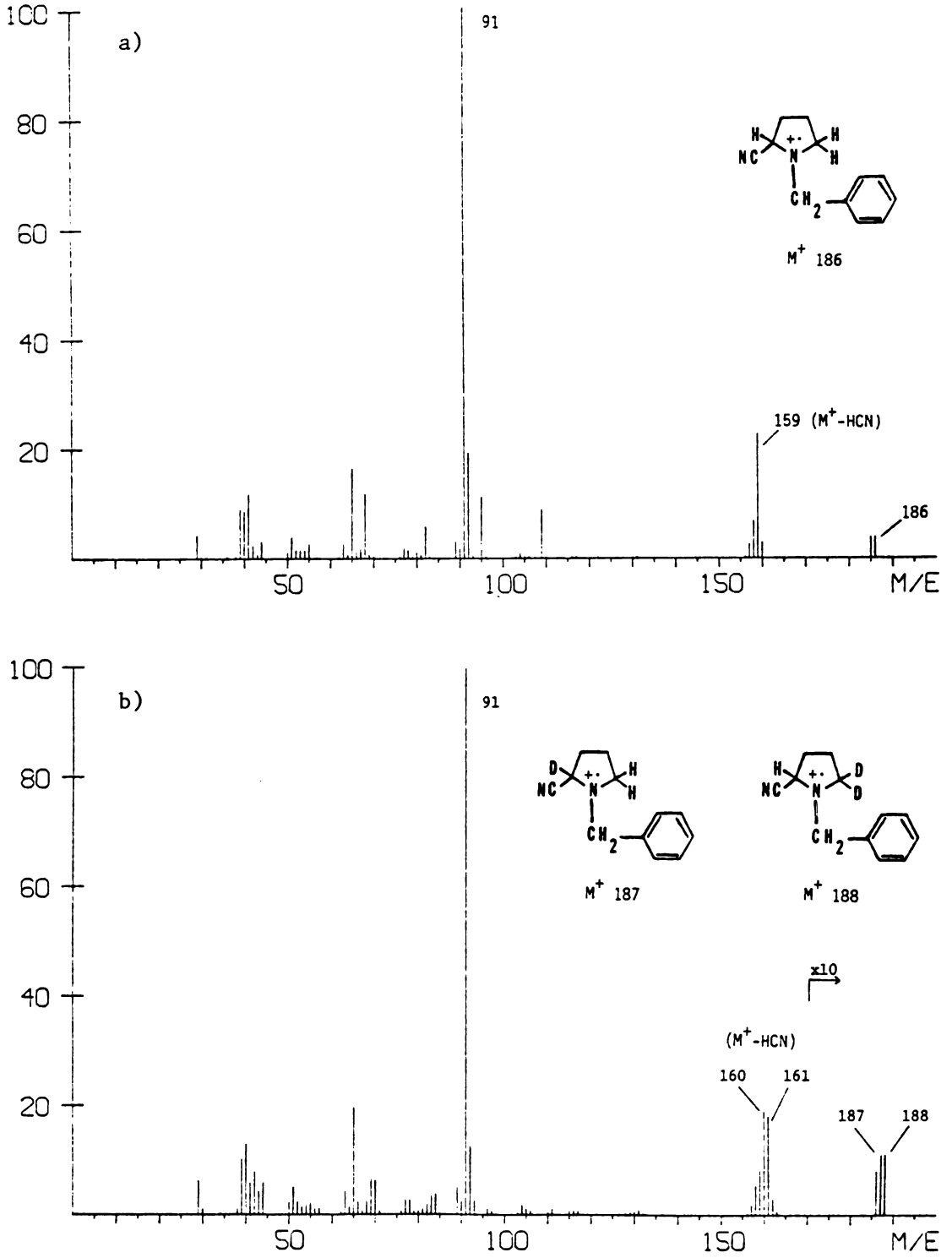


Figure 10. The gc-eims of a) 1-benzyl-2-cyanopyrrolidine (223) from the metabolism of 1 in the presence of cyanide; b) 223-d_1 and 223-d_2 from the metabolism of 1-2,2-d_2 in the presence of cyanide.

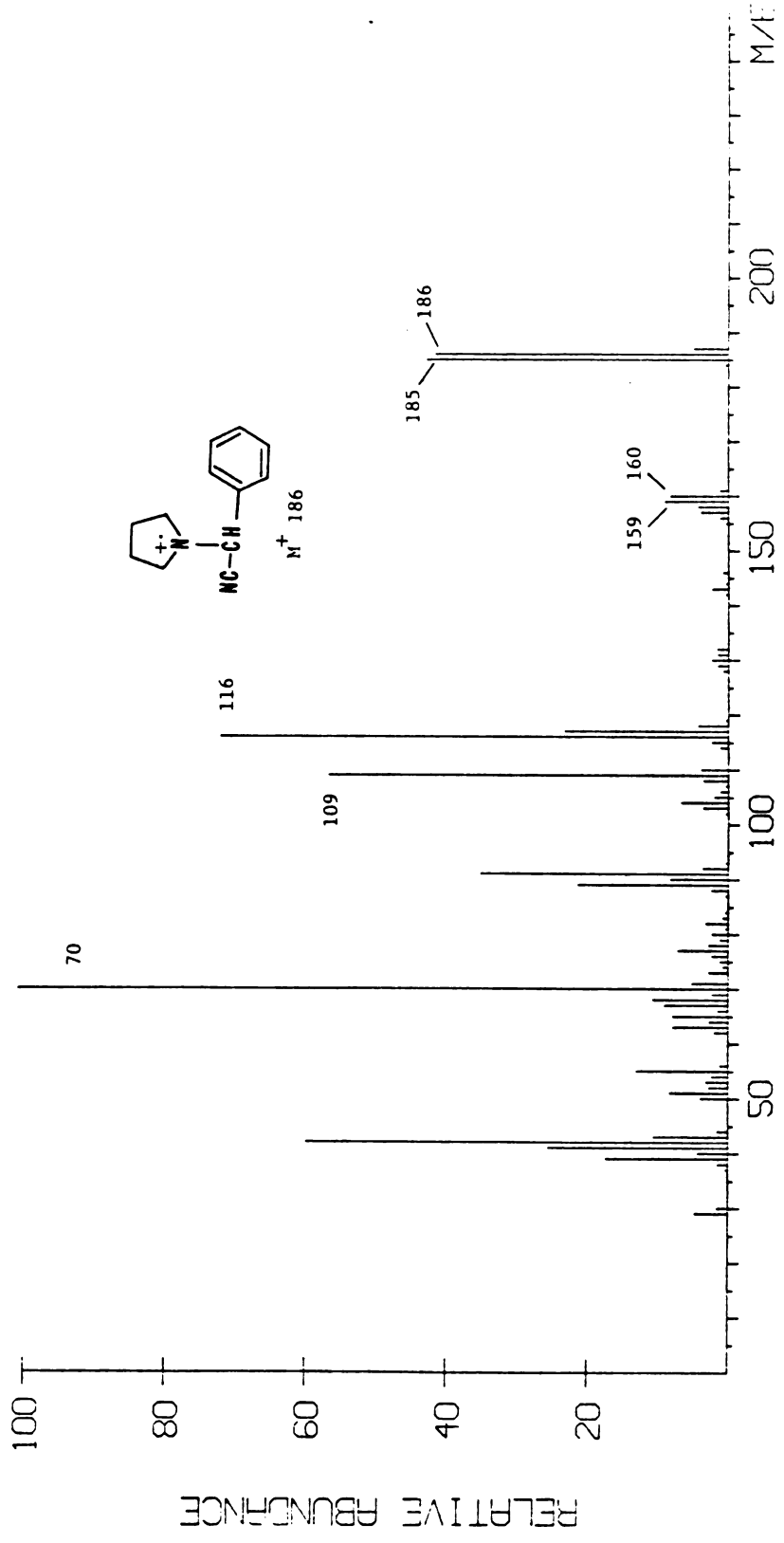
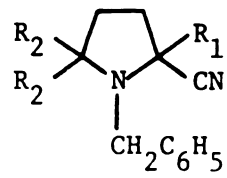


Figure 11. The gc-eims of synthetic α-1-pyrrolidinylphenylacetonitrile (218).

of pyrrolidine, benzaldehyde, and sodium cyanide[169] did not correspond to any of the products isolated from the postincubates of 1 with sodium cyanide.



	R_1	R_2
223	H	H
223-d_1	D	H
223-d_2	H	D

The location of the cyano group was established with the aid of 1-benzylpyrrolidine-2,2-d_2(1-2,2-d_2). The gc-eims of the monocyano product (Figure 10b) isolated from the co-incubation of 1,2,2-d_2 and sodium cyanide displayed two weak parent ions at M^+ 187 and 188 [1-benzyl-2-cyanopyrrolidine-2-d_1 (223-d_1) and 1-benzyl-5-cyanopyrrolidine-2,2-d_2 (223-d_2), respectively]. Additionally, fragment ions which correspond to the deuterated analogs of 223ii$^+$ (Table III) generated by the loss of HCN from the d_1 and d_2 products are present at m/e 160 and 161. Final verification of the structure was based on comparison of the gc and gc-eims characteristics of the metabolically derived product with those of the fully characterized synthetic 223 (Figure 12) prepared by the oxidation of 1-benzylpyrrolidine with mercuric acetate followed by treatment of the iminium intermediate with sodium cyanide.[121]

Two of the remaining three new peaks gave identical mass spectra upon gc-eims analysis (Figure 13a) which suggested they were geometric isomers. The appearance of parent ions at M^+ 211 suggests the introduction of two cyano groups; other than the tropylium ion (m/e 91) the only significant fragment ion appeared at m/e 157. These products were shown to be the

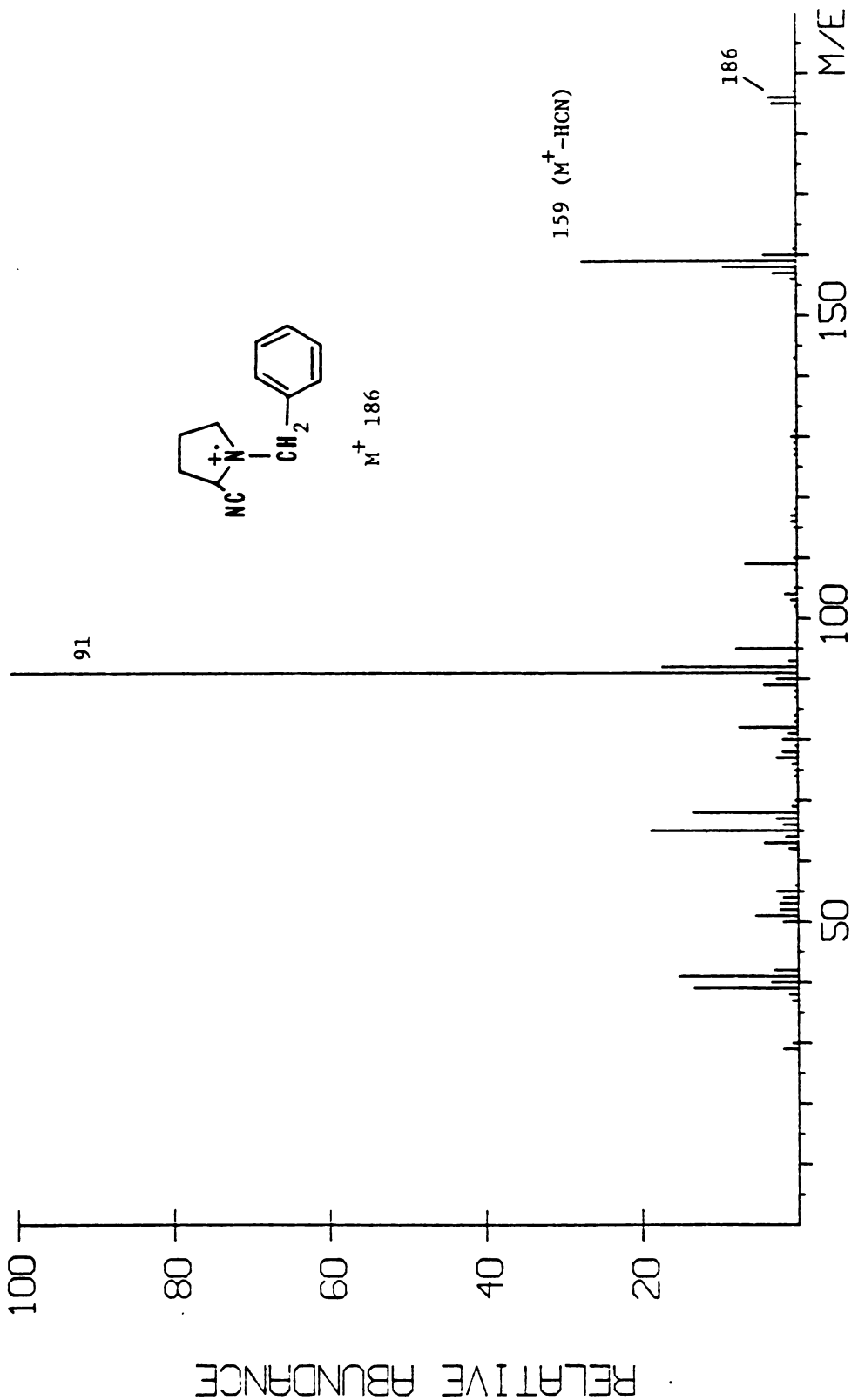


Figure 12. The gc-eims of synthetic 1-benzyl-2-cyanopyrrolidine (223).

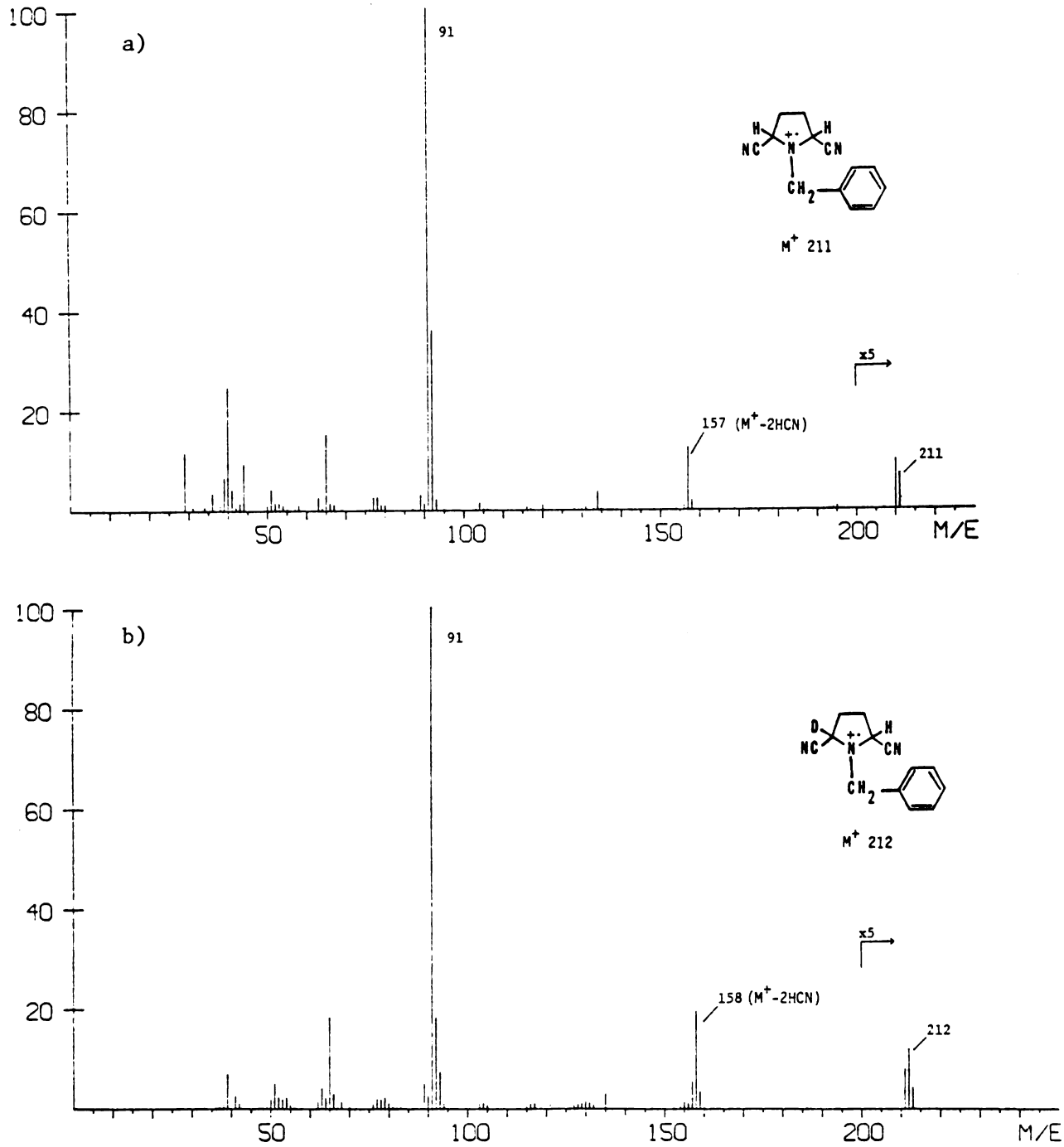
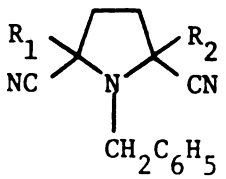


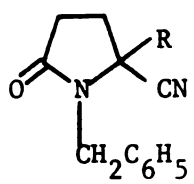
Figure 13. The gc-eims of a) 1-benzyl-2,5-dicyanopyrrolidine (234) from the metabolism of 1 in the presence of cyanide; b) 234-d_1 from the metabolism of 1-2,2-d_2 in the presence of cyanide.

cis and trans-isomers of 1-benzyl-2,5-dicyanopyrrolidine (234a and 234b) since the corresponding metabolites (234a-d_1 and 234b-d_1) isolated from the postincubates of 1-2,2-d_2 both retained one deuterium atom (Figure 13b). The retention of the deuterium atom is observable in the parent ions (M^+ 212) and the deuterated fragment ion (m/e 158) corresponding to 234ii$^+$ (m/e 157, Table III). The only structure assignment consistent with the retention of only one deuterium atom in 234a and 234b requires the introduction of the two cyano groups at C-2 and C-5.



	R_1	R_2
234	H	H
234-d_1	D	H

The final product characterized in these incubations has been assinged the structure 1-benzyl-5-cyano-2-pyrrolidinone (235). This assignment is based on the nominal masses of the parent ion at M^+ 200 (Figure 14a) and the expected fragment ions 235i$^+$ (M^+ - HCN) and 235ii$^+$. Incubation of substrate 1-2,2-d_2 (Figure 14b) led to the two expected products 235 and 1-benzyl-5-cyano-2-pyrrolidinone-5-d_1 (235-d_1, M^+ 201). Analogous fragment ions derived from 235 and 235-d_1 appear at m/e 173 and 174 (M^+-HCN, cf 234i$^+$) and at m/e 146 (235ii$^+$). The presence of only



235	R = H
235-d_1	R = D

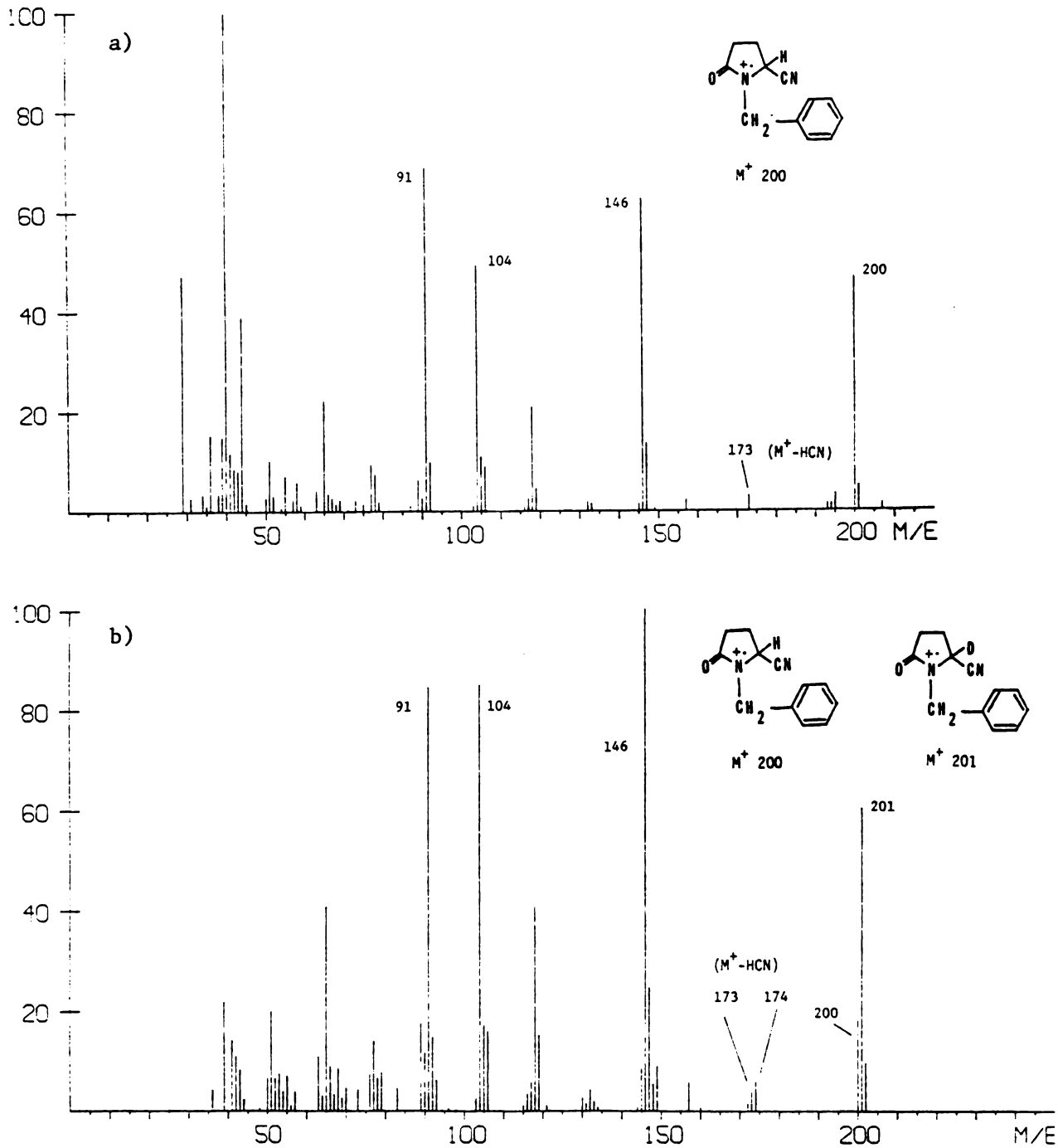
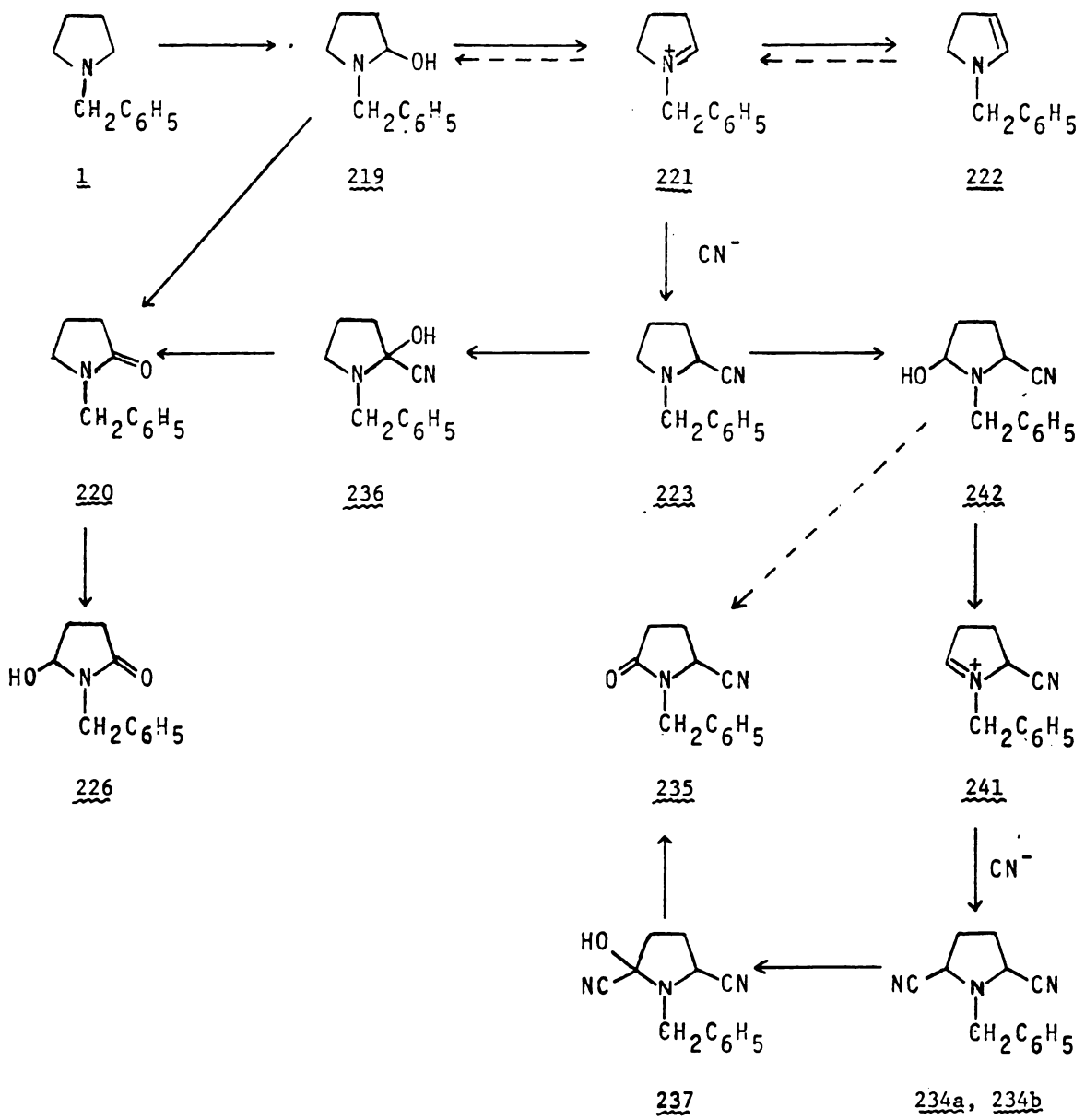


Figure 14. The gc-eims of a) 1-benzyl-5-cyano-2-pyrrolidinone (235) from the metabolism of 1 in the presence of cyanide; b) 235 and 235-d_1 from the metabolism of 1 in the presence of cyanide.

deuterium free 235ii$^+$ is consistent with the formation of this ion by the fragmentation process proposed for 1-benzyl-2-pyrrolidinone.

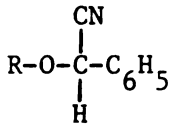
3. THE NATURE OF THE CYANO ADDUCTS

It is likely that the cyano adducts 223, 234a, and 234b result from cyanide ion attack on reactive iminium intermediates which are most probably formed by ionization of the carbinolamine precursors. These same carbinolamines may act as substrates for lactam formation. It is also possible that the lactams 220 and 235 characterized from cyanide-containing incubations could result from mixed function oxidation at the carbon atoms bearing the cyano group to form the corresponding cyanohydrins 236 and 237, respectively. As illustrated in Scheme 1, these cyanohydrins would be expected to breakdown spontaneously to the lactams 220 and 235. This question was addressed by examining the metabolism of 1-benzyl-2-cyanopyrrolidine (223) and of 1-benzyl-2-pyrrolidinone (220) in the absence and presence of cyanide ion. The lactam substrate 220 was metabolized (10%) to the carbinolamide product 226; in the presence of added sodium cyanide, no cyanolactam 235 was formed from 220. On the other hand, the cyanopyrrolidine was extensively (20-40%) metabolized in the presence or absence of cyanide with lactam 220 being the major product (48% of total metabolites). Surprisingly, in addition to 220, significant amounts of the dicyano compounds 234a (20% of total metabolites), 234b (17%) and the cyanolactam 235 (15%) were generated from the metabolism of 223 even though inorganic cyanide had not been added to the incubation mixture. A number of α-aminonitriles such as amygdalin (238), α-N-alkylaminophenylacetonitriles 239, and 2-N-phenethylaminoalkanenitriles 240 are known to liberate cyanide upon non-

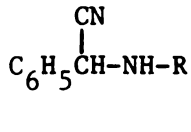


Scheme 1. Proposed pathways to the various compounds characterized from microsomal incubates of 1-benzylpyrrolidine in the presence and absence of cyanide.

enzymatic dissociation at pH 7.3 and 37°.[170] In the present case, the formation of 223, 234a, 234b, and 235 from the incubation of the cyanopyrrolidine compound was found to require NADPH, implying that the liberation of cyanide is an enzymatic reaction.



238 R = Glucoside



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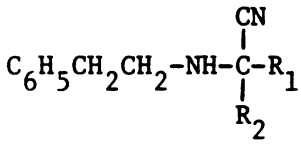

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R, R_1, R_2 = H or Alkyl

The remarkable trapping efficiency observed with the metabolically generated cyanide ion suggested that the concentration of cyanide ion used in earlier experiments (10 mM) was unnecessarily high. A series of experiments to determine the optimal cyanide ion concentration for the formation of 223 from 1-benzylpyrrolidine (1 mM) incubations and to determine the effect of varying cyanide concentration on the metabolism of 1 were conducted. The results summarized in Table V establish that maximum trapping efficiency (in terms of cyano adduct 223 formed) is achieved at a concentration of 1 mM inorganic cyanide. At higher concentrations of cyanide ion, the metabolism of 1-benzylpyrrolidine is effectively inhibited and the yields of the cyano adduct are lower. Although 1 mM sodium cyanide is considerably higher than the estimated maximum concentration of cyanide ion released in the incubation of 223 (< 0.2 mM), lipoidal membrane barriers not encountered by metabolically generated cyanide ion from 223 may limit the access to metabolic sites of added inorganic cyanide.

Table V. Effect of cyanide ion concentration on the formation of 223 from microsomal incubations of 1 (1.0 mM).

NaCn (mM)	0	0.2	0.4	0.8	1.0	2.0	5.0	10.0
% 1 Metabolized	70	78	58	68	61	61	53	51
Cyano adduct 223 formed (% of total metabolites)	--	7	25	40	40	36	20	17

The evidence accumulated thus far indicates that 1-benzylpyrrolidine undergoes an NADPH dependent microsomal oxidation to generate intermediate species which in the presence of cyanide are converted to isolable cyano adducts. Scheme 1 attempts to summarize the various biotransformation pathways proposed for 1-benzylpyrrolidine and its metabolic products. The monocyano adduct 223 may be further metabolized at C-2 to the lactam 220 or, by oxidative attack at C-5, it may form a second electrophilic intermediate 241 that leads to the cis- and trans-dicyano adducts 234a and 234b. The further metabolism of these dicyano adducts may result in the cyano lactam 235.

The key steps in the formation of cyano adducts 223 and 234 are the ionization of the putative precursor carbinolamines 219 and 242 to the electrophilic iminium ions 221 and 241. Although no direct evidence for the presence of these iminium intermediates is available, they provide a reasonable rationalization for the formation of the isolated cyano adducts. The fact that the enamine 222 was detected only in those incubations not containing cyanide ion suggests that the iminium ion 221 is a precursor to both the enamine and the monocyano adduct 223.

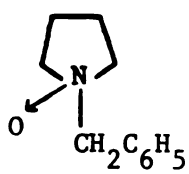
In an effort to further characterize the pathway leading to 223, 1-benzylpyrrolidine (1 mM) was incubated with microsomes and NADPH in the absence of cyanide for one hour and then sodium cyanide (10 mM) was added and the extent of cyano adduct 223 formation was measured over time. A previous experiment had established that 30 minutes after the addition of NADPH no further metabolism of the substrate 1 occurred. If the iminium ion is a common precursor to both enamine 222 and cyano adduct 223, one would expect to see the disappearance of the enamine upon the addition of cyanide with the concurrent appearance of the cyano adduct over time.

Inexplicably the gc tracing of an aliquot of the incubation mixture analyzed at the end of the one hour preincubation period but before the addition of sodium cyanide did not show the enamine product. Nevertheless, the amount of lactam 220 observed at this time accounted for only 15-20% of the metabolized substrate 1, leaving unaccounted 80-85% of the metabolites formed. We speculate that the "missing" metabolites can be accounted for as the now undetected enamine species or some non-extractable but reversible adduct formed with an endogenous nucleophile. Following the addition of sodium cyanide to this mixture, the formation of cyano adduct 223 was monitored (Table VI). After 30 minutes 70% of the 1-benzylpyrrolidine metabolized could be accounted for as cyano adduct 223. It seems quite evident that the mixed function oxidation of 1 generates a relatively stable species (carbinolamine ?) which undergoes conversion to an electrophilic intermediate (iminium ion?) that is trapped by cyanide ion.

Table VI. Formation of 1-benzyl-2-cyanopyrrolidine (223) after addition of sodium cyanide to a 1 hour postincubate of 1-benzylpyrrolidine (1).

Minutes After Addition of Sodium Cyanide	0	15	30	45	60
Cyano Adduct 223 Formed (% of 1 Metabolized)	--	49	70	71	61

The possible involvement of an N-oxidation metabolic pathway in the formation of the cyano adducts was examined with the aid of 1-benzylpyrrolidine-N-oxide (243) synthesized by the m-chloroperoxybenzoic acid oxidation of 1.[171] Gas chromatographic analysis of the base extract obtained from the postincubate of 243 with the 100,000 x g microsomal fraction in the presence of inorganic cyanide showed no trace of the cyano adducts 223, 234, or 235. In addition, attempts to characterize, by gc analysis, a metabolic product corresponding to 243 from the incubation of 1 with the 100,000 x g microsomal fraction failed. These results suggest N-oxide formation is not involved in the metabolism of 1 or in the formation of the putative iminium intermediates and the isolated cyano adducts.



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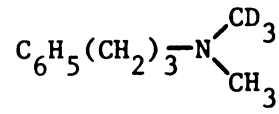
4. DEUTERIUM ISOTOPE EFFECT STUDIES

It appears that α-carbon oxidation is the initial step in the metabolism of 1-benzylpyrrolidine (Scheme 1). The metabolism of the deuterated analog 1-2,2-d_2 presents an unique opportunity to examine the deuterium isotope effect on α-carbon hydroxylation in a compound with both protio and deuterio atoms on the same molecule. The deuterium isotope effect of α-carbon hydroxylation on the pyrrolidine ring (if it exists) will be seen only if the energy barrier to rotation about the benzylic carbon-nitrogen bond is low (free rotation) relative to the energy barrier for proton abstraction (carbon hydroxylation). If rotation about this bond is slow or not allowed, there will just be a statistical probability (1:1) that either the labeled or unlabeled side of the pyrrolidine ring will be at the active site and no isotope effect will be seen.

Deuterium isotope effects on α-carbon hydroxylation have been reported for metabolic N-dealkylation reactions. Most of the reported kinetic deuterium isotope effects (k_H/k_D) are less than 2.0. The minimum theoretical k_H/k_D value is the square root of the ratio of the masses m_D/m_H which equals 1.4.[172] These observations have prompted speculation that primary deuterium isotope effects are involved in N-dealkylation and that cleavage of the α-C-H bond is the rate determining step.[173,174] Based on formaldehyde formation, Elison, et al. found morphine (244) to be N-demethylated at a rate 1.4 times as fast as morphine-N-methyl-d_3 (244-d_3) in rat liver preparations.[175] In rat liver microsomes, the average deuterium isotope effect (k_H/k_D) was 1.25 for the N-demethylation of ethylmorphine-N-methyl-d_3 (164-d_3).[176] Abdel-Monem compared the oxidative N-demethylation of a protiomethyl group with the demethylation of a deuteriomethyl group on the same molecule. The k_H/k_D value for the

demethylation of 245 was found to be 1.45 with mouse microsomes and 1.32 with uninduced rat microsomes.[173] A primary isotope effect value (k_H/k_D) of 1.49 was measured for the N-dealkylation of lidocaine-d_4 (55-d_4).[174] However, when a mixture of nicotine (60) and nicotine-5',5'-d_2 (60-d_2) was incubated with sodium cyanide in a rabbit liver microsomal preparation, the ratio of the isolated unlabeled to labeled 5'-cyanonicotine products was found to be 0.96.[177] The findings of this study argues against α-C-H bond cleavage as the rate determining step.



245

1-Benzylpyrrolidine (1-2,2-d_2) can be hydroxylated α to nitrogen at C-2 or C-5 on the ring. The ratio of the amounts of dideuterio to monodeuterio products (i.e., 222-d_2/222-d_1, and 223-d_2/223-d_1) should reflect the deuterium isotope effect of the initial α-carbon hydroxylation. The ratio of the amounts of labeled to unlabeled 220, 226, or 235 should also provide information on the deuterium isotope effect of the other oxidative reactions involved in the metabolism of 1.

The base extract obtained from the incubation of 1-2,2-d_2 was analyzed by gc-eims. The k_H/k_D ratios of the various metabolic products are shown in Table VII. These ratios were obtained by measuring the peak heights (gc-eims) of appropriate ions in the mass spectra of the compounds in question after correcting for natural isotopic contributions.

Table VII. Isotope effect k_H/k_D

222 (222-d_2/222-d_1)	1.1
220 (220-5,5-d_2/220-d_0)	2.4
226 (226-d_1/226-d_0)	0.9
223 (223-d_2/223-d_1)	1.1
235 (235-d_1/235-d_0)	3.2

The data indicate no deuterium isotope effect in the formation of 222 or 223. The iminium ion 221 is the common precursor to these two products; in turn the carbinolamine 219 is the precursor to 221. The absence of an isotope effect in the formation of the enamine 222 or cyano adduct 223 suggests that the α-carbon hydroxylation process which generates 219 is not affected by deuterium substitution. On the other hand, an isotope effect is observed for the formation of 220 (k_H/k_D = 2.4) and 235 (k_H/k_D = 3.2). These results can be rationalized by assuming deuterium isotope effects on the oxidation of the initially formed cyano adduct 223. The data indicates that 223-d_2 and 223-d_1 are formed in equal quantities. However, hydroxylation at C-2 of 223 to produce the cyanohydrin 236 and, subsequently, the lactam 220 occurs at different rates for 223-d_2 and 223-d_1. Apparently, 223-d_2 is preferentially hydroxylated over 223-d_1 at C-2 to give an enrichment of 220-d_2. Similarly, 223-d_1 is preferentially hydroxylated at C-5 to give an enrichment of 235-d_1 over 235-d_0. The k_H/k_D value for the formation of 220 might also be explained by assuming a deuterium isotope effect on the

direct oxidation of the carbinolamine 219 to 220. The k_H/k_D ratio observed for 220 is not affected by cyanide ion. The possibility of a deuterium isotope effect on the oxidation of 219 to 220 cannot be accurately assessed because the relative amounts of 220 formed from the oxidation of 219 or 223 has not been determined. The observed ratio for 226 is hard to explain, but the rest of the data can be rationalized according to the pathways proposed in Scheme 1.

5. MICROSOMAL PROTEIN BINDING OF 1-BENZYLPYRROLIDINE METABOLITES

The capability of the proposed metabolically generated iminium intermediates to alkylate macromolecules was tested in microsomal protein binding studies with radiolabeled 1-benzylpyrrolidine. Since products resulting from metabolic oxidation at the benzylic carbon of 1 were not detected the tritium label was introduced at the benzylic position. The radiolabeled substrate, 1-benzylpyrrolidine-α,α-$^3H_2$ (1-α,α-$^3H_2$, 1.97 mCi/mmole) was synthesized by $LiAl^3H_4$ reduction of 1-benzoylpyrrolidine (227). After the incubation of 1-α,α-$^3H_2$ with rabbit liver microsomal preparations, the postincubates were repeatedly homogenized with ethanolic $HClO_4$ or EtOH (until washes showed a constant level of radioactivity) to remove any non-covalently bound metabolites or substrate. The results (Table VIII) show that the binding of 1-α,α-$^3H_2$ to the microsomal macromolecular fraction was 7 to 8 times greater in incubations performed in the presence of NADPH than in the absence of NADPH. The level of radiolabel incorporated into the macromolecular fraction was low compared to the amounts of cyano adducts isolated from the sodium cyanide coincubates. However, the level of incorporation found in these experiments is comparable to that reported in the literature for other metabolically

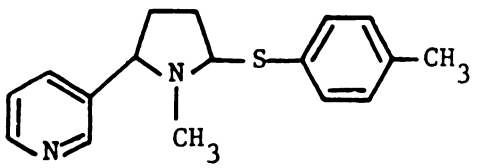
activated cytotoxic xenobiotics (e.g., acetaminophen[37] and isoniazid[50]). Since incorporation of the label is dependent on the presence of NADPH, the species binding to macromolecular microsomal components are likely to be formed by the mixed function oxidation of the substrate. Further experiments have shown that sodium cyanide does not block the observed binding.

Table VIII. Incorporation (nmoles/mg protein) of 1-α,α-$^3H_2$ into microsomal macromolecules

Washing Procedure	With NADPH	Without NADPH
$HClO_4$/EtOH	.17	.02
	.19	.02
EtOH	.29	.04
	.36	.05

The low level of incorporation of label in the microsomal macromolecular fraction (< 1% of total substrate incubated) compared to the level of cyano adduct formation (30-50% of 1 incubated) could be due to the instability of thio, aza, or oxo adducts expected to result from attack of the iminium species by the corresponding nucleophilic functionalities on macromolecules. As we have seen in Chapter III, iminium ions are chemically reactive with almost all nucleophiles but in many cases the products are highly unstable and decompose. Attempts to isolate thio and oxo adducts from the reaction of simple nucleophiles with iminium salts in chemical systems have met with difficulty because of product

instability.[120] However, the thio adduct 246 was characterized from the addition of p-methylbenzenethiol (247) to the reduction production of cotinine (63).[112] Reduction of 63 with $Na(CH_3OCH_2CH_2O)_2AlH_2$ apparently gives rise to the nicotine $\Delta^{1'(5')}$ iminium ion (65) which can be trapped as a stable adduct by the nucleophilic 247. Nevertheless, one would expect that the instability of thio and oxo adducts in general would be compounded many-fold by various factors in biological systems rendering the isolation of such adducts extremely difficult.



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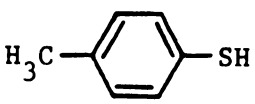

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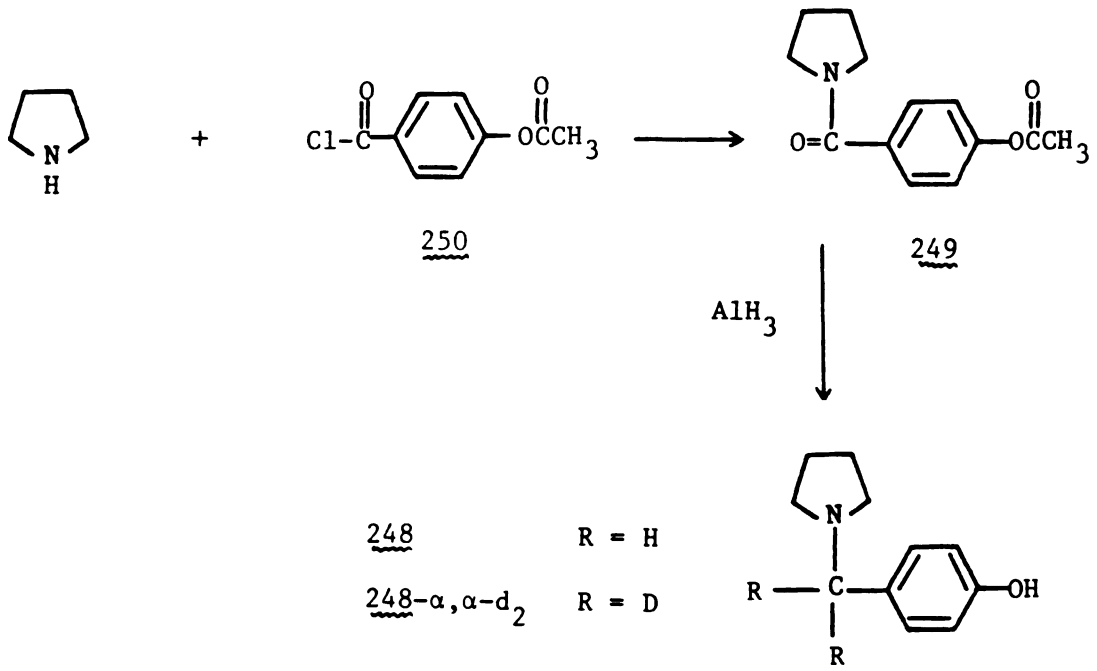
Another possible explanation for the low incorporation would implicate the participation of another electrophilic intermediate other than the postulated iminium species. A likely candidate would be an arene oxide intermediate. Since arene oxide formation would be expected to produce the corresponding para-phenolic metabolite, microsomal postincubates of 1-α,α-d_2 were analyzed for the presence of 1-(4-hydroxylbenzyl)pyrrolidine-α,α-d_2 (248-α,α-d_2). The deuterium free internal standard 248 was synthesized by AlH_3 reduction of 1-(4-acetoxybenzoyl)pyrrolidine (249) which in turn was prepared by acetylation of pyrrolidine with p-acetoxybenzoyl chloride (250).



Microsomal postincubates of 1-α,α-d_2 (1 mM) were first treated with 10 μg of 248 and then extracted with ethyl acetate (at pH 9). Following derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA), gc-eims analysis was performed. Both the parent ion (M^+ 249) and base peak (m/e 179) of the internal standard (Figure 15) were readily detected by multi-channel averaged narrow scans. The intensity of the ion at mass 251 for the proposed metabolite 248-α,α-d_2 (m/e 251 to m/e 249 was 0.0621 ± 0.0058) were indistinguishable from that observed with pure unlabeled 248 (m/e 251 to m/e 249 was 0.0590 ± 0.032). Less than 0.05% of the substrate is estimated to be present as the p-hydroxy compound in the postincubate. Based on these results it appears unlikely that arene oxide formation is responsible for the observed macromolecular binding of tritium-labeled 1. These data suggest that metabolically generated iminium ions are capable of alkylating nucleophilic functionalities present on microsomal macromolecules.

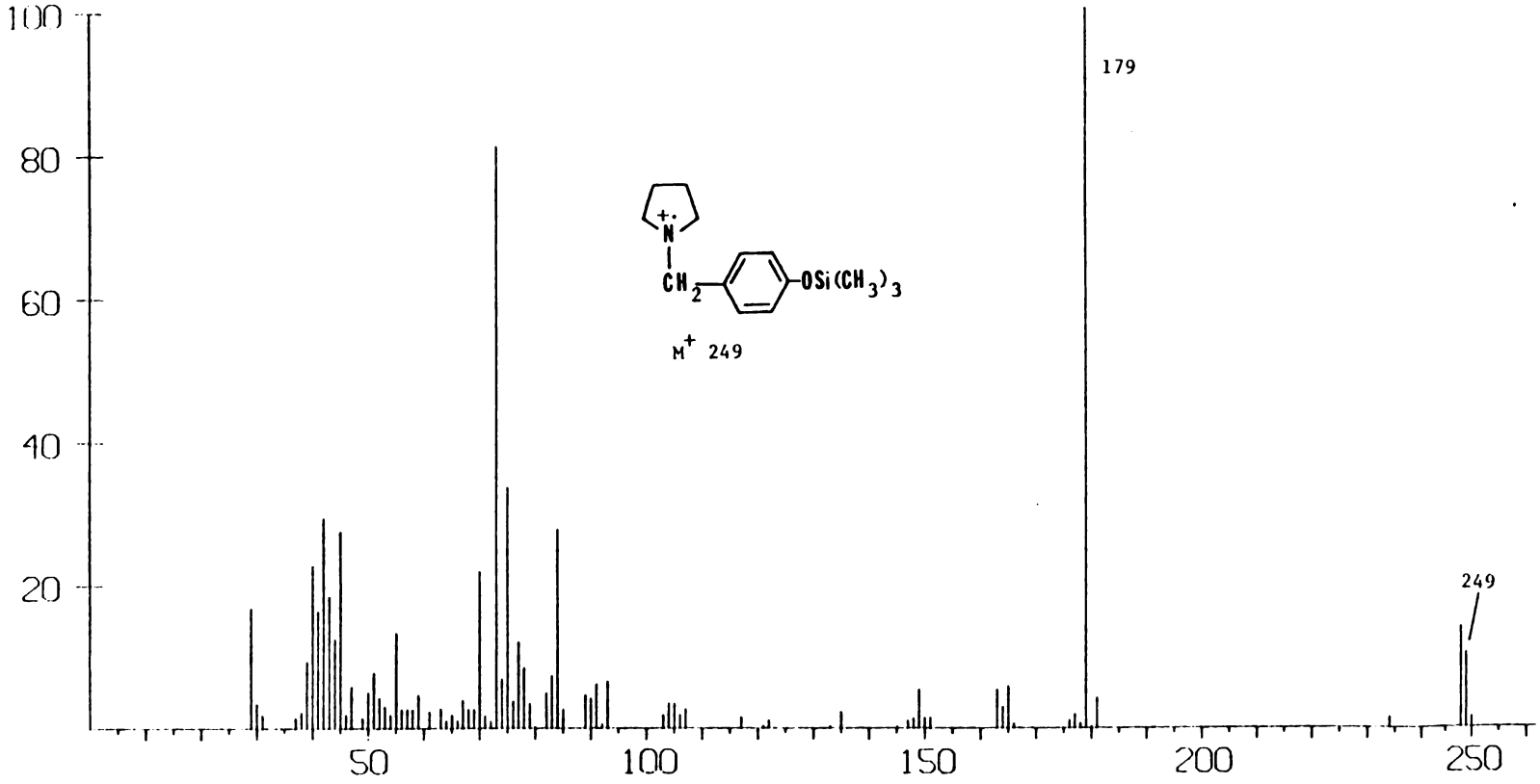


Figure 15. The gc-eims of the product resulting from the treatment of 1-(4-hydroxybenzyl)-pyrrolidine (248) with BSTFA.

CHAPTER V

METHAPYRILENE

A. INTRODUCTION*

Many alkaloids, food additives, pesticides, and numerous drugs encountered by man are tertiary amines.[178] The histamine H_1-receptor antagonists (antihistamines) are a particularly important group of tertiary amines. Because of their common usage, the chemistry, metabolism, and toxicology of antihistamines are of considerable concern to humans.

Antihistamines have been found to be of considerable use because of their therapeutic effects. However all H_1 antagonists elicit side effects even in therapeutic doses. These side effects are rarely serious and disappear with continued therapy. At times, they can be troublesome and may even lead to withdrawal of the drug from therapy. Some of the frequent, non-severe side effects include sedation and other central actions (i.e., dizziness and tinnitus), digestive tract irritation (i.e., nausea and vomiting), and allergic reactions.[179] Grave complications such as leukopenia and agranulocytosis occur rarely; in addition, the piperazine compounds, cyclizine (251), chlorcyclizine (252), and meclizine (253), have been shown to be teratogenic.[179] In May, 1979, the FDA took regulatory action on the over the counter antihistamine agent methapyrilene [N,N-dimethyl-N'-2-pyridyl-N'-(2-thienylmethyl)-1,2-ethanediamine, 2].

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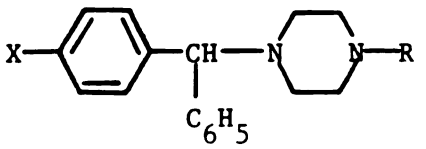
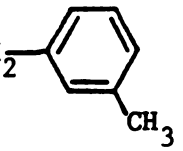
*The work described in this chapter was performed in collaboration with Dr. René Ziegler currently with Sandoz A.G., Basel, Switzerland. (Please see Acknowledgment)

This action was based on animal studies which showed methapyrilene to be a potent hepatocarcinogen in rats and as such, a potential hazard to humans.[180]



251 X = H; R = CH_3

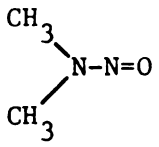
252 X = Cl; R = CH_3

253 X = Cl; R = 

288 X = Cl; R = H

1. THE FORMATION OF N-NITROSO DERIVATIVES

A number of tertiary amines react with nitrous acid in mildly acidic pH (3.0 - 6.5) solutions to form dialkylnitrosamines.[178] Methapyrilene has been demonstrated to undergo reaction with nitrous acid at pH 3.4 - 3.5 (90°, 1 hour) to give the potent carcinogenic dimethylnitrosamine (254) in 14% yield.[181] Under the same conditions, the antihistamine chlorpheniramine (255) gives rise to 254 in 1.8% yield. At 37°, 255 and 2 produce dimethylnitrosamine in 0.07% and 0.7% yield, respectively. These studies indicate that tertiary amines and methapyrilene, in particular, can react with nitrous acid under conditions existing in the stomach (mildly acidic) to produce possible carcinogenic N-nitroso compounds. This chemical finding was confirmed by studies which showed a significant incidence of liver tumors and other liver lesions in rats fed an aqueous solution of methapyrilene plus sodium nitrite.[182] It was postulated that the hepatocarcinogenicity was due to formation of dimethylnitrosamine in the stomach.



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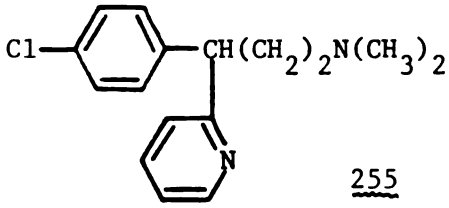

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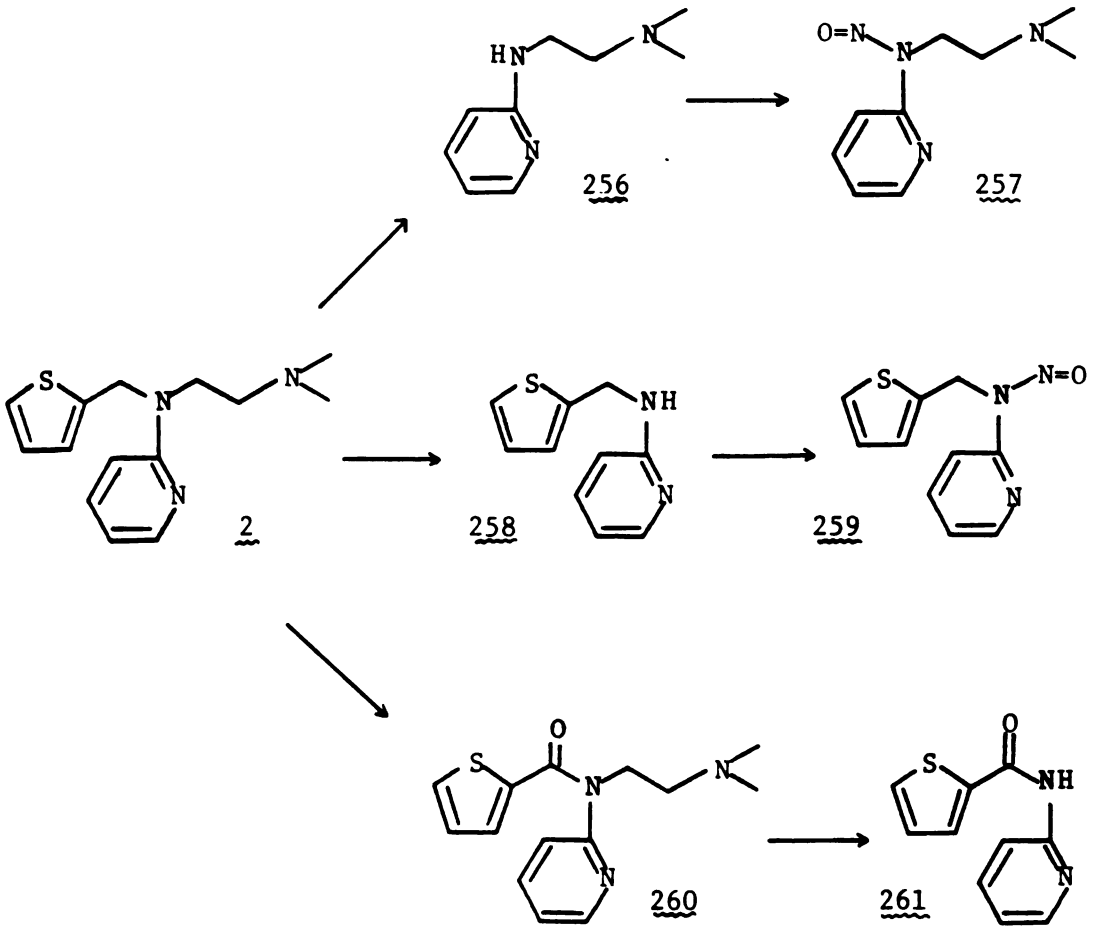
More recent studies have indicated that the reaction of 2 with nitrous acid is complex.[183] Under conditions simulating gastric digestion (i.e., 4 hours at 37° and pH 3.7), only small amounts of dimethylnitrosamine were found in incubations of 2 with nitrous acid. The secondary amine N-(2-pyridyl)-N',N'-dimethylethanediamine (256) and the corresponding nitroso derivative 257 were isolated from incubations of methapyrilene with sodium nitrite at 25° and pH 6.6. It is believed that 256 is formed through the nitrite oxidation of the thiophene activated methylene bridge of 2. The secondary amine 2-(2-thienylmethylamino)pyridine (258) and its N-nitroso derivative 259 were detected under similar conditions. When methapyrilene was treated with a large excess of sodium nitrite at pH 3.7 and 90°, the major reaction product was found to be the amide 260 formed from the nitrite oxidation of the methylene bridge. Other dealkylation products observed at 90° were 258 and 261. The results clearly demonstrate that the nitrosation reaction of methapyrilene cannot be regarded simply in terms of dimethylnitrosamine formation.



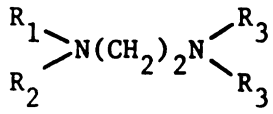
Chronic studies indicate that in rats treated with methapyrilene (1000 ppm) or with methapyrilene (1000 ppm) plus nitrite (2000 ppm), the incidence of liver tumors is the same.[180] Liver tumors were found in rats treated with methapyrilene alone at the end of a 26 week subchronic dose study.[180] These recent results imply that the nitrosation of methapyrilene is not required for its carcinogenic activity. If N-nitroso derivatives of methapyrilene are not the responsible carcinogenic agents, then it is very likely that methapyrilene itself is converted to other toxic species. It is possible that the pyridyl ring can undergo aromatic oxidation to reactive arene oxide species or the thiophene moiety can be oxidized to a reactive epoxide species. Our interests

thus far have focused on the formation of iminium intermediates. As will be discussed below, methapyrilene has four sites available for α-carbon hydroxylation and subsequent formation of electrophilic iminium intermediates.

Methapyrilene has been used therapeutically for over thirty years but no report on its metabolism has appeared in the literature. The following discussion is a brief review of the metabolism of structurally related antihistaminic agents and of thienyl and pyridyl substituted compounds.

2. THE METABOLISM OF H_1-HISTAMINE ANTAGONISTS

Most of the effective histamine H_1-receptor antagonists may be described by the general structures 262-264. The R_1 and R_2 substituents on the generalized β-aminoethyl side chain are aryl groups or arylmethyl groups.[186] The R_3 substituents are usually methyl groups. The other moiety on the generalized β-aminoethyl side chain may be nitrogen (ethanediamine, 262), a saturated carbon (dialkylaminopropane, 263), or a saturated (sp^3) carbon-oxygen moiety (β-dialkylaminoethers, 264).[186]



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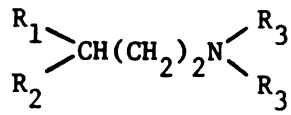

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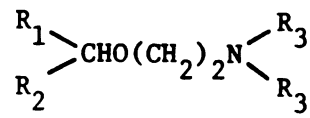


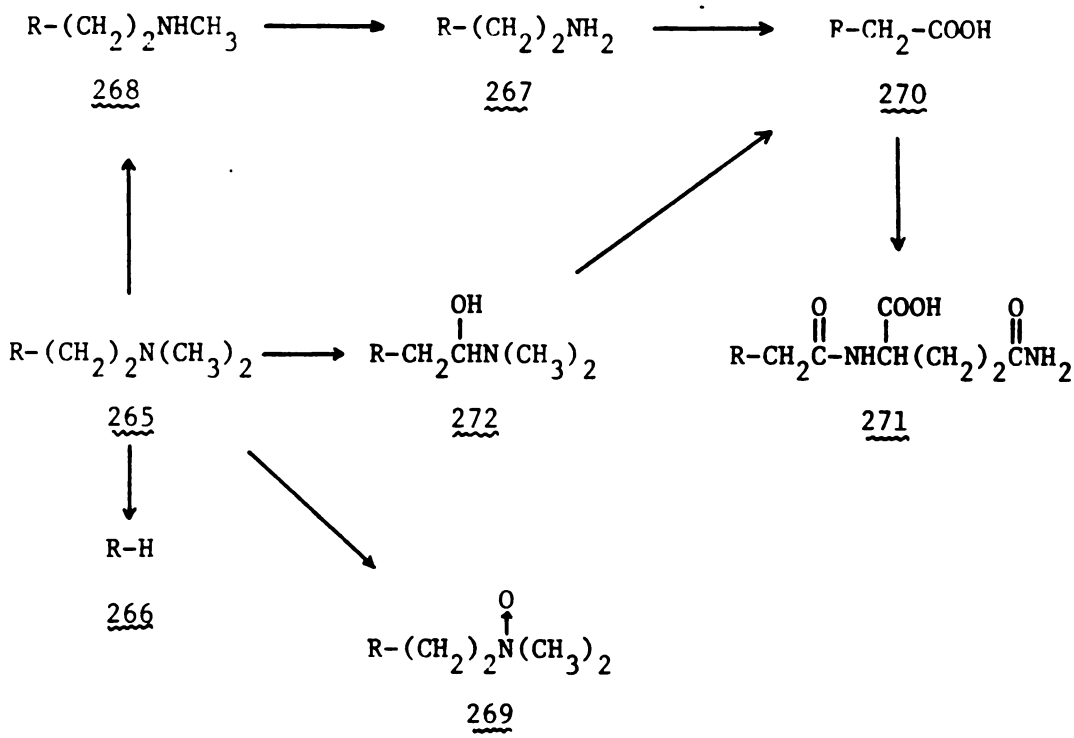
264

Most H_1 antagonists are readily absorbed from the gastrointestinal tract and parenteral sites of administration. Studies on the metabolic fate of a limited number of antihistamines have shown that these drugs

are extensively metabolized and that the main site of these transformations is the liver.[179]

The β-dimethylamino ether, diphenhydramine (265), was found to be extensively metabolized by rats in studies utilizing ^{14}C-labeled diphenhydramine (radiolabel at the benzyl carbon atom) as a tracer.[187] Glazko and Dill showed 265 to be metabolized by minced rat livers to non-basic metabolites. These authors rationalized the formation of these products by assuming cleavage of the ether linkage to yield dimethylaminoethanol and benzhydrol (266).[188] Liver tissue was found to be most effective in the degradation of 265. Oxygen is required for metabolic activity. The optimal pH for the degradation of 265 by rat liver is 7.3; the reaction can be inhibited by various reagents but sodium cyanide at 2.0 mM concentrations has little or no effect.[188] Drach and Howell employed tritiated diphenhydramine with ^{3}H in the diphenylmethoxy moiety to aid in the identification of the urinary metabolites of 265 from the rhesus monkey.[189] The primary (3-6% of total metabolites) and secondary (5-7%) amine analogs of diphenhydramine (267 and 268, respectively), diphenhydramine N-oxide (269, 7-13%), and benzhydrol (266, 1-2%) were identified as urinary metabolites of 265 by comparison with authentic compounds. But diphenylmethoxyacetic acid (270, 10-20%) and its glutamine conjugate (271, 45-50%) accounted for the majority of the urinary metabolites. The characterization of 270 and 271 as metabolites indicates that the major site of metabolic oxidation is at the methylene carbon atom alpha to the dimethylamine nitrogen atom. It is not clear whether the acid 270 arises from the direct oxidation of 265 or from the metabolites 268 and 267. It is possible that oxidative attack on 265 leads to the carbinolamine intermediate 272 which can subsequently give rise to 270 and its conjugate 271. The following scheme attempts to summarize the observed

pathways of diphenhydramine metabolism.



R = $OCH(C_6H_5)_2$

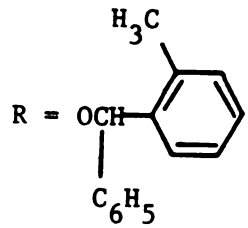
The structurally similar compound, orphenadrine (273), also undergoes N-demethylation and O-dealkylation. The primary and secondary amine analogs of 273 (274 and 275, respectively) and the substituted benzhydrol 276 have been identified as metabolites of orphenadrine in the urine of rats.[190]

$R-(CH_2)_2N(CH_3)_2$ 273

$R-(CH_2)_2NHCH_3$ 275

$R-(CH_2)_2NH_2$ 274

R-H 276



The metabolism of the aminopropyl compound, chlorpheniramine (255), has been studied in the dog, rat, and man.[191-193] The desmethyl and bisdesmethyl analogs (277 and 278, respectively) have been characterized

as urinary metabolites of 255 in man but 68% of the administered drug remains unaccounted for in these studies.[191] Later studies on the metabolism of chlorpheniramine in man utilizing tritium-labeled drug showed that a large portion of the administered radioactivity (25-55%) was excreted as unidentified polar metabolites.[192] Chlorpheniramine was similarly metabolized in the dog and the rat.[193] The primary amine 278 was found to be a major metabolite in the urine of dogs and rats (8% and 20%, respectively). In both species, a high percentage of the recovered radiolabel exists as unidentified polar metabolites which are not susceptible to enzymatic or acidic hydrolysis.[193] A recent study utilizing ^{14}C-labeled chlorpheniramine has shown that in addition to the N-desmethyl metabolites 277 (5% of total recovered metabolites) and 278 (17%), three polar metabolites can be isolated from the urine of dogs.[194] One of these polar metabolites (12%) was identified as the alcohol 279 by comparison of mass spectral characteristics with the authentic compound. Acid hydrolysis of the most polar metabolite (18%) indicated that it was a conjugate but not a glucuronide or sulfate derivative. The hydrolysis product was identified as 2-(p-chlorobenzyl)-2-(2-pyridyl)propionic acid (280) by comparison of mass spectral characteristics with the authentic compound.[194] The least abundant polar metabolite was not identified. The acid product 280 is apparently conjugated with an amino acid *in vivo* to form a polar product. The alcohol 279 may result from the reduction of the initially formed aldehyde 281. Such an aldehyde could easily result from an oxidative N-dealkylation reaction; the authors suggest that the unidentified metabolite might be the aldehyde but could not confirm this possibility.[194] In addition, no evidence for an N-oxide product was found.

$R\text{-}(CH_2)_2N(CH_3)_2$ — 255, 282

$R\text{-}(CH_2)_2NHCH_3$ — 277, 283

$R(CH_2)_2NH_2$ — 278, 284

$R\text{-}(CH_2)_2OH$ — 279

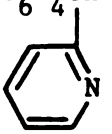
$R\text{-}CH_2COOH$ — 280, 286

$R\text{-}CH_2CHO$ — 281

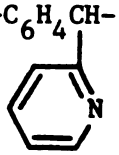
$R\text{-}(CH_2)_2\overset{\uparrow}{N}(CH_3)_2$ (O) — 285

$R\text{-}CH_2CONHCH_2COOH$ — 287

255, 277 - 281 R = p-Cl-C_6H_4CH-



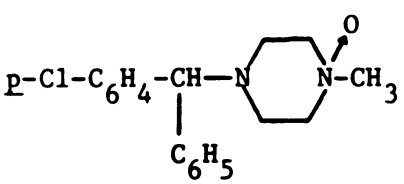
282 - 287 R = p-Br-C_6H_4CH-



The metabolism of brompheniramine (282) in the dog and human was studied by Bruce, et al. utilizing ^{14}C-brompheniramine.[195] Comparison of tlc characteristics with authentic compounds has led to the identification of five metabolites in dog urine and four metabolites in the urine of humans.[195] The principal route of metabolism in both species is N-dealkylation to give the mono and bisdesmethyl derivatives (283 and 284, respectively). The N-oxide product 285 was found as a minor metabolite only in the dog. Oxidative dealkylation resulted in the isolation of 2-(p-bromophenyl)-2-(2-pyridyl)propionic acid (286) as a metabolite in both species. The glycine conjugate 287 was also identified as a minor metabolite.

Studies on the metabolism of benzhydrylpiperazine antihistaminic

agents have shown that N-dealkylation is a major metabolic pathway.[196,197] Piperazine type antihistamines have been shown to exhibit strong teratogenic potential in rats. Cyclizine (251), chlorcyclizine (252), and meclizine (253) can induce malformations in rats at doses of 50-75 mg/kg.[198] Norchlorcyclizine (288), a major metabolite of 252 and 253, produces these same malformations at a lower dose (25 mg/kg).[198] In this study, methapyrilene showed no teratogenic potential even at doses up to 250 mg/kg. Chlorcyclizine is metabolized in rats and dogs to norchlorcyclizine.[196] Chlorcyclizine N-oxide (289) has been characterized as a metabolite of 252 in the urine of rats and humans.[199] No norchlorcyclizine could be characterized from the incubation of the N-oxide 289 with 9,000 x g rat liver supernatant. These results indicate that in this case the N-oxide is not a direct intermediate in the formation of the demethylated product 288. Another metabolite resulting from the cleavage of the piperazine ring has been detected in the tissues of rats treated with chlorcyclizine. The structure of this metabolite which accumulates in tissues has been identified as N-(p-chlorobenzhydryl)-1,2-ethanediamine (290) by comparison with the synthetic compound.[200] The teratogenic compound meclizine also has been demonstrated to give rise to the demethylated product 288 and the ethanediamine 290.[197,200]



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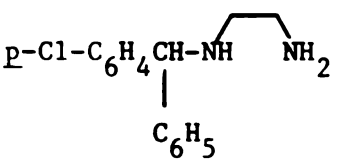
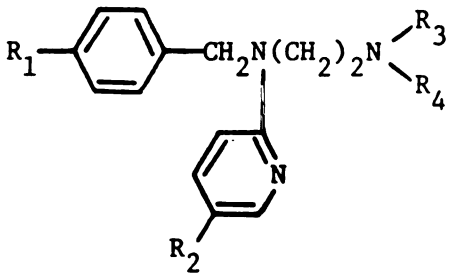


290

The metabolism of the ethanediamine antihistamine tripelennamine (291) has been studied in the guinea pig. Tripelennamine labeled

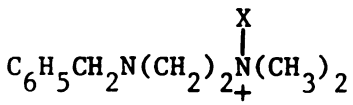
with ^{14}C in the benzyl carbon was found to be metabolized to several products which were excreted in the urine. Indirect evidence suggested that the majority of the metabolites were glucuronide conjugates of tripelennamine. There were also indications that the dimethylamino group had been modified metabolically.[201] Four polar metabolites have been isolated and characterized from the urine of human subjects treated with tripelennamine. The N-oxide (292) was identified as a minor metabolite by comparison with the authentic compound. Two of the other metabolites were shown to be O-glucuronide conjugates after hydrolysis with β-glucuronidase. The identity of the two hydrolyzed products were determined by nmr and mass-spectral studies. The major metabolite is the glucuronide of hydroxytripelennamine 293 and the other is the corresponding O-glucuronide of nortripelennamine 294.[202] Apparently aromatic hydroxylation



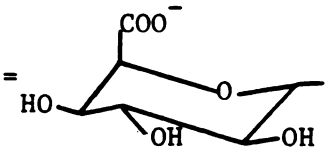
291 $R_1=R_2=H$, $R_3=R_4=CH_3$

293 $R_1=H$, $R_2=$ O-glucuronide, $R_3=R_4=CH_3$

294 $R_1=R_3=H$, $R_2=$ O-glucuronide, $R_4=CH_3$



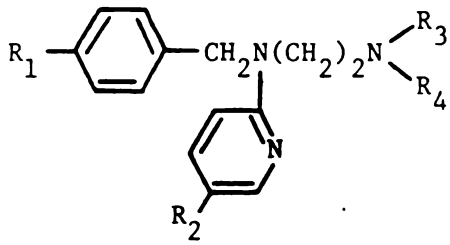
292 X = O^-

295 X = 

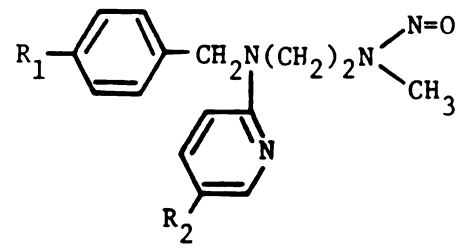
of the pyridine ring is favored over hydroxylation of the benzene ring and N-demethylation is a relatively minor pathway in man. The final major product was identified as a quaternary ammonium N-glucuronide of 291; structure 295 was deduced on the basis of chemical behavior and nmr characteristics.[202]

Tripelennamine can react with sodium nitrite *in vivo* to form N-nitrosodesmethyltripelennamine (296). In rats treated orally with tripelennamine and sodium nitrite, 6% of the administered drug is converted to 296 in the stomach and the nitroso product could be detected in the urine.[203] Oral administration of tripelennamine, N-nitrodesmethyltripelennamine, or tripelennamine with sodium nitrite at doses of 100 mg/kg failed to produce kidney or liver necrosis in rats in short-term toxicity studies.[203] The binding of tripelennamine and its N-nitroso derivative to rat liver microsomal protein was examined with the aid of benzyl-^{14}C and N,N-dimethyl-^{3}H labeled derivatives.[204] Both tripelennamine (291) and its N-nitroso derivative (296) exhibit covalent binding to liver microsomal protein. This binding requires an NADPH-generating system and oxygen and is inhibited by a carbon monoxide-oxygen atmosphere. The covalent binding of 296 was ten times greater than that of 291 whereas *in vitro* demethylation of 291 was greater than that of 296. When equimolar mixtures of benzyl-^{14}C and N,N-dimethyl-^{3}H derivatives of 296 were incubated with rat liver microsomal protein, the covalently bound metabolite was found to retain both radiolabels in exactly the same amounts, indicating that N-dealkylation is not required for the formation of the reactive metabolite from 296.[204] Similarly, the covalently bound metabolite of 291 retains the benzyl moiety and both methyl groups. Even though tripelennamine is rapidly N-demethylated, it appears that this metabolic reaction is not responsible for the formation of the reactive

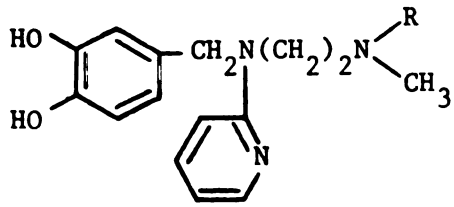
metabolite(s).



	R_1	R_2	R_3	R_4
297	H	H	H	H
298	OH	H	H	CH_3
299	H	H	H	CH_3
300	OH	H	CH_3	CH_3
301	OH	OH	CH_3	CH_3
302	OH	OH	H	CH_3



	R_1	R_2
296	H	H
304	OH	H
305	OH	OH



303 R = H

306 R = N=O

Further in vivo studies on the metabolism of 291 and 296 in the rat have demonstrated that the level of covalent binding of these two compounds to tissue and plasma proteins is low.[205] Most of the urinary metabolites of 291 and 296 were excreted as glucuronide and sulfate conjugates, and thus were subjected to enzyme hydrolysis before characterization by gc-ms. Tripelennamine underwent extensive N-demethylation and aromatic hydroxylation, but no evidence for the formation of the bisdesmethyl derivative 297 was found. On the other hand, N-nitrosodesmethyltripelennamine only gave rise to aromatic hydroxylation products. The

major urinary metabolites of tripelennamine were found to be the p-hydroxybenzyl derivative of desmethyltripelennamine (298, 38% of total urinary metabolites).[205] Other urinary metabolites of tripelennamine include the desmethyl derivative (299, 3%), the p-hydroxylbenzyl product (300, 8%), the p-hydroxybenzyl-5-hydroxypyridyl derivatives (301, 2%, and 302, 4%, respectively) and the catechol derivative of desmethyltripelennamine (303, 2%).[205] Similarly, 304, 305, and 306 were isolated as urinary metabolites of N-nitrosodesmethyltripelennamine. The characterization of phenols and catechols from the metabolism of 291 is evidence for the involvement of an arene oxide intermediate in the metabolism of tripelennamine or its N-nitroso derivative. It is possible that arene oxides are the reactive intermediates responsible for the observed covalent binding of 291 and 296.

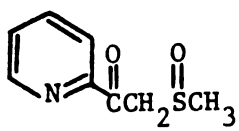
3. THE METABOLISM OF DERIVATIVES OF PYRIDINE AND THIOPHENE

In the preceding discussion, we have seen that the pyridine ring moiety of chlorpheniramine (255) and brompheniramine (282) apparently does not undergo metabolic oxidative attack. To the contrary, products resulting from metabolic attack on the pyridine component of tripelennamine have been isolated and characterized.[202,205] Metabolic oxidation of the pyridine ring does not appear to be a major metabolic pathway for tripelennamine.

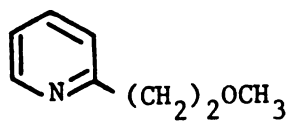
From literature reports on the metabolism of compounds containing the pyridyl moiety, it appears that this aromatic ring does not undergo metabolic attack. However, the products of this metabolic reaction did not come under consideration in most of these studies.

The metabolism of the immunosuppressive agent, oxisuran (307),

has been examined in the pig and the rabbit. The metabolites characterized from *in vivo* and *in vitro* studies are the products of carbonyl reduction to the alcohol or sulfur oxidation to the sulfone.[206,207] *In vivo* studies on the fate of methyridine (308), an anthelmintic agent, in animals have shown that this compound primarily undergoes metabolic attack on the side chain to give rise to an acetic acid derivative.[208] No metabolites resulting from oxidative attack on the pyridine ring of phenazopyridine (309) have been detected in the urine of humans or rabbits treated with 309.[209]



307



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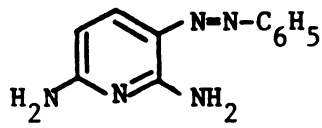
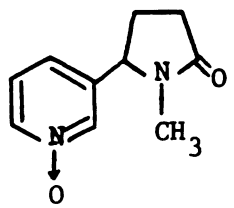


309

In addition to tripelennamine, another compound known to undergo metabolic oxidation on the pyridine ring is cotinine (63). Cotinine is reported to undergo N-oxidation to cotinine N-oxide (310).[210] Nicotinic acid (311) and nicotinamide (312) are extensively metabolized in rats and mice. Among others, nicotinamide-N-oxide (313), N'-methyl-2-pyridone-5-carboxamide (314), N'-methyl-4-pyridone-3-carboxamine (315), 6-hydroxynicotinic



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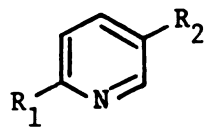
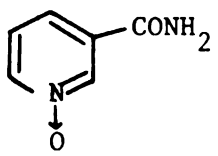


	R_1	R_2
311	H	COOH
312	H	$CONH_2$
316	OH	COOH
317	OH	$CONH_2$



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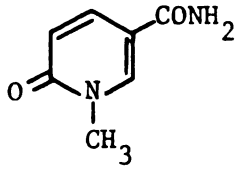

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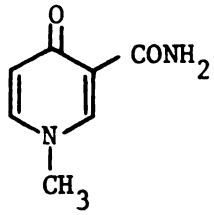
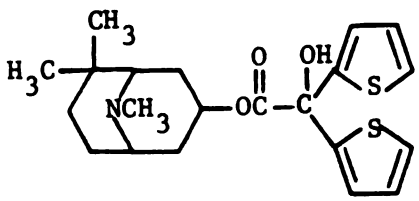


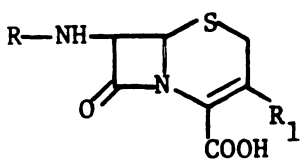
315

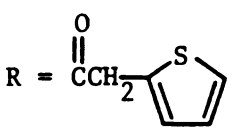
acid (316), and 6-hydroxynicotinamide (317) have been identified as urinary metabolites of 311 and 312.[211]

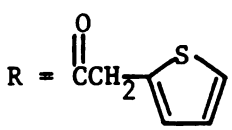
Reports on the metabolism of compounds containing a thiophene moiety are similar to those concerning the pyridyl ring. Metabolites in which the thiophene ring has been altered have not been detected yet such metabolites were not considered in these studies. The metabolic fate of the potential antiparkinsonian agent PG-501 (318) was examined in the rat; nine urinary metabolites were isolated and characterized but no evidence of metabolic attack on the thiophene ring was obtained in these studies.[212] Two antibiotics of the cephalosporin group contain the thiophene moiety.



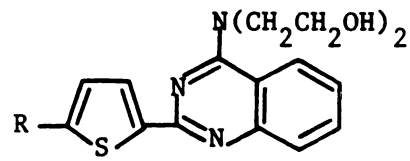
318



319 R =  $R_1 = CH_2OCCH_3$

320 R =  $R_1 = CH_2-N^+$

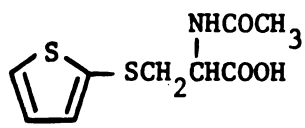
Cephalothin (319) is known to cause occasional renal damage whereas cephaloridine (320) is a potent nephrotoxin.[213] These drugs are not well absorbed and are rapidly excreted.[213] The fate of orally administered 319 and 320 has been studied in the rat. Only two products containing the intact thiophene ring and an unidentified polar compound could be isolated as urinary metabolites.[214] Several 5-nitrothiophene compounds are known to be carcinogenic but the thienyl moiety is not necessarily involved in the formation of the toxic intermediate. This is most clearly illustrated by comparing the carcinogenic potential of 321 and its 5-nitro analog 322. The 5-nitrofuran 322 was found to be a strong carcinogen in rats whereas 321 was devoid of carcinogenic activity.[215]



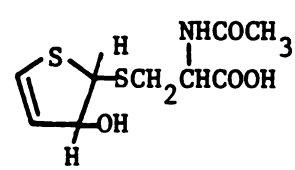
321 R = H

322 R = NO_2

Thiophene itself is metabolized and excreted in the urine of the rat and rabbit as two mercapturic acids. The first is 2-thienylmercapturic acid (323) and the second is the premercapturic acid, 3-hydroxy-2, 3-dihydro-2-thienylmercapturic acid (324).[216] It is postulated that thiophene is initially epoxidized and then conjugated with glutathione at C-2. The glutathione conjugate is then further transformed to the premercapturic acid 324 which can dehydrate to yield 323.



323



324

B. THE IN VITRO METABOLISM OF METHAPYRILENE

It is now generally accepted that most, if not all, chemical carcinogens are converted into toxic reactive species via metabolic transformation pathways. Available evidence suggests that the reported carcinogenic properties of methapyrilene are due to some reactive species not related to the interaction of methapyrilene with nitrite. Thus it seems likely that the toxic intermediate of methapyrilene results from either aromatic oxidation or α-carbon hydroxylation processes.

We have seen evidence for arene oxide intermediates in the metabolism of the structurally related tripelennamine. Direct oxidation of the sulfur or nitrogen atoms of methapyrilene might lead to the formation of reactive intermediates. However, the evidence obtained from cyanide

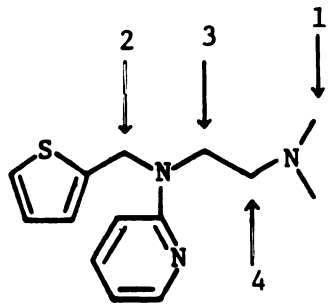
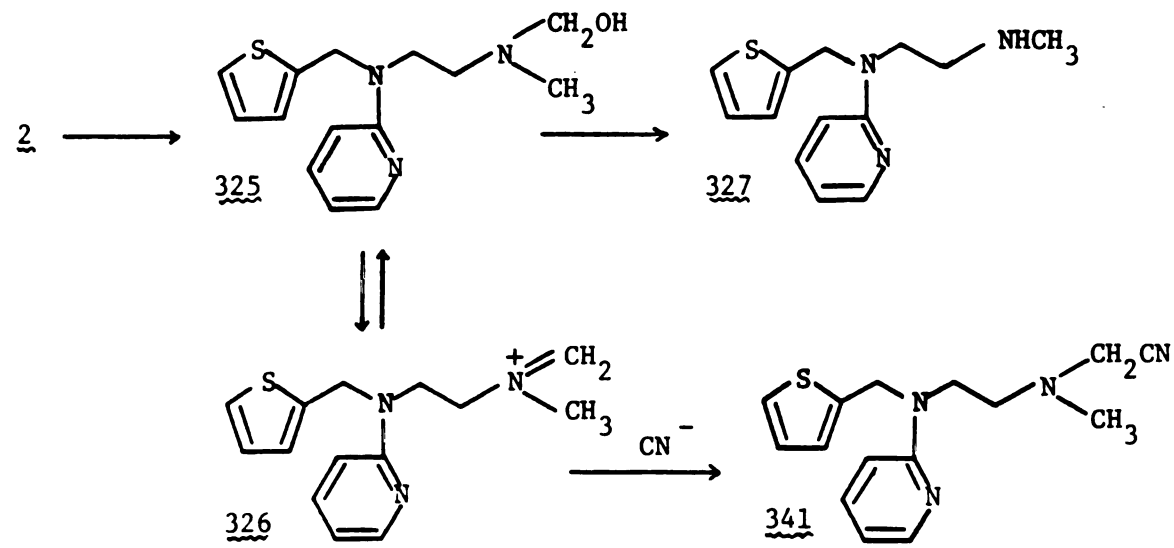


Figure 16. Sites of α-carbon hydroxylation on methapyrilene [N,N-dimethyl-N'-2-pyridyl-N'-(2-thienylmethyl)-1,2-ethanediamine, 2]

trapping experiments involving nicotine and 1-benzylpyrrolidine suggests the potential role of iminium ions as reactive metabolic intermediates. Methapyrilene has four carbon atoms alpha to nitrogen which are susceptible to metabolic hydroxylation (see Figure 16). The resulting carbinolamines can ionize to form unstable iminium ions which can react with nucleophilic functionalities present on biomacromolecules. Close exami-

nation of the putative iminium intermediates (vide infra) reveals that intramolecular stabilization is possible for several of these intermediates. Intramolecular stabilization of these electrophilic iminium ions should promote the formation of these possibly toxic reactive intermediates. In addition, the stabilization of an otherwise very unstable species might lead to the more efficient reaction with biomacromolecular nucleophiles. It is also possible that the stabilized species might have the right properties to facilitate its transport from the site of formation to, perhaps, crucial sites on nucleic acids or proteins.

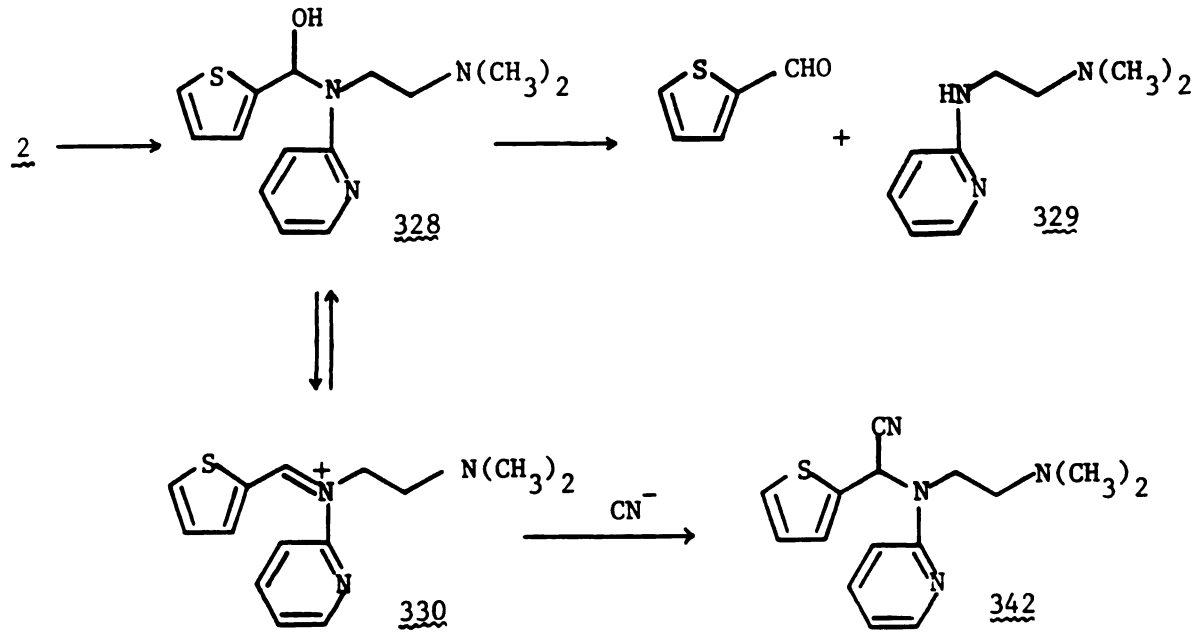
Metabolic oxidation at the N-methyl position (Scheme 2) would give rise to the hydroxymethyl intermediate 325 which could subsequently ionize to form the iminium species 326 or cleave to give the demethylated product 327 and formaldehyde. The isolation of a methyleniminium intermediate similar to 326 as a cyano adduct has been described for nicotine.[72]



Scheme 2

Oxidation at the methylene carbon adjacent to the thienyl moiety (Scheme 3) would give rise to the hydroxy intermediate 328. This

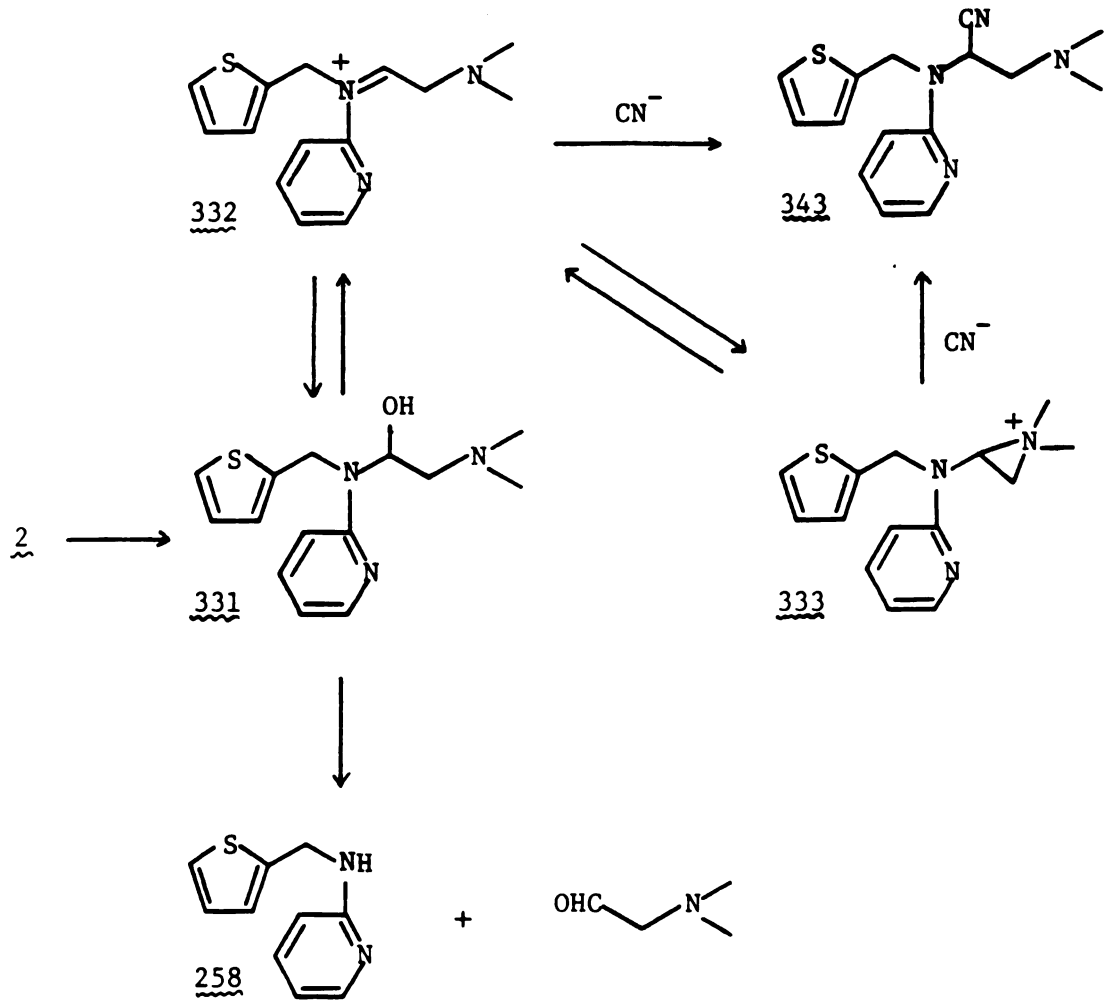
carbinolamine can either fragment to amine 329 and 2-thiophenecarboxaldehyde or ionize to the iminium intermediate 330. This methylene carbon is doubly activated towards oxidative metabolism in that it is "benzylic"



Scheme 3

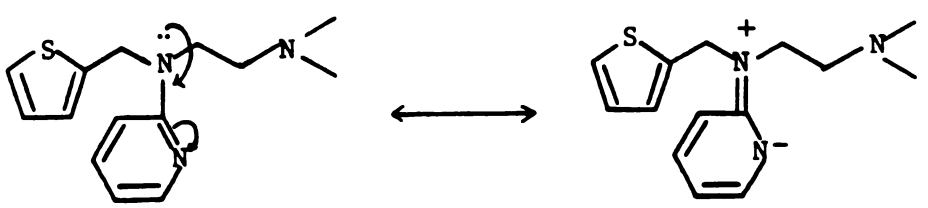
and alpha to a heteroatom. This position is very similar to the benzylic carbon of tripelennamine; no evidence for metabolic attack at this carbon was found in the metabolism of tripelennamine.[201-205] Furthermore, as noted in Section IV.B., products resulting from metabolic oxidation at the benzylic carbon of 1-benzyl-pyrrolidine could not be detected. Steric factors might be responsible for the resistance of the benzylic position to metabolic attack.

Metabolic hydroxylation described in Scheme 4 would give the carbinolamine derivative 331 which could ionize to the iminium species 332 or cleave to the secondary amine 258 and dimethylaminoacetaldehyde. In this case the iminium ion 332 can be stabilized intramolecularly as shown by the tautomeric aziridinium species 333. The formation of imi-



Scheme 4

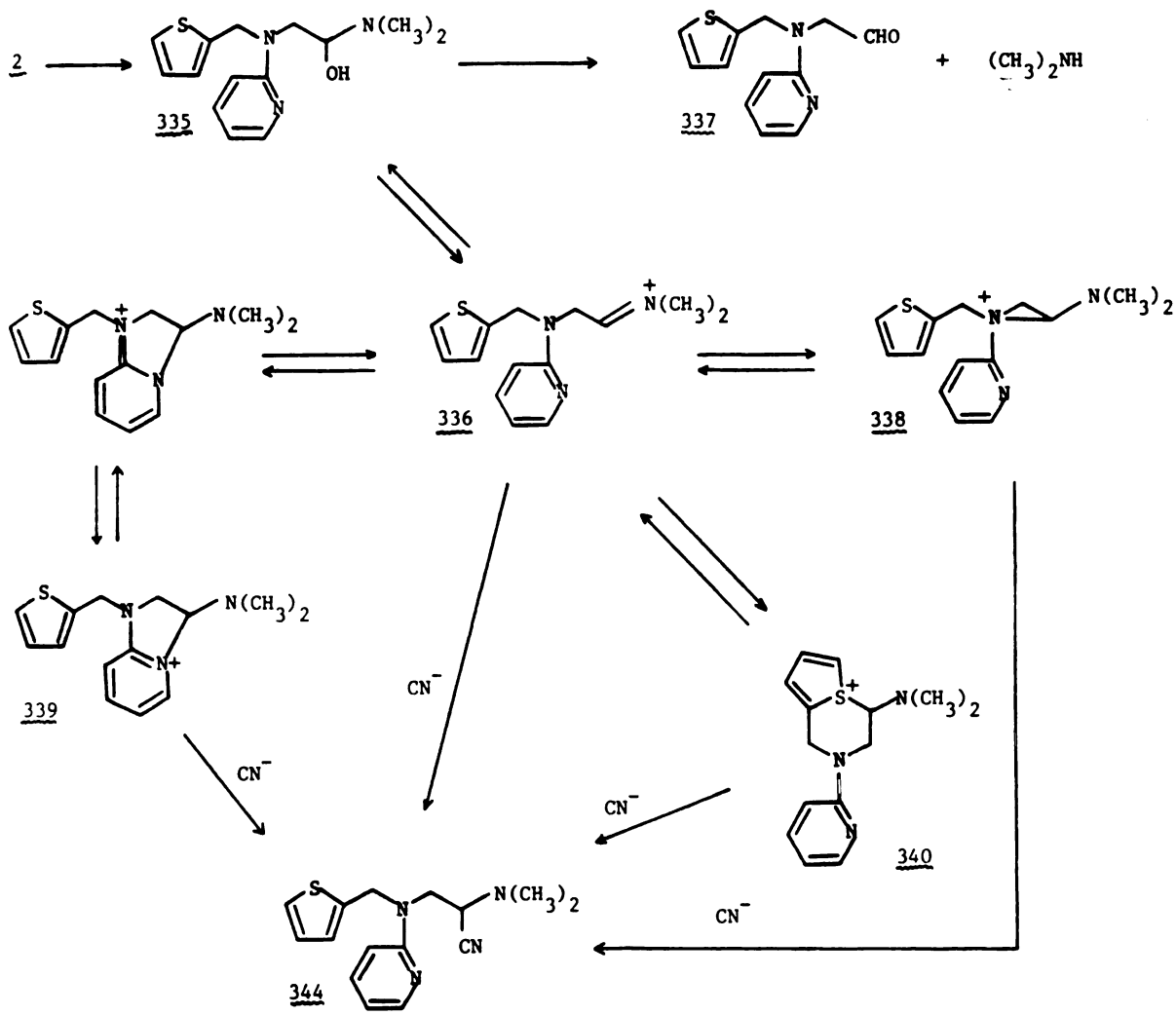
nium species 330 or 332 requires the participation of the lone pair electrons of the 2-amino nitrogen atom. The low basicity of 2-amino-pyridine (pKa^{217} = 6.71) indicates that the availability of the lone pair is decreased by delocalization into the pyridine ring as shown in



334

the charge resonance form 334 of methapyrilene. Consequently, the probability of forming iminium ions 330 or 332 is decreased.

The carbinolamine 335 that would result from metabolic hydroxylation at C-1 of the ethanediamine chain (Scheme 5) may ionize to the iminium ion 336 or cleave to the substituted acetaldehyde 337 and dimethyl-



Scheme 5

amine. The iminium species 336 can be stabilized intramolecularly by the 2-amino nitrogen atom, the pyridyl nitrogen atom, or the thienyl sulfur atom to give, respectively, the 3-, 5-, or 6-membered ring species 338, 339, and 340.

The feasibility of trapping metabolically generated iminium inter-

mediates with cyanide ion *in vitro* has been established. If similar iminium intermediates are generated in the *in vitro* metabolism of methapyrilene, it should be possible to trap these intermediate as their cyano adducts. The following section describes our efforts to isolate and characterize the possible cyano adducts 341-344 from the incubation of methapyrilene with sodium cyanide. Section V.B.2. summarizes our attempts to isolate and characterize the *in vitro* metabolites of methapyrilene; section V.C. describes the synthesis and chemistry of postulated methapyrilene metabolic products.

1. THE CHARACTERIZATION OF THE CYANO ADDUCT(S)

The ability of cyanide ion to trap electrophilic intermediates resulting from the metabolism of methapyrilene was explored by incubating methapyrilene with ^{14}C-sodium cyanide. These incubations were conducted with 100,000 x g liver microsomal preparations obtained from phenobarbital-induced Sprague-Dawley rats or untreated Dutch rabbits. As seen in Table IX, the base fraction (CH_2Cl_2 extract) isolated from incubations of 1.0 mM methapyrilene with 1.0 mM ^{14}C-labeled sodium cyanide showed incorporation of ^{14}C-label into the organic layer. The level of radiolabel isolated from incubations with NADPH present as a cofactor was always higher than the level observed in parallel incubations without added NADPH. This result indicates that the incorporation of ^{14}C-label into the organic fraction is metabolically dependent (no incorporation was observed in control experiments with NADPH in the absence of substrate 2). The ratio of ^{14}C-label incorporated into the organic soluble fraction in the presence of NADPH *versus* the absence of NADPH ($^{+}$NADPH/-NADPH) is

Table IX. ^{14}C Radiolabel isolated from the incubation of methapyrilene with ^{14}C-sodium cyanide

Species	Expt	No. of incubations	^{14}CN/incubation (dps)	^{14}CN isolated*/incubation (dps) + NADPH, -NADPH	% incorporation (+ NADPH, as % of ^{14}CN incubated)	+NADPH/-NADPH
Rat	--	10	2.69×10^4	322, 83	1.2	3.9
	A	3	7.15×10^4	713, 173	1.0	4.1
Rabbit	A	3	2.77×10^4	3258, 50	11.8	65.2
	B	2	4.89×10^4	2726, 28	5.6	97.6
	C	2	6.6×10^4	3324, 91	5.0	36.5
	D	2	8.46×10^4	3183, 38	3.8	84.0
	F	4	2.49×10^4	571, 48	2.3	12.2

*Values reported are mean values from all incubations in each experiment.

an indication of the amount of electrophilic intermediates trapped by cyanide ion. The amount of isolated organic soluble ^{14}C label can also be expressed as a fraction of the initial ^{14}C-labeled cyanide incubated (% incorporation). This figure can be used as a rough estimate of the amount of cyano adduct isolated. These data also provide a rough measure of the magnitude of cytochrome P-450 mediated enzymatic activity.

These two indicators of cyanide incorporation are appreciably higher for the rabbit as compared to the rat. If the incorporation of cyanide results primarily from α-carbon hydroxylation and iminium ion formation, the observed trend is consistent with reported differences in metabolic pathways for the rat and the rabbit. In oxidative O-demethylation reactions which presumably proceed via a carbinol intermediate, the rabbit displays a greater dealkylating ability than the rat.[218] The rabbit also displays a greater tendency than the rat to metabolize nicotine to cotinine via the presumed α-carbon hydroxylation pathway.[218] In a study on the comparative metabolism of amphetamine and related compounds, the rat showed marked aromatic oxidation ability while the rabbit displayed a pronounced tendency towards oxidative deamination, possibly via a carbinolamine intermediate.[218] The greater amounts of the cyano adducts formed with rabbit liver preparations led us to conduct further experiments with rabbit liver microsomal preparations only. Later experiments showed the metabolic profile of methapyrilene to be qualitatively similar in both the rat and the rabbit.

The results from a preliminary study on the kinetics of ^{14}C-labeled cyanide incorporation into the organic soluble fraction are summarized in Table X. The data indicate that the radiolabel is rapidly incorporated into the organic soluble fraction. The level of incorporation builds up

to a maximum after 20 to 30 minutes and gradually declines to a slightly lower level at the end of 60 minutes. It appears that the electrophilic metabolite trapped by cyanide ion is formed rapidly and that the cyano adduct(s) is subject to further metabolic or chemical decomposition processes.

Table X. The incorporation of ^{14}C radiolabel into the base extracts of methapyrilene postincubates

Time(mins) after addition of NADPH	0	5	10	20	30	60
^{14}CN isolated (+NADPH/-NADPH)	1	11	16	19	23	32
% incorporation (+NADPH, as % of ^{14}C incubated)	0.1	0.9	1.8	2.5	1.9	1.8

Large scale microsomal incubations of methapyrilene were required to obtain sufficient amounts of the radiolabeled material for structural studies. Rabbit liver microsomal incubation mixtures (20-64 incubations) each containing 1.0 mM methapyrilene and an equivalent amount of ^{14}C-labeled sodium cyanide were combined after the 60 minute incubation period. The postincubates were made basic (pH 10) and extracted with

CH_2Cl_2. The organic extracts were washed with 10% NaCl solution to remove any inorganic material, dried, filtered, and evaporated to dryness. The amount of ^{14}C radioactivity found in the organic residue usually corresponded to the "% incorporation" figures presented in Table IX. Most of the radioactivity found in the residue could be recovered even after partitioning the residue into acid and then base. In addition to purifying the desired material, the acid/base partitioning process established that the radioactive residue was organic and basic in nature.

The organic residue obtained from the base extracts of the large scale postincubates was initially purified by preparative scale thin layer chromatography (tlc). The plates (2000μ silica gel) were developed with $CH_2Cl_2/CH_3CN/Et_3N$ (90/10/1). The radioscan of the plates showed most of the radioactivity was localized in a zone which was also strongly UV-absorbing ($R_f \sim 0.55$). The radioactive zone was scraped from the plate and extracted to yield a material which accounted for 50-80% of the radioactivity applied to the tlc plate. The loss of radioactivity is probably due to inorganic or highly polar organic ^{14}C labeled material at the origin of the tlc plate or inefficient extraction of organic material from the silica.

Further purification of the isolated radioactive material by preparative tlc (250 μ silica gel) gave a radioactive compound which was pure by gc analysis; however, normal phase and reverse phase hplc analysis of the purified material indicated the presence of two UV-active components. Scintillation counting of the hplc eluents (Table XI) showed most of the radioactivity to be present in the larger of the two UV-absorbing components. CIMS analysis of these components reveals largely the presence of phthalate impurities. Acid/base partitioning of the

Table XI. Radioactivity of the eluent fractions obtained from the HPLC[a] analysis of the product isolated from incubations of 2 with $Na^{14}CN$.

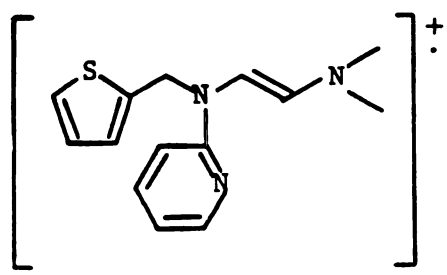
Fraction #	1	2	3	4	5	6	7	8	9	10	11	12
Radioactivity (cpm)	16	14	33	102	64	22	86	1138	57	21	20	29
UV absorption[b] (240 nm)	--	--	--	+++	++	+	++	+++	+	--	--	--

[a]Reverse phase (C-8), 1.32 ml/fraction.

[b]+++ = strong UV absorption, ++ = moderate, + = weak.

material finally gave a radioactive compound which was pure by reverse phase hplc analysis. Upon cims analysis, the purified product gave rise to a protonated molecular ion (MH^+) at m/e 287 corresponding to the substitution of one hydrogen atom in methapyrilene (MW = 261) by a cyano group.

Analysis of the gc-eims of the purified radioactive compound (Figure 17) allowed us to make a conjecture as to the structure of the cyano adduct. The parent ion at m/e 286 is consistent with a cyano substituted methapyrilene structure; by comparison with the gc-eims of methapyrilene (see Section V.B.2), the presence of fragment ions at m/e 97 and 203 exclude 342 and 343 as possible structures for the cyano adduct. One would expect 344 to lose HCN upon gc-eims analysis to give rise to a strong fragment ion at m/e 259 (345^+); this fragment ion is not present to any appreciable extent in the mass spectrum of the isolated product. By elimination, 341 appears to be the most likely structure for the isolated product. As described in Section V.B.2., this structure was confirmed by comparison of its mass spectral characteristics with those synthetic N-cyanomethylnormethapyrilene (341, Fig. 18). In addition, the proton nmr spectrum of authentic 341 (Fig. 19) obtained from the reaction of 327 with CH_2O and NaCN is identical to the nmr spectrum of the metabolically derived 341 (Figure 20).



345^+

In addition to the metabolic route discussed above (Scheme 2), N-cyanomethynormethapyrilene (341) also may originate from the *in vitro*

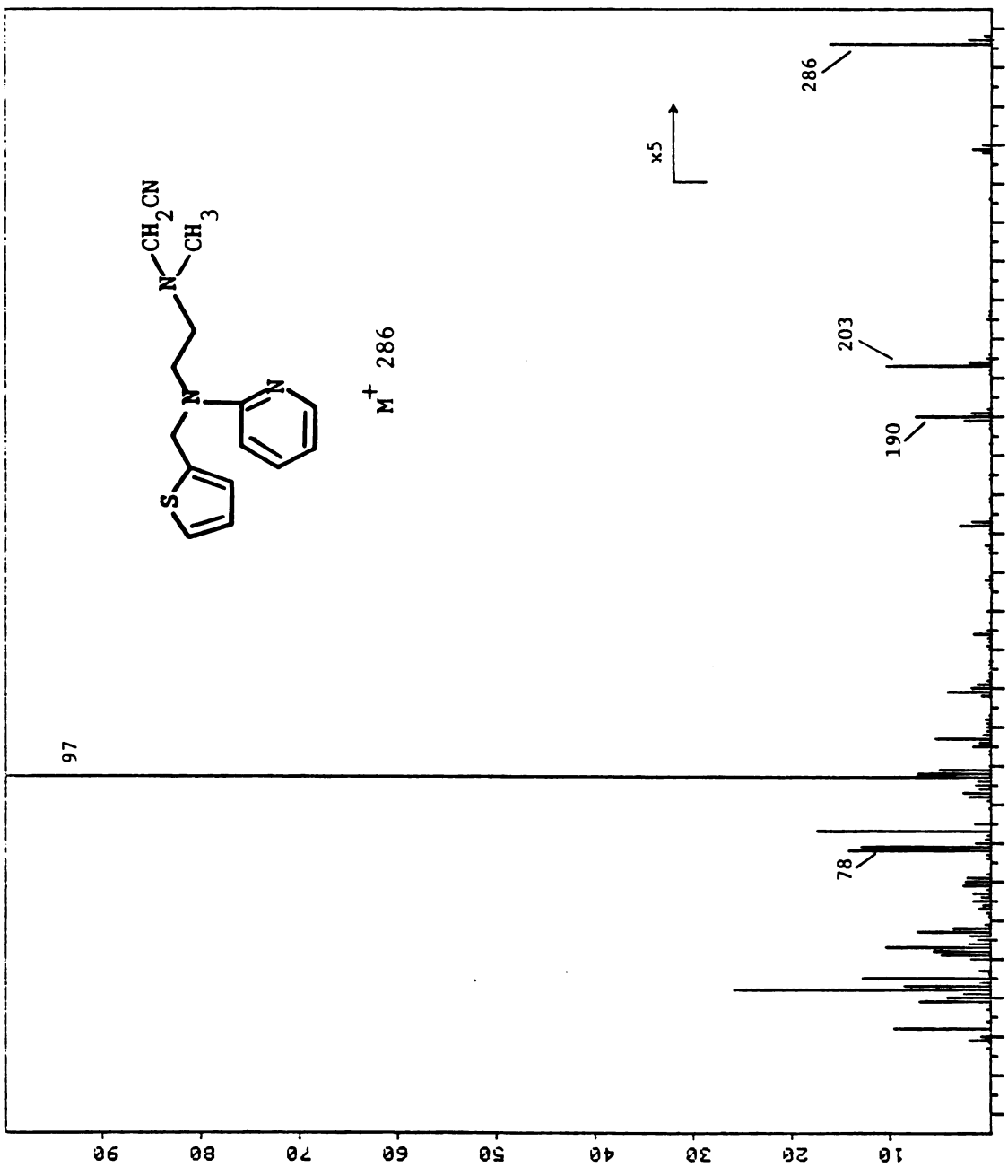


Figure 17. The gc-eims of 341 from the metabolism of 2 in the presence of cyanide.

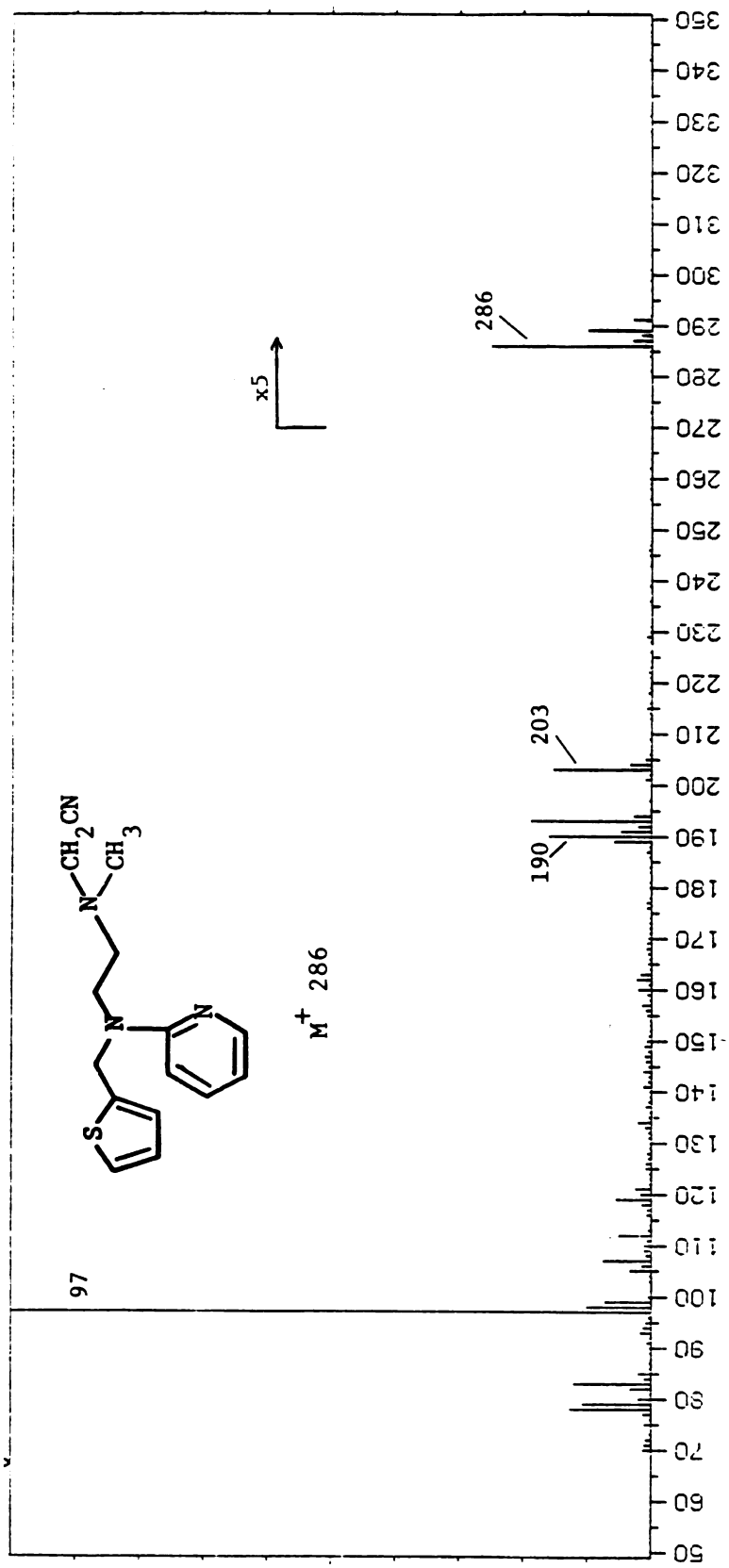


Figure 18. The eims of synthetic 341.

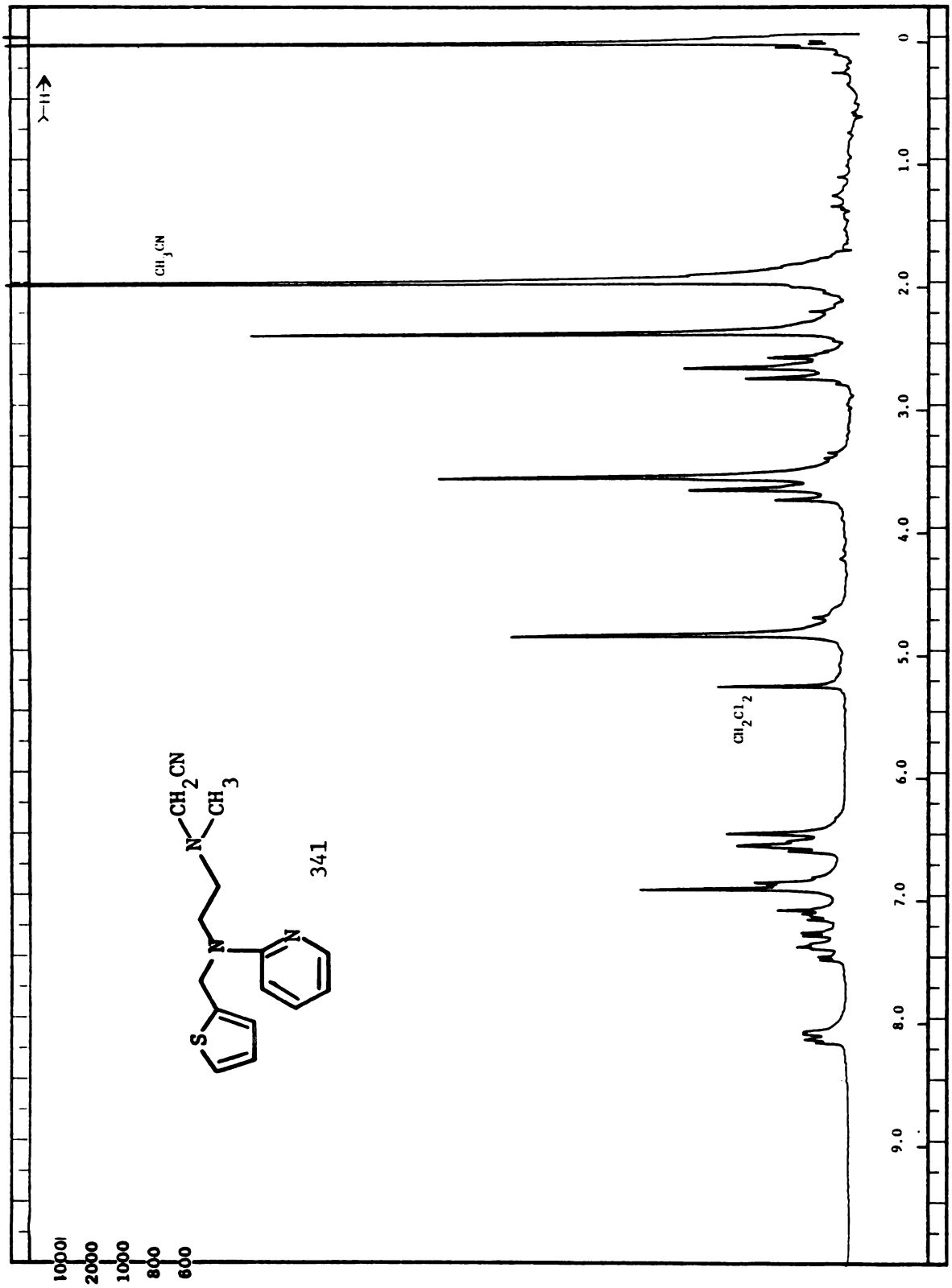


Figure 19. The nmr spectrum of synthetic 341.

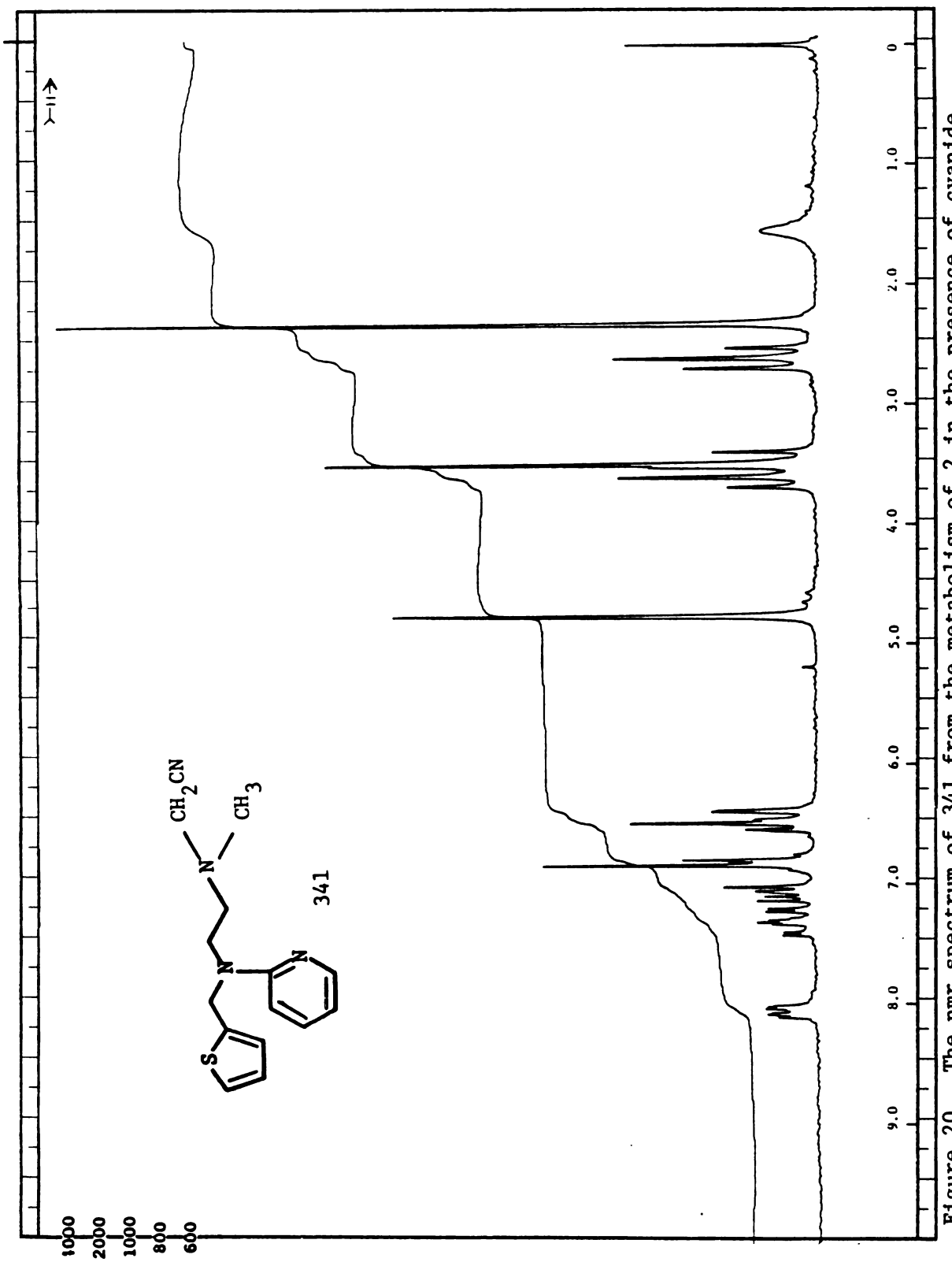


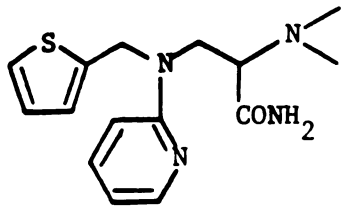
Figure 20. The nmr spectrum of 341 from the metabolism of 2 in the presence of cyanide.

reaction of metabolically generated normethapyrilene 327 with formaldehyde (a product of N-demethylation reaction or contaminant) and NaCN. Our synthesis of 341 from 327 (see Section V.C.2.b.) demonstrates the facile nature of this reaction. Similarly, the formation of cyanomethylnornicotine from the incubation of nicotine with sodium cyanide has been demonstrated to result in part from prior cleavage of the C-N bond.[72] Thus, the characterization of 341 from the incubation of 2 with cyanide does not necessarily imply the direct formation of the methyleniminium ion 326 without prior C-N bond cleavage.

In order to properly analyze the microsomal incubations for the presence of 344, we decided to obtain a synthetic sample of this postulated cyano adduct. It was felt that the full characterization of the properties of authentic 344 would allow us to optimize the analytical methods used to detect its presence in incubations of 2 with sodium cyanide. The synthesis and chemical behavior of the α-cyanoamine 344 will be described in Section V.C.

All attempts thus far to isolate a compound corresponding to synthetic 344 from incubations of 2 with cyanide have failed. Studies with authentic 344 have indicated that the isolation of 344 (if formed) in microsomal postincubations would be extremely difficult if not impossible because of the instability of 344 under incubation and work-up conditions. The α-cyanoamine 344 could not be detected in the base extracts obtained from microsomal preparations to which 344 had been added. It has been demonstrated that 344 is readily converted to the secondary amine 258 and the amide 346 (see Section V.C.3.). Compounds 258 and 346 have been isolated and characterized (tlc, cims) from the base extracts obtained from microsomal incubations of authentic 344.

Despite intensive efforts (continuous extraction and extraction of postincubates at pH 4-8 and Amberlite XAD-2 clean-up; see Section V.B.2 for details), the expected hydrolysis product of 344, amino acid 347, has not yet been isolated from microsomal postincubates of 2 with cyanide.



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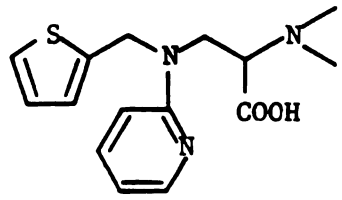


347

As noted above, the nitrile 344 would be expected to lose HCN to form the enamine 345 upon the thermal conditions of gc or mass spectral analysis. The cims of synthetic 344 displays a protonated molecular ion MH^+ 287 and a cluster of ions resulting from the loss of HCN and unexplained proton transfers at m/e 259-261. The probe eims of 344 (Figure 21) does not show a molecular ion at m/e 286 and displays only a weak parent ion resulting from the loss of HCN at m/e 259. The gc-eims analysis of the same compound (Figure 22) again displays no parent ion at m/e 286; a major ion resulting from the loss of HCN is present at m/e 259. These observations indicate that under our conditions, the gc-eims analysis of any metabolically-generated 344 would show only a compound with a parent ion at m/e 259 not at m/e 286. In one instance, a compound with a parent ion at M^+ 259 (Figure 23) was characterized from the incubation of 2 with cyanide. The gc-eims of this product (Figure 23) is very similar to the gc-eims of the synthetic α-cyanoamine (Figure 22). The nominal mass, the electron impact-induced fragmentation pattern, and high resolution

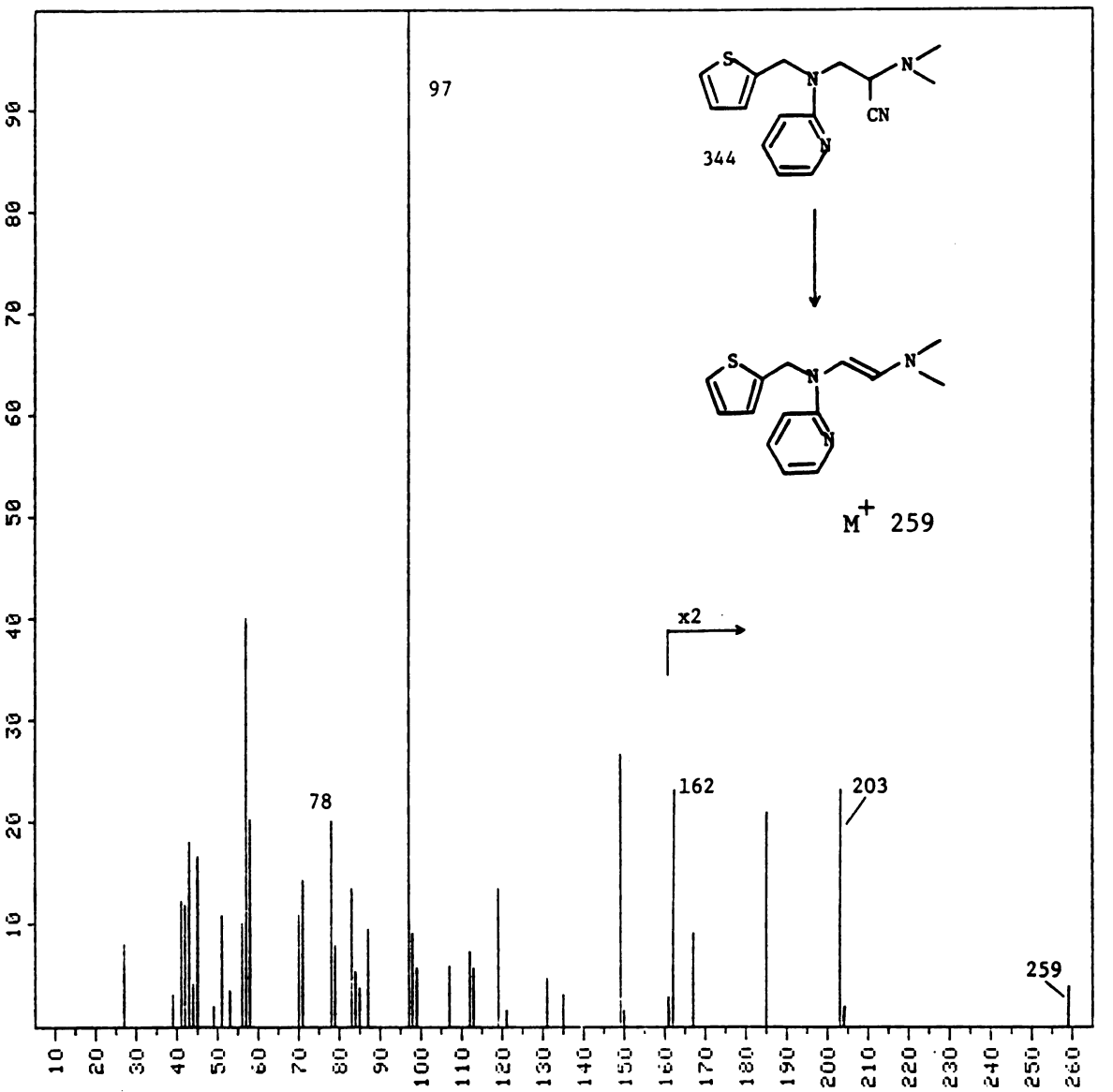


Figure 21. The direct probe eims of synthetic 344.

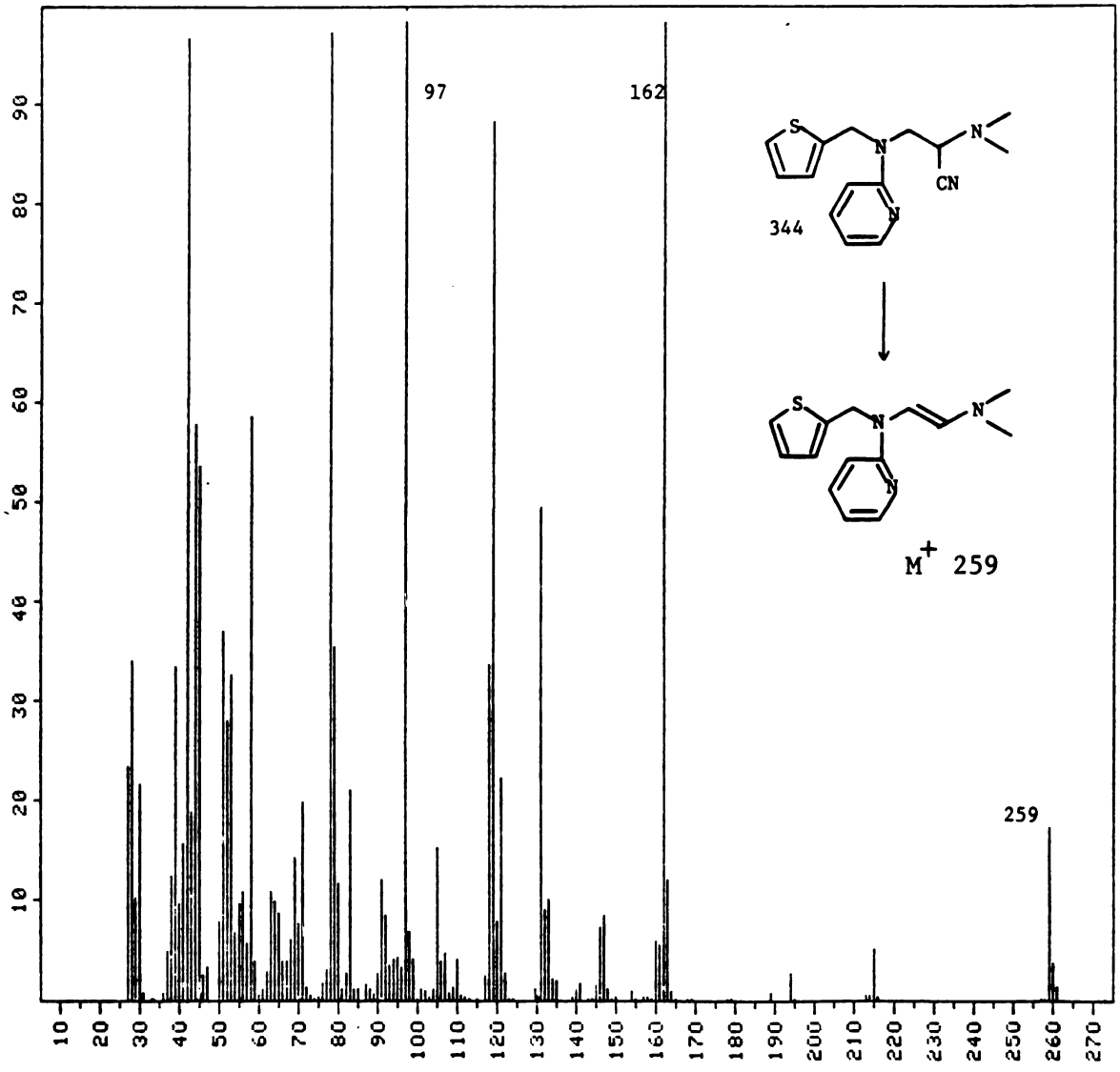


Figure 22. The gc-eims of synthetic 344.

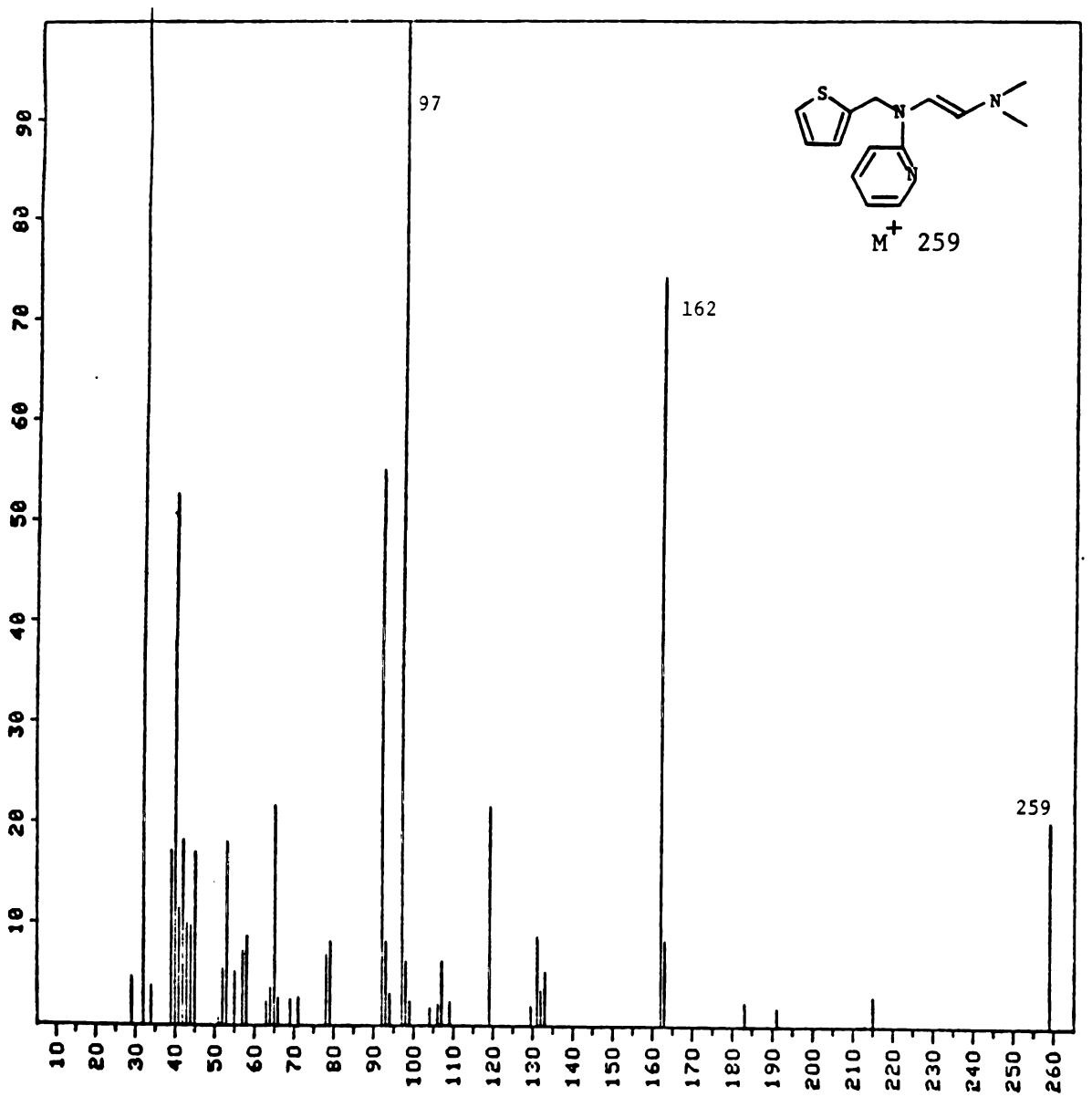
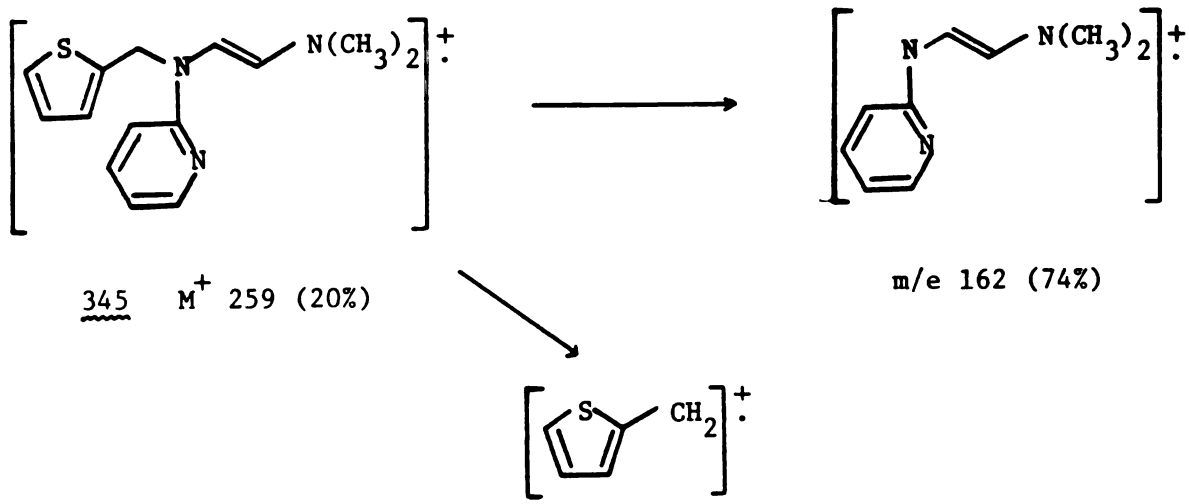


Figure 23. The gc-eims of 345 from the metabolism of 2 in the presence of cyanide.

eims analysis are consistent with the postulated enamine structure 345. By comparison with the ei-induced fragmentation pattern of methapyrilene (Section V.B.2.), the absence of prominent fragment ions at m/e 58, 71, and 203 in the mass spectrum of the putative enamine are consistent with a structure containing an altered dimethylaminoethyl chain. The observed major fragment ions at m/e 97 (100%) and 162 (74%) can be rationalized by the cleavage of the thienylmethyl moiety. The fragment ions of 345 may be assigned as shown below. The characterization of 345 from incubations of 2 with cyanide is evidence for an enamine species which could result from the elimination of HCN from the α-cyanoamine 344 or the elimination of water from the carbinolamine 335.



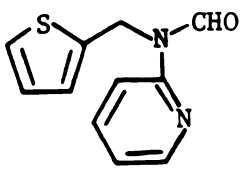
345 M^+ 259 (20%)

m/e 162 (74%)

m/e 97 (100%)

As depicted in Scheme 5, the carbinolamine 335 is the initial intermediate in the formation of the cyanoamine 344. The carbinolamine may also cleave to the aldehyde 337. Studies on the chemical behavior of synthetic aldehyde 337 (see Section V.C.2.) have demonstrated the unstable nature of this compound. This aldehyde has been shown to decompose in air to the secondary amine 258 and the N-formyl compound 348. Both of

these decomposition products have been characterized from basic extracts of methapyrilene postincubates. Further evidence for the formation of the carbinolamine intermediate 335 comes from the characterization of the amide 349 as an *in vitro* metabolite of methapyrilene (see Section V.B.2.). One would expect that the amide 349 results from the oxidation of the carbinolamine intermediate 335.



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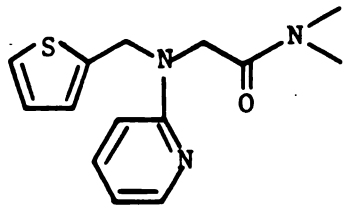


349

The evidence presented here argues for the intermediacy of the carbinolamine 335 which would be directly involved in the formation of the cyano adduct 344. This finding in addition to the characterization of the enamine and the chemical instability of 344 have led us to speculate that our failure to isolate metabolically generated 344 lies primarily in the labile chemical nature of this product.

2. CHARACTERIZATION OF METHAPYRILENE METABOLIC PRODUCTS

In addition to our attempts to trap reactive iminium metabolites of methapyrilene as their cyano adducts, we felt a detailed metabolic profile of methapyrilene would prove valuable in our attempts to elucidate its mechanism of toxicity. The identity of the observed metabolites could be established by gc-eims analysis and comparison of chromatographic and spectral characteristics with synthetic compounds when possible. The gc-eims analysis of the basic extracts obtained from microsomal postincubates of methapyrilene provided preliminary

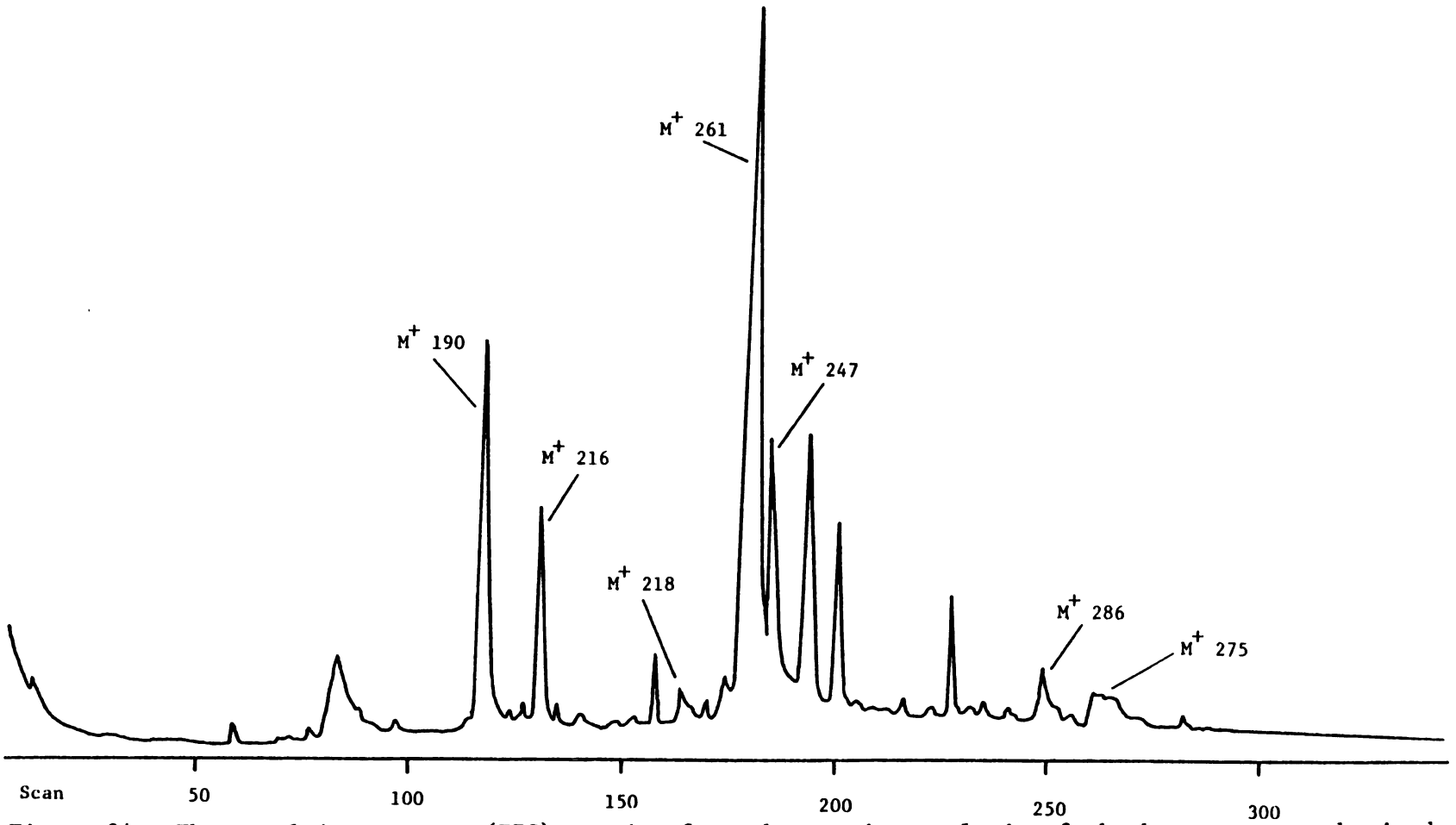


Figure 24. The total ion current (TIC) tracing from the gc-eims analysis of the base extract obtained from the incubation of 2 with cyanide in the presence of NADPH.

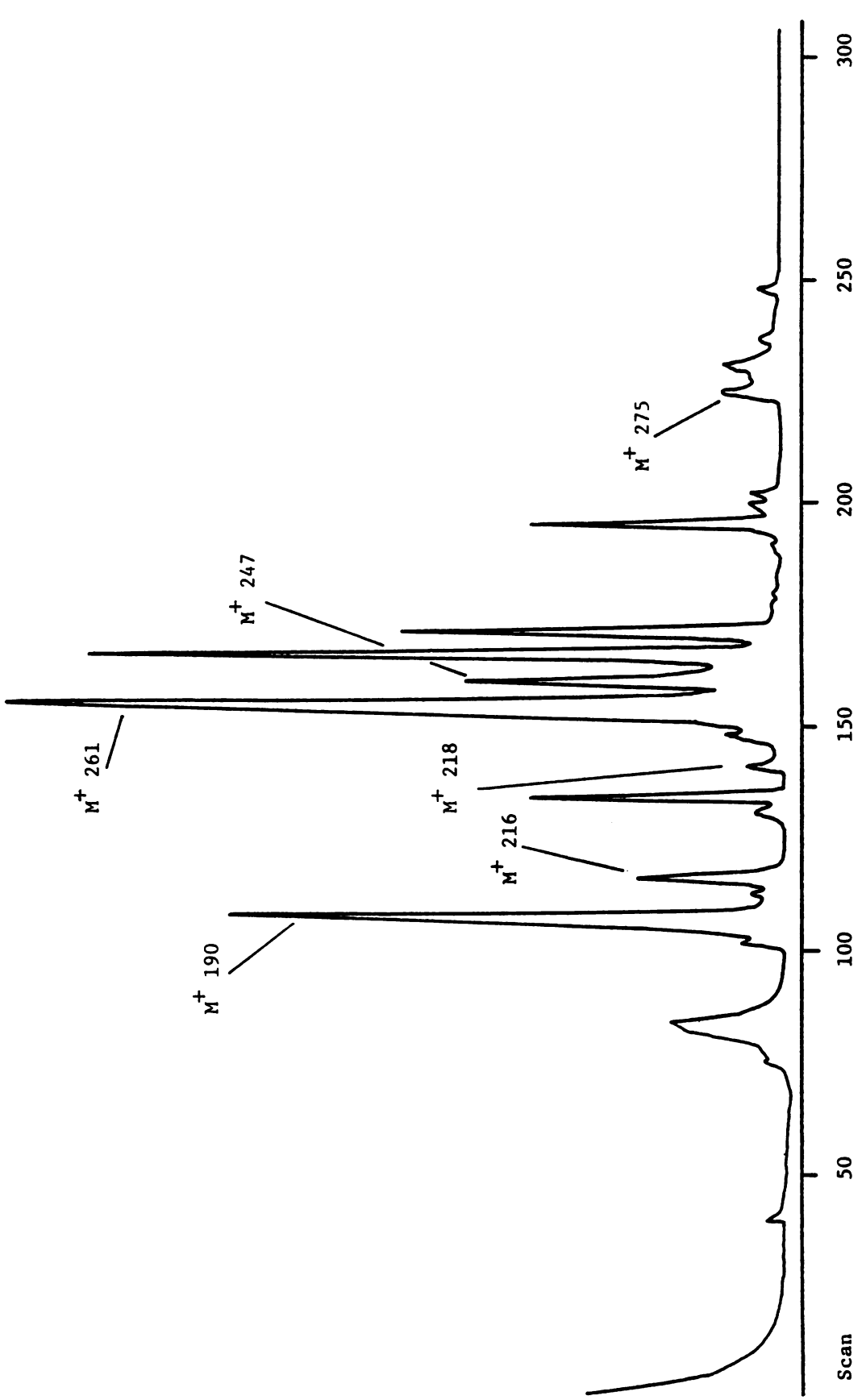


Figure 25. The TIC tracing from the gc-eims analysis of the base extract obtained from the incubation of 2 with NADPH.

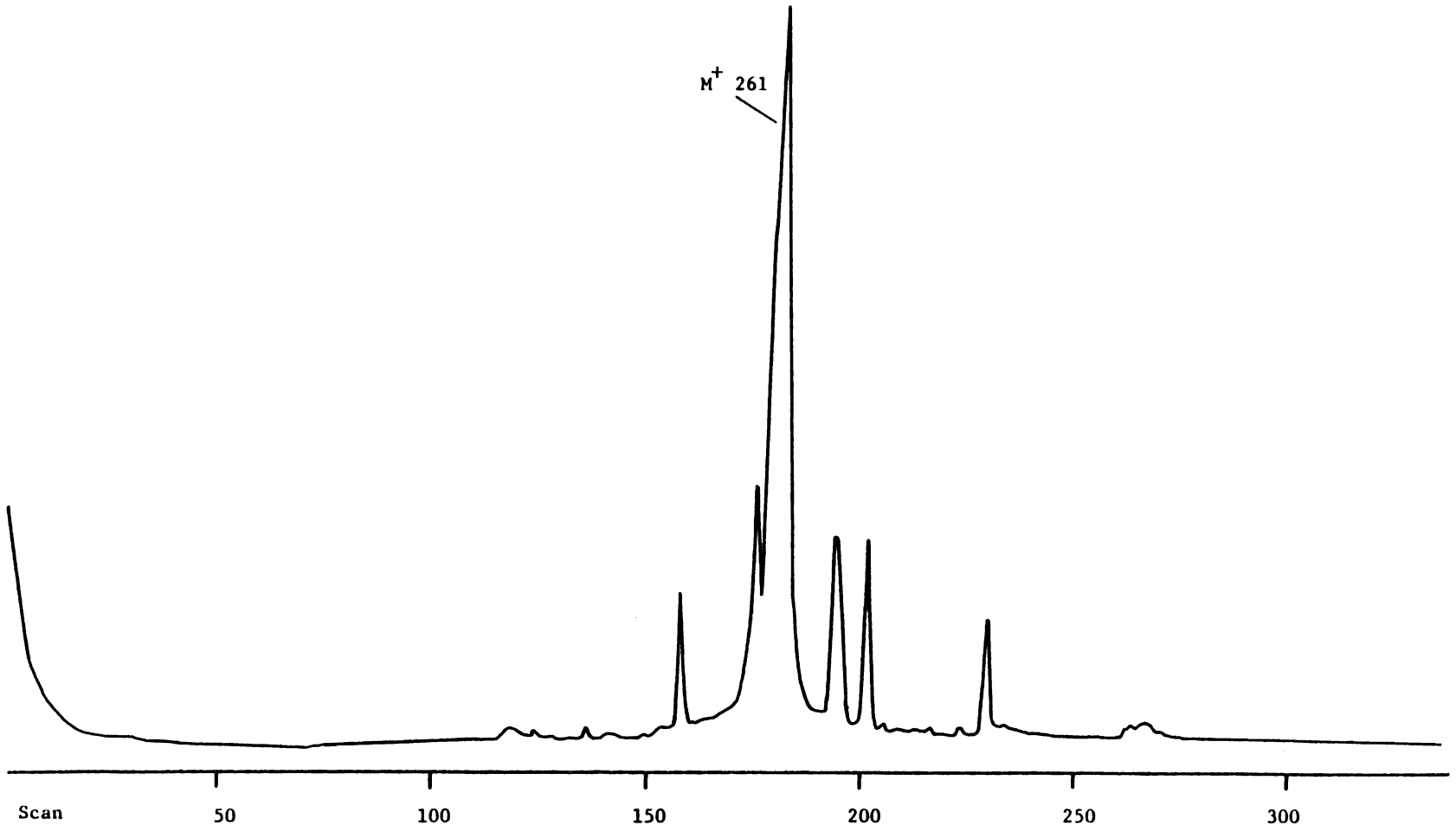
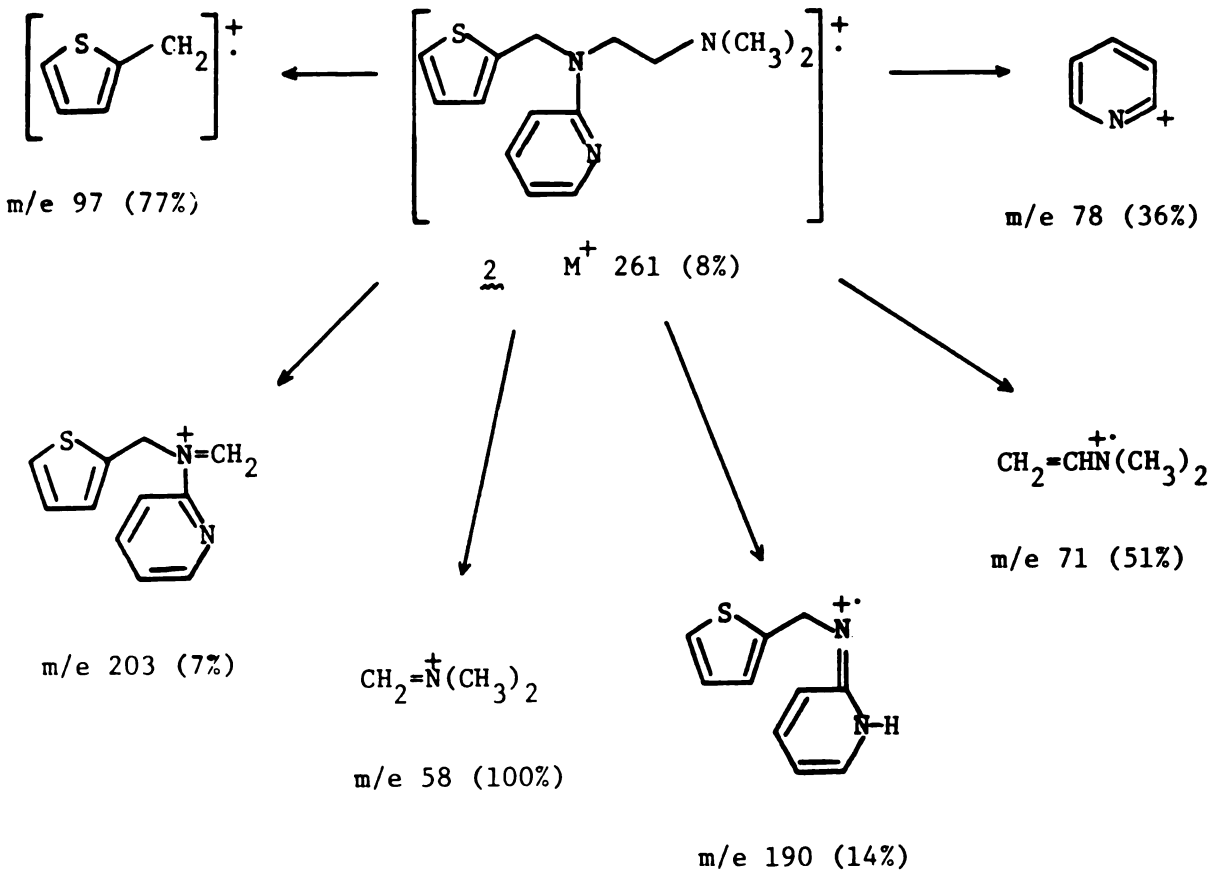


Figure 26. The TIC tracing from the gc-eims analysis of the base extract obtained from control incubations of 2 (-NADPH, -CN).

information as to the structure of the isolated metabolites. In some cases, the metabolites could be isolated as fairly pure compounds by tlc.

The total ion current (TIC) tracing from the gc-eims analysis of the base extract obtained from the incubation of methapyrilene with sodium cyanide in the presence of NADPH is shown in Figure 24. The corresponding incubation conducted in the absence of sodium cyanide gives rise to a TIC tracing (Figure 25) which is identical except for the peak with M^+ 286 which is absent. Figure 26 shows the TIC tracing from the gc-eims analysis of the basic extract obtained from the control incubation of methapyrilene with sodium cyanide in the absence of NADPH. The extraneous peaks in Figure 26 and their counterparts in Figures 24 and 25 are believed to result from either impurities in the substrate or extractable organic materials in the liver preparations.

The major component isolated from the postincubates proved to be unmetabolized methapyrilene. The gc-eims of the isolated compound (Figure 27) is virtually identical to that of the authentic compound (Figure 28). Since the electron impact-induced fragmentation pattern of authentic methapyrilene provides useful structural information on metabolites and cyano adducts, it will be informative to examine this spectrum (Figure 28) in more detail. The fragment ions of methapyrilene may be assigned as follows:



The fragment ion m/e 97 results from the cleavage of the thienylmethyl moiety from the amino nitrogen; the presence of this fragment ion would indicate the intact nature of this moiety in metabolites. Substitution of the pyridine ring will undoubtedly cause shifts in the pyridyl fragment ion at m/e 78. The fragment ion m/e 190 is likely to arise as shown below. Substituents introduced on the pyridyl group might be detected by this fragment. This process also gives rise to the fragment ion m/e 71; this fragment ion reveals the nature of the dimethylaminoethyl side chain.

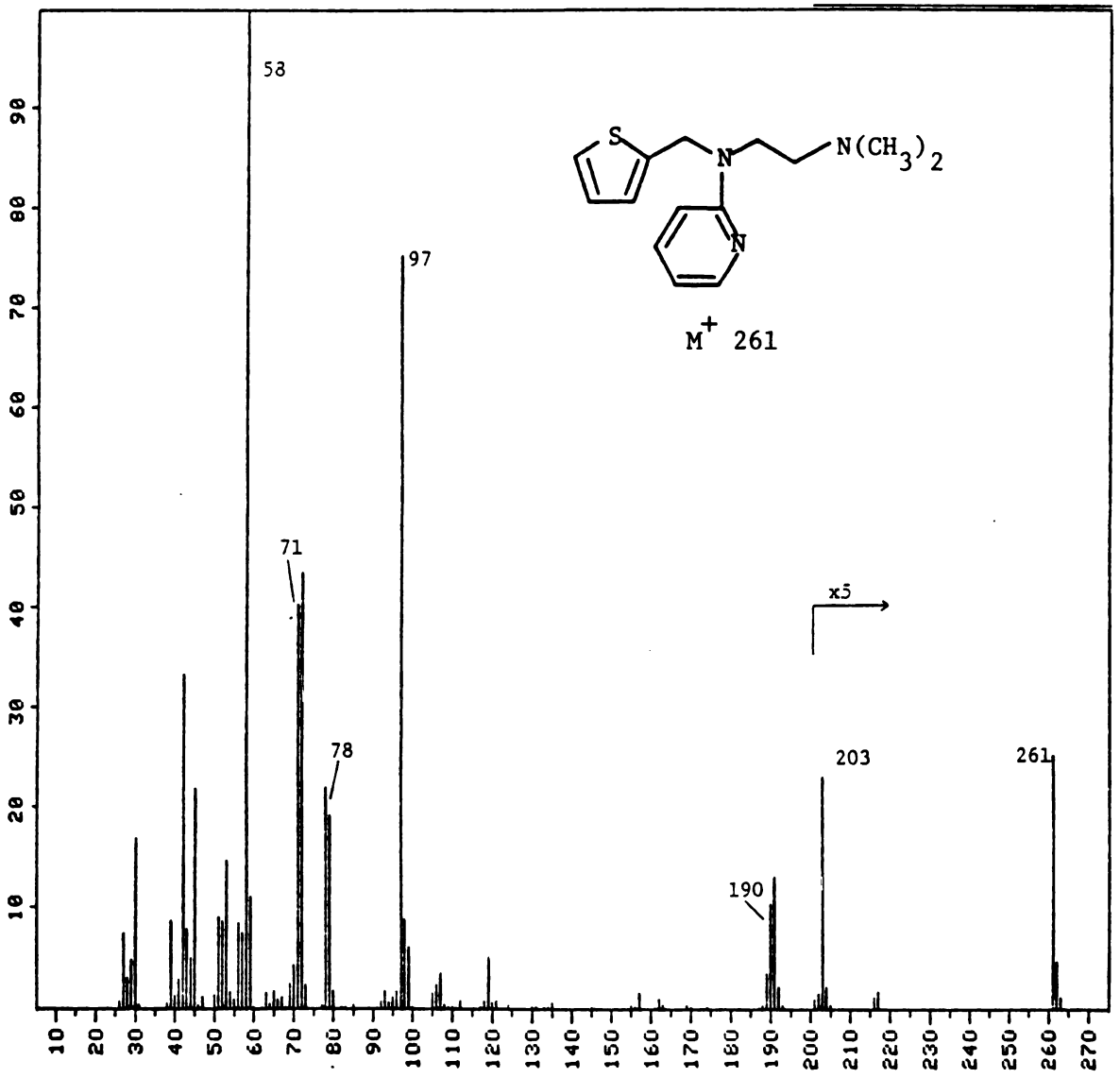


Figure 27. The gc-eims of 2 isolated from the base extracts of methapyrilene postincubates.

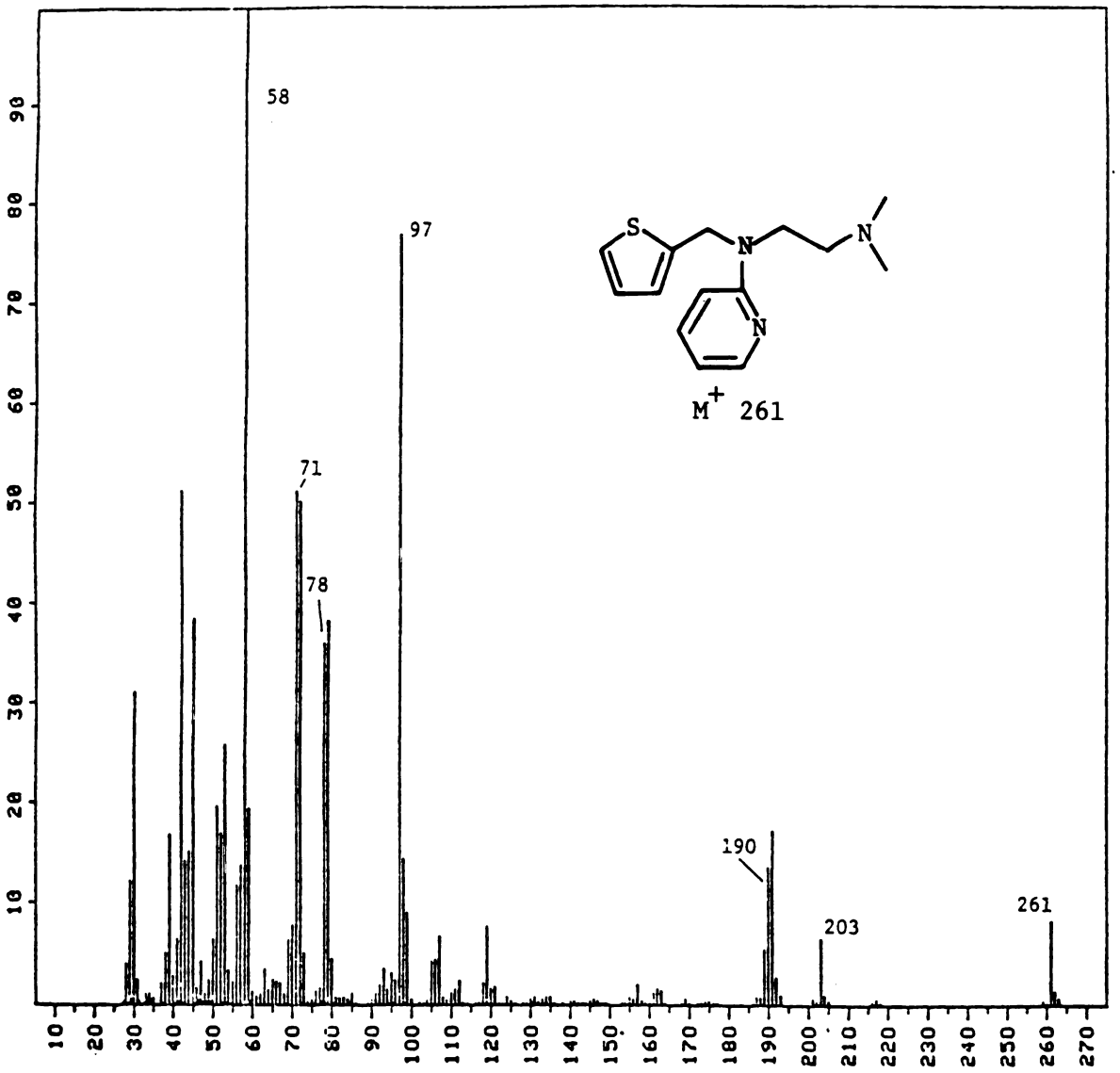
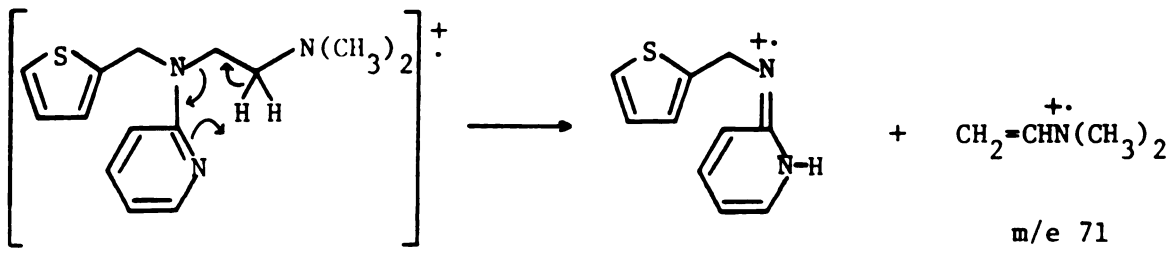


Figure 28. The gc-eims of authentic methapyrilene (2).



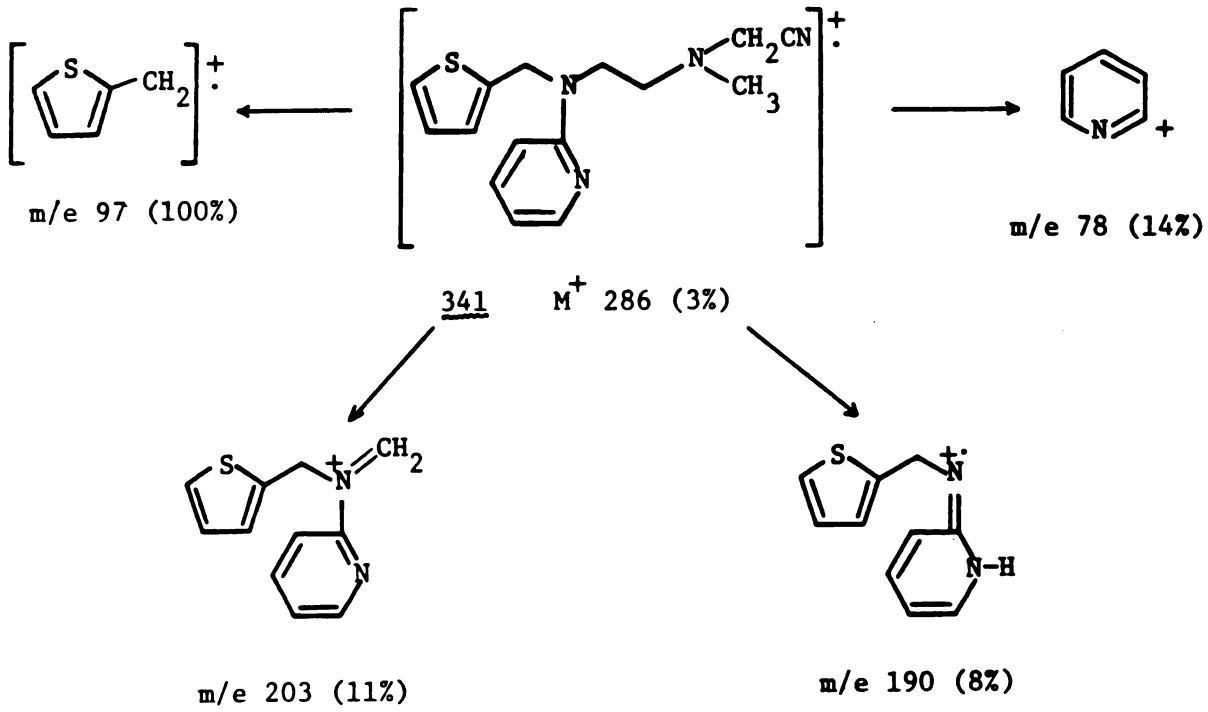
M^+ 261 m/e 190 m/e 71

Similarly, cleavage of the ethyl chain gives rise to fragment ions m/e 203 and m/e 58 (base peak). This cleavage may provide additional information concerning the nature of the substitution pattern on the ethyl moiety. Comparison of the electron impact mass fragmentation pattern of methapyrilene with that of unknown metabolic products has assisted us in making a preliminary structure assignment for the cyano adduct and in structure assignments for the other metabolites.

a. N-(2-pyridyl)-N-(2-thienylmethyl)-N'-cyanomethyl-N'-methyl-1,2-ethanediamine (341)

The gc-eims analysis of the radioactive material isolated by tlc (Figure 17) was essentially identical to the eims of the synthetic cyanomethyl compound (341, Figure 18). The gc-eims of the metabolic product displays a molecular ion at 286, a base peak at m/e 97, and fragment ions at m/e 78, 190, and 203. By comparison with the methapyrilene mass spectrum, the fragment ions m/e 78, 97, and 190 indicate the intact nature of the pyridyl and thienylmethyl moieties. Similarly, the fragment ion at m/e 203 indicates the intact nature of the C-2 position adjacent to the 2-aminopyridyl nitrogen atom. The absence of the fragment ion m/e 58 is consistent with the introduction of a substituent into the dimethylamino

moiety. These data and the nmr spectrum of the metabolic product (Figure 20) are consistent with the cyanomethyl structure 341.



b. Normethapyrilene (327)

The compound eluting immediately after methapyrilene on gc-eims analysis displays a parent ion at M^+ 247 (Figure 29). The nominal mass (247 amu) and the mass fragmentation pattern are consistent with normethapyrilene (327). On the basis of gc-eims and cims analysis, the same compound has been isolated by thin layer chromatography of the incubation extracts. The gc-eims of the nor metabolite (Figure 29) displays a base peak at m/e 97 and prominent fragment ions at m/e 204, 191, 107, and 78. As evidenced for methapyrilene, the fragment ions 97 and 78 indicate the intact nature of the thienylmethyl and pyridyl moieties. In addition, the fragment ions at m/e 191 and 204, which probably result from intramolecular proton trans-

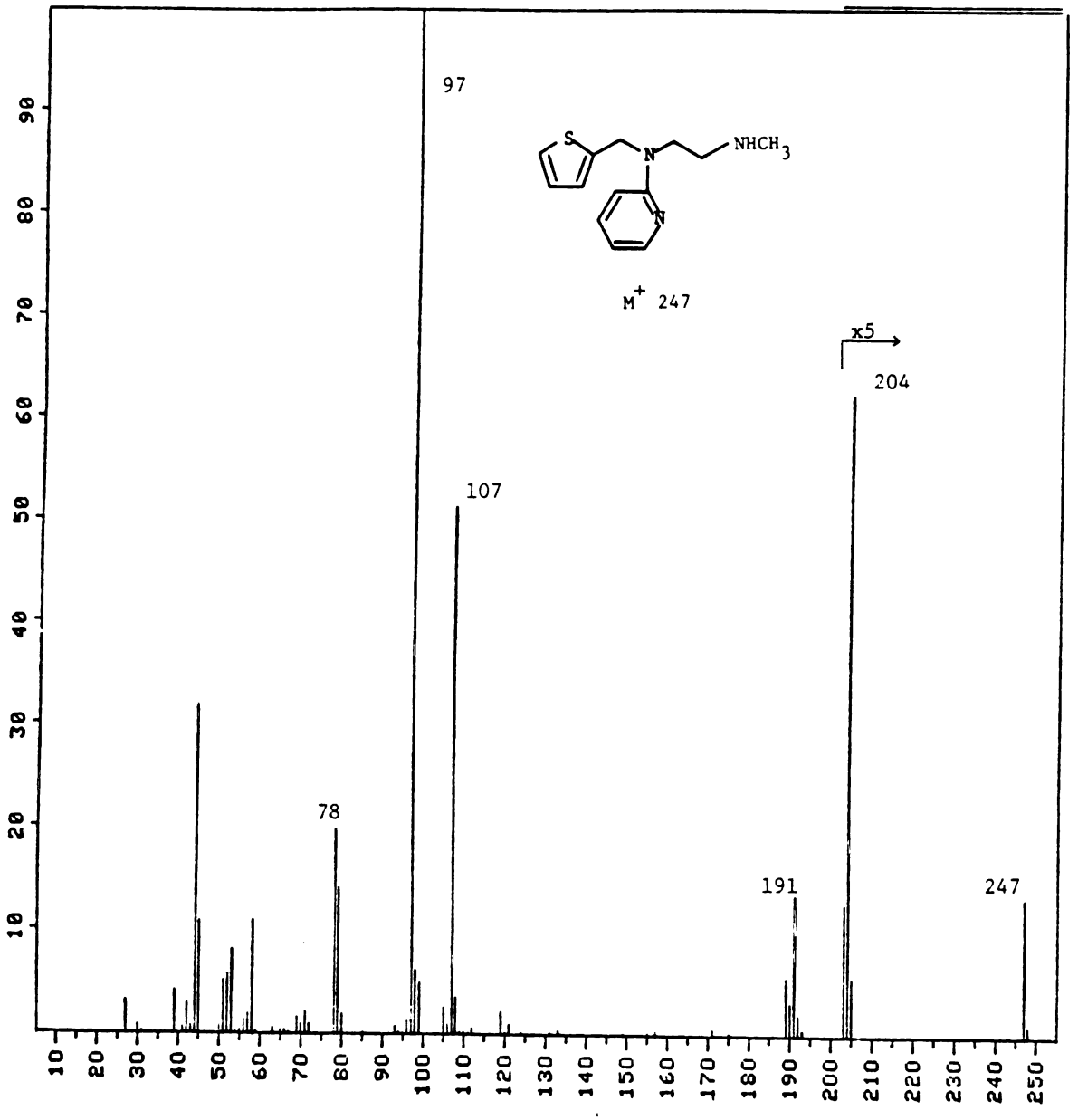
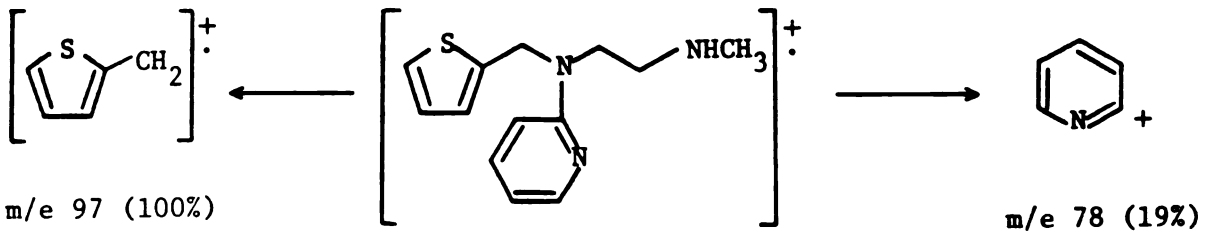


Figure 29. The gc-eims of normethapyrilene (327) from the metabolism of 2.

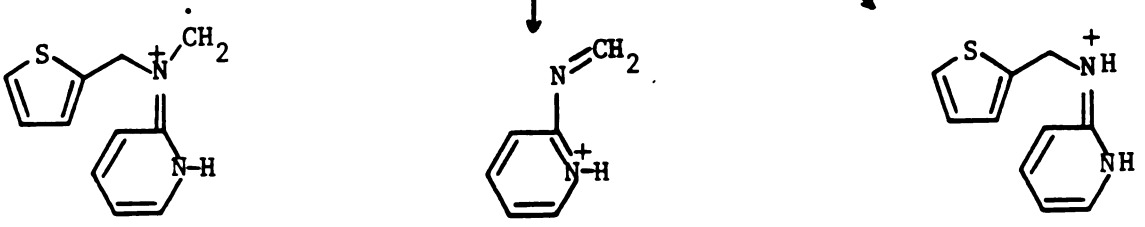
fers, correspond to the fragment ions m/e 190 and 203 of methapyrilene. Further evidence for this structure was obtained by comparing the gc retention times of the metabolically derived product and synthetic 327 (see Section V.C.2.). No evidence for the primary amine metabolite 350 has been found.



m/e 97 (100%)

m/e 78 (19%)

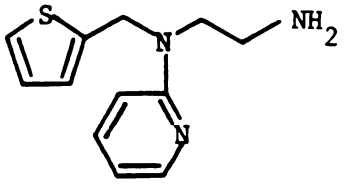
327 M^+ 247 (3%)



m/e 204 (12%)

m/e 107 (51%)

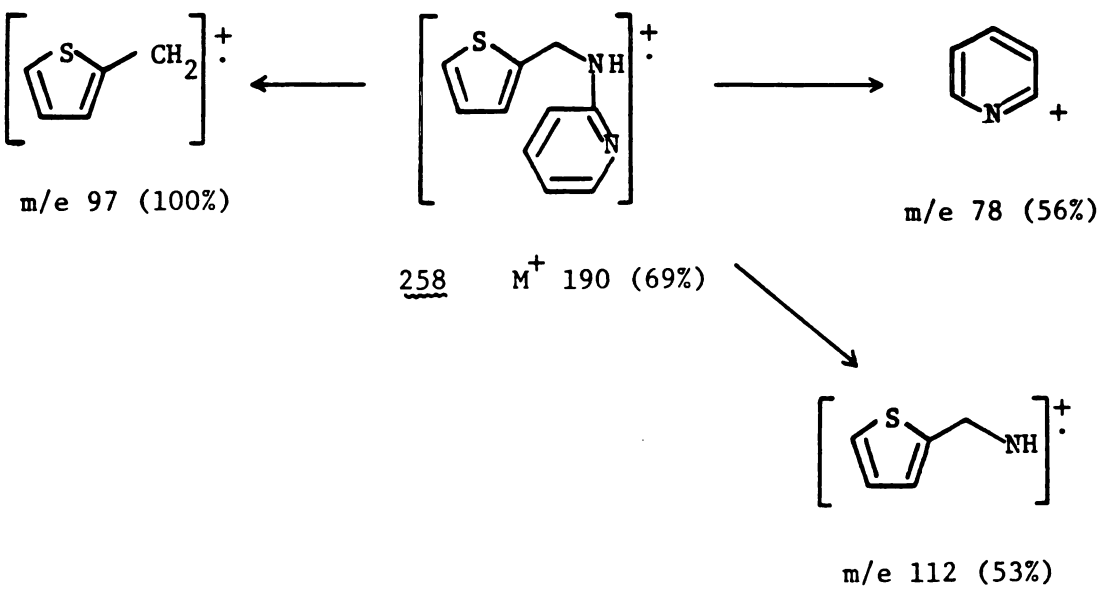
m/e 191 (13%)



350

c. 2-(2-Thienylmethylamino)pyridine (258)

One of the major components found upon gc-eims analysis of the base extract of the methapyrilene postincubates displays a molecular ion at M^+ 190. The gc-eims of this metabolic product is shown in Figure 30. These data suggest the structure of the product to be 258. The results of gc-eims and cims analyses of the compound after purification by tlc are consistent with this structure. The structure assignment of this metabolically derived product was confirmed by comparison of gc retention times and ei-induced fragmentation patterns with synthetic 258 (Fig. 31). The fragmentation pattern of 258 can be accounted for as follows:



The characterization of 258 from microsomal incubations of methapyrilene does not necessarily mean 258 is formed as a direct metabolite of methapyrilene as indicated in Scheme 4. This secondary amine has been found as a decomposition product of the α-cyanoamine compound 344 and the

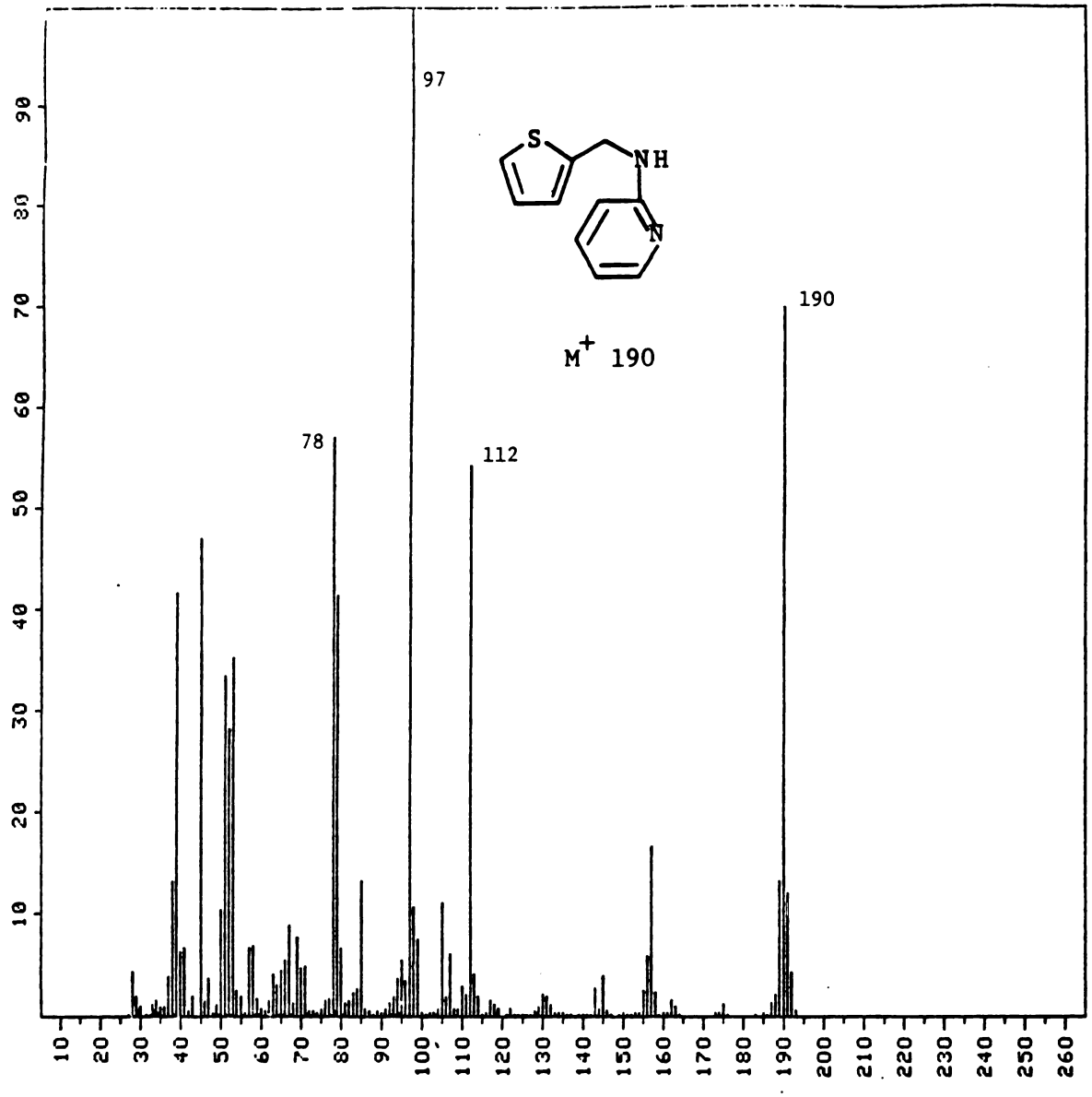


Figure 30. The gc-eims of 258 from the metabolism of 2.

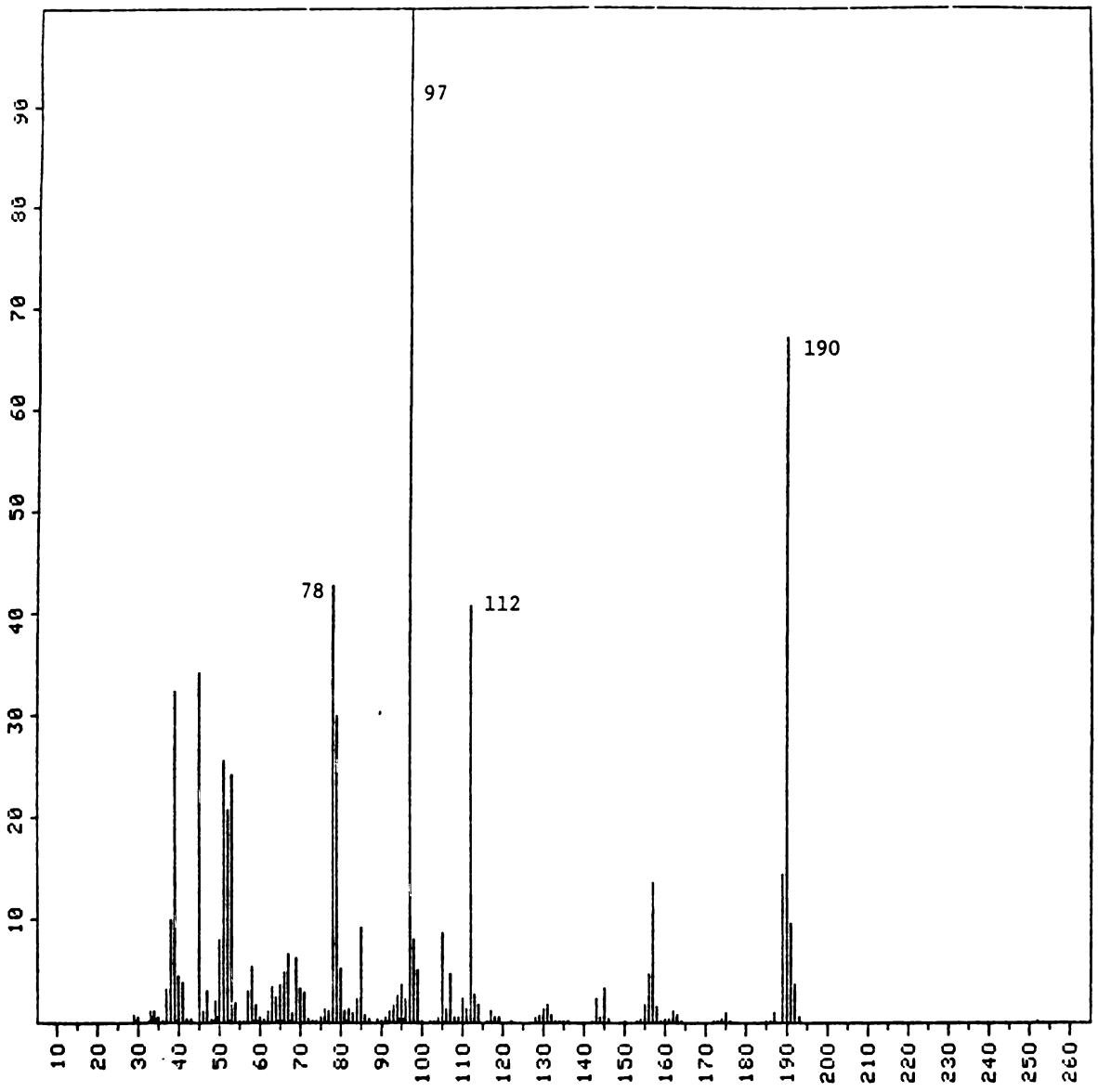
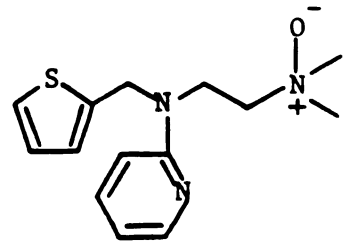


Figure 31. The gc-eims of synthetic 258.

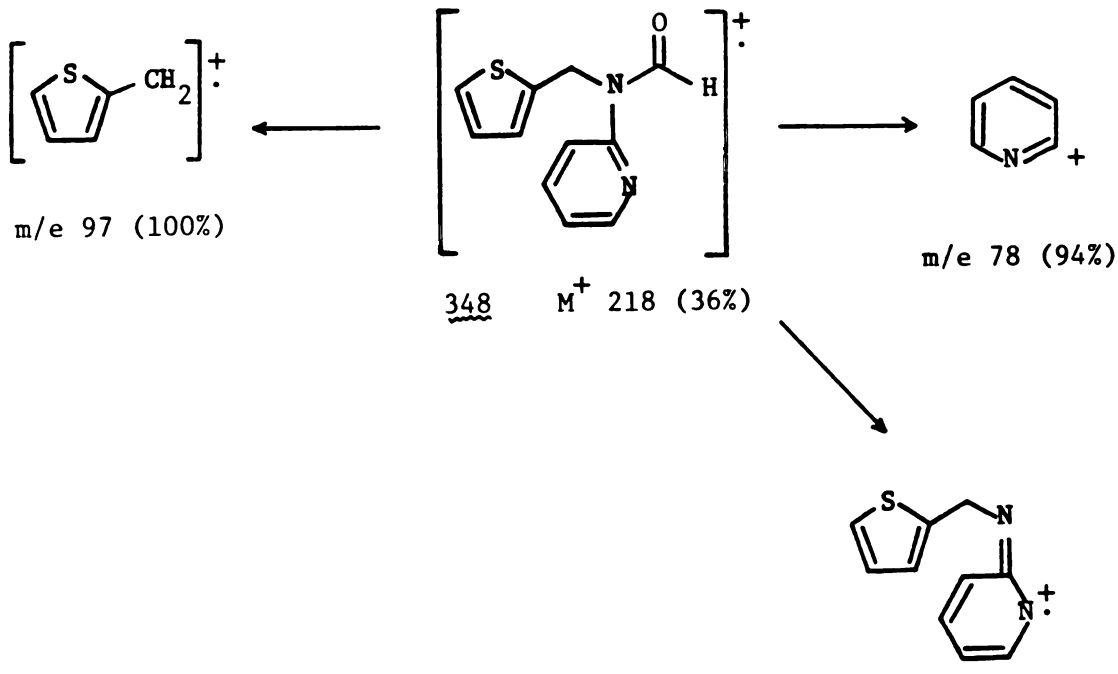
aldehyde 337. As we shall discuss later, methapyrilene N-oxide (351) can also give rise to 258 upon thermolysis.



351

d. N-Formyl-N-(2-thienylmethyl)-2-aminopyridine (348)

A compound with a molecular ion at M^+ 218 is present as a minor component in the TIC tracing of the methapyrilene postincubate (Figure 24).



m/e 97 (100%)

348 M^+ 218 (36%)

m/e 78 (94%)

m/e 189 (75%)

The nominal mass (218 amu) and gc-eims fragmentation pattern (Figure 32) suggests the compound has the N-formyl structure 348. Again, the presence of fragment ions at m/e 78 and 97 reveal the intact nature of the pyridyl and thienylmethyl moieties. The fragment ion at m/e 189 (M^+ - 29) result-

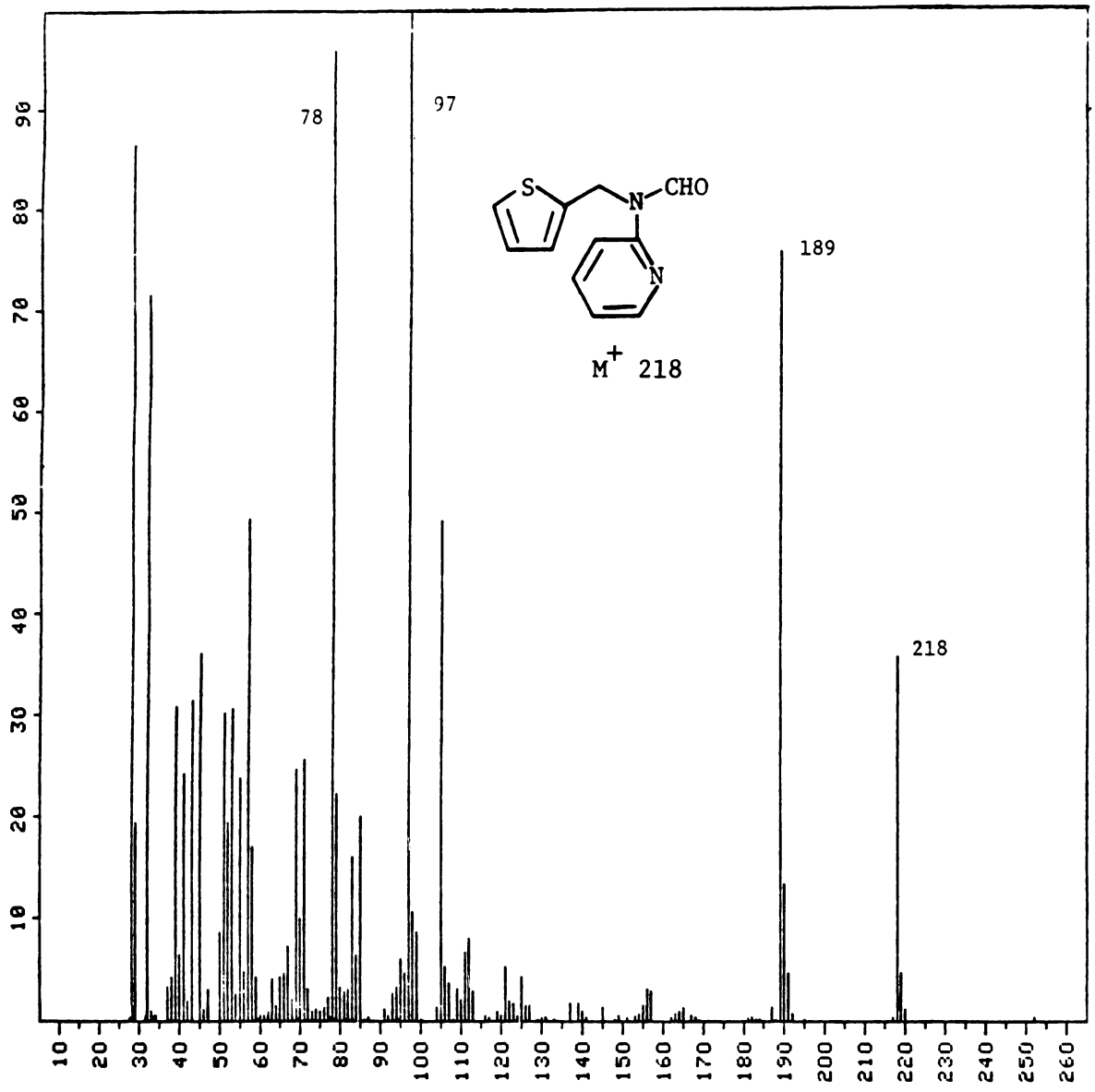
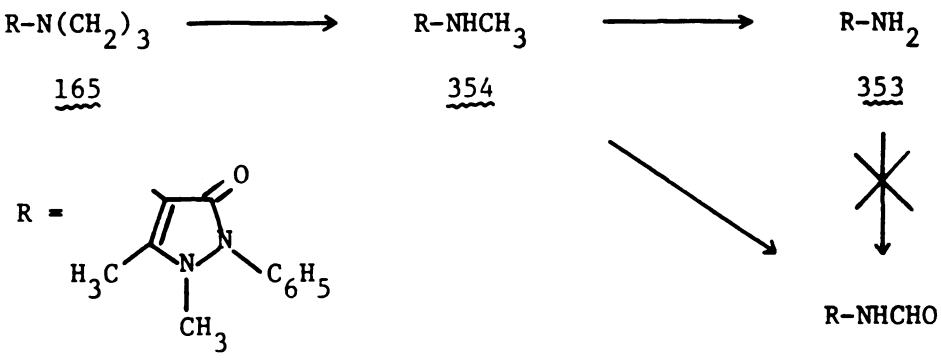


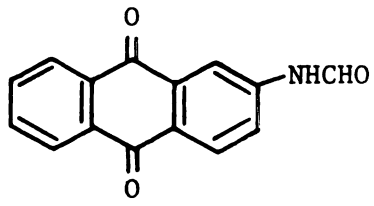
Figure 32. The gc-eims of 348 from the metabolism of 2.

ing from the loss of CHO from the parent ion is consistent with the N-formyl structure. Finally, the high resolution gc-eims analysis (Calc'd for $C_{11}H_{10}N_2OS$: M^+ 218.0512; Found: M^+ 218.0495) of this product is consistent with the elemental composition of the postulated structure.

It is difficult to rationalize the formation of 348 from the *in vitro* metabolism of methapyrilene but there are a few examples of N-formyl metabolites in the literature. The N-formyl metabolite 4-formylaminoantipyrine (352) has been detected in the urine of man and other animals after the oral administration of aminopyrine (165).[219,220] Further studies on the metabolic formation of this product have demonstrated that 352 is not formed by the formylation of the primary amine



165 354 353 352



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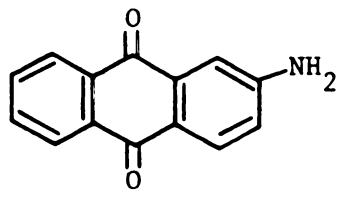


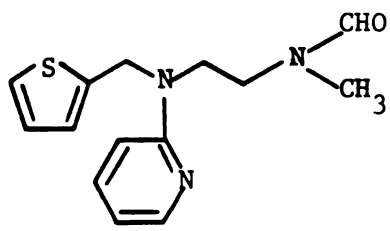
356

4-aminoantipyrine (353) but *via* oxidation of the N-methyl group of 4-methylaminoantipyrine (354).[220] On the other hand, a formylation reac-

tion is believed to be responsible for the formation of N-formyl-2-aminoanthraquinone (355) as a urinary metabolite of 2-aminoanthraquinone (356) in rats.[221] In the case of methapyrilene, the N-formyl compound 348 has also been detected as a decomposition product of the aldehyde 337 upon exposure to air. If the same decomposition reaction occurs under metabolic conditions, then the isolated N-formyl product may be an artifact derived from metabolically generated aldehyde 337.

e. N-(2-pyridyl)-N-(2-thienylmethyl)-N',N'-dimethyl-1-oxo-1,2-ethanediamine (349)

Another minor component present in the TIC tracing (Figure 24) of the methapyrilene postincubate displayed a molecular ion at M^+ 275. The gc-eims of the compound is shown in Figure 33; the nominal mass (275 amu) is consistent with the introduction of a carbonyl substituent into the methapyrilene molecule. The presence of fragment ions at m/e 78, 97, and 189 indicate the absence of substituents on the pyridyl or thienylmethyl moieties. Additionally, the fragment ion m/e 203 implies intactness of the C-2 position adjacent to the 2-aminopyridyl nitrogen atom. These data indicate that the carbonyl function may have been introduced at the N-methyl carbon atom to give 357 or at the carbon atom alpha to the dimethylamino nitrogen atom to give 349. One would expect a fragment ion



357

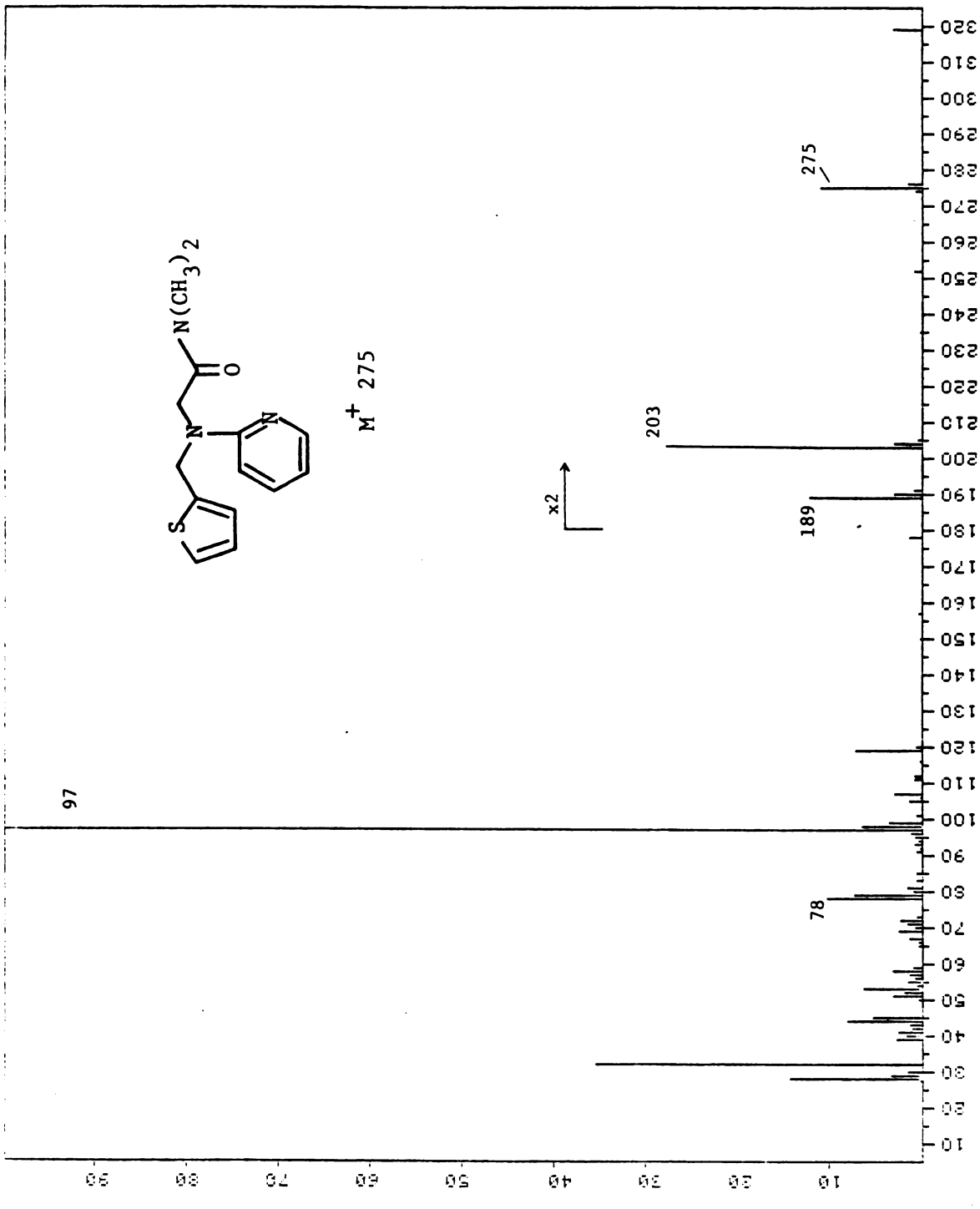
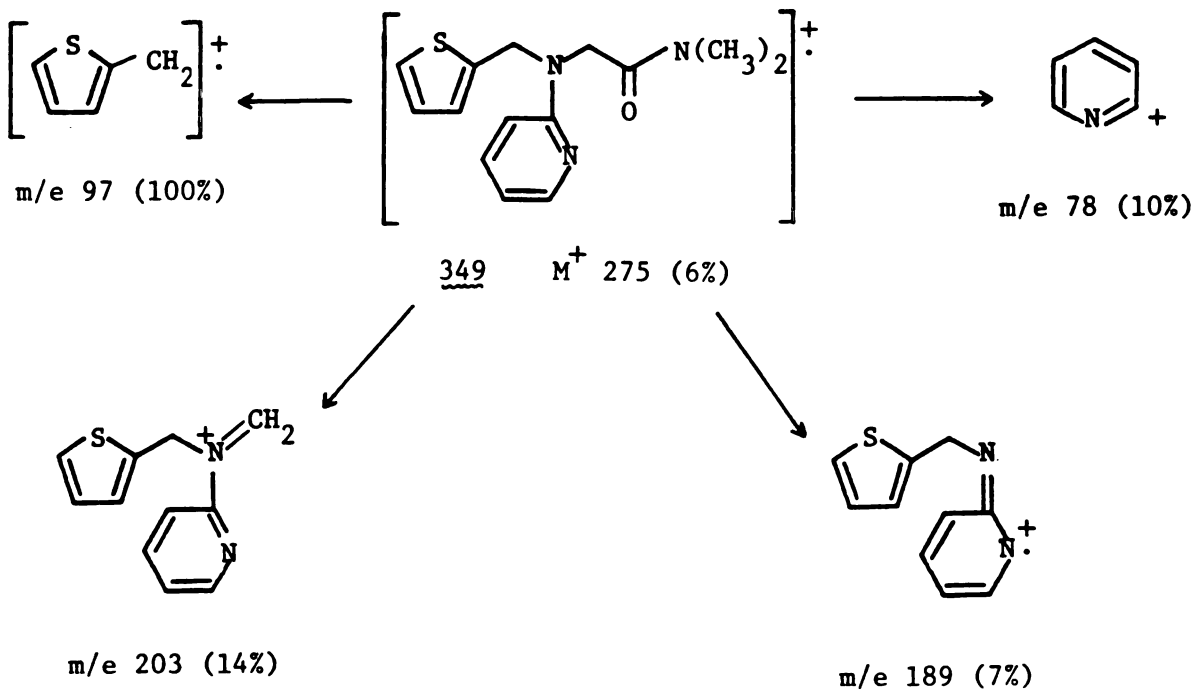


Figure 33. The gc-eims of 349 from the metabolism of 2.

resulting from the loss of CHO (M^+-29) or CO (M^+-28) from the parent ion for the N-formyl compound 357 under gc-eims conditions. A fragment ion corresponding to the loss of CHO was observed in the gc-eims of the putative N-formyl compound 348 (Figure 32) and a prominent fragment ion resulting from the loss of CO was observed for N-formyl-2-amino-anthraquinone (355).[220] No evidence for the loss of CHO or CO was seen in the gc-eims of the M^+ 275 metabolic product. Therefore, the structure of this compound was assigned as 357. This product has also been isolated in a fairly pure form by thin layer chromatography of extracts of methapyrilene postincubates. The high resolution eims (Calcd for $C_{14}H_{17}N_3OS$: M^+ 275.1089; Found: M^+ 275.1105) and gc-eims analysis of the purified product is consistent with the assigned structure. This amide metabolite may result from enzymatic oxidation of the carbinolamine 335 (Scheme 5).



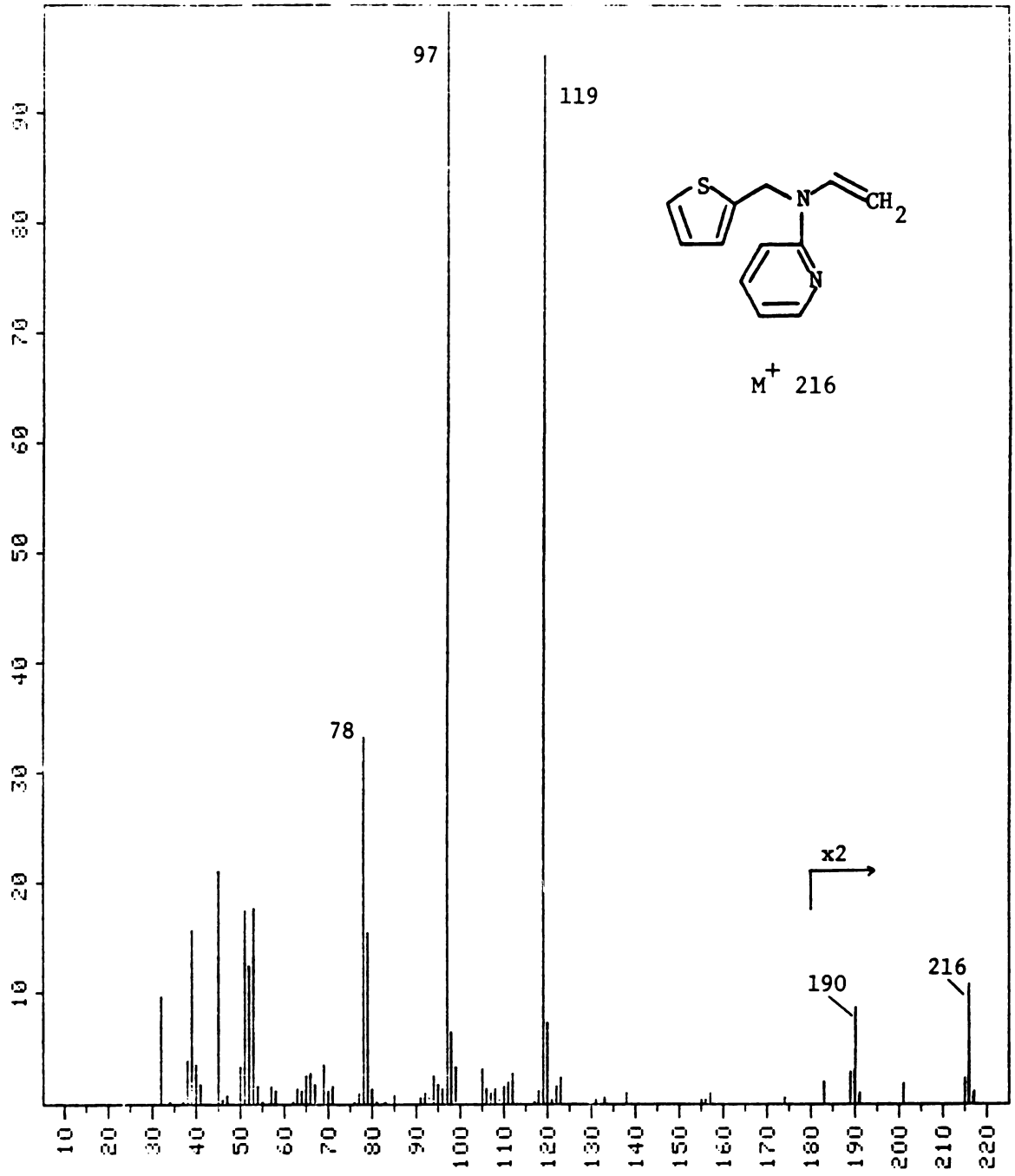
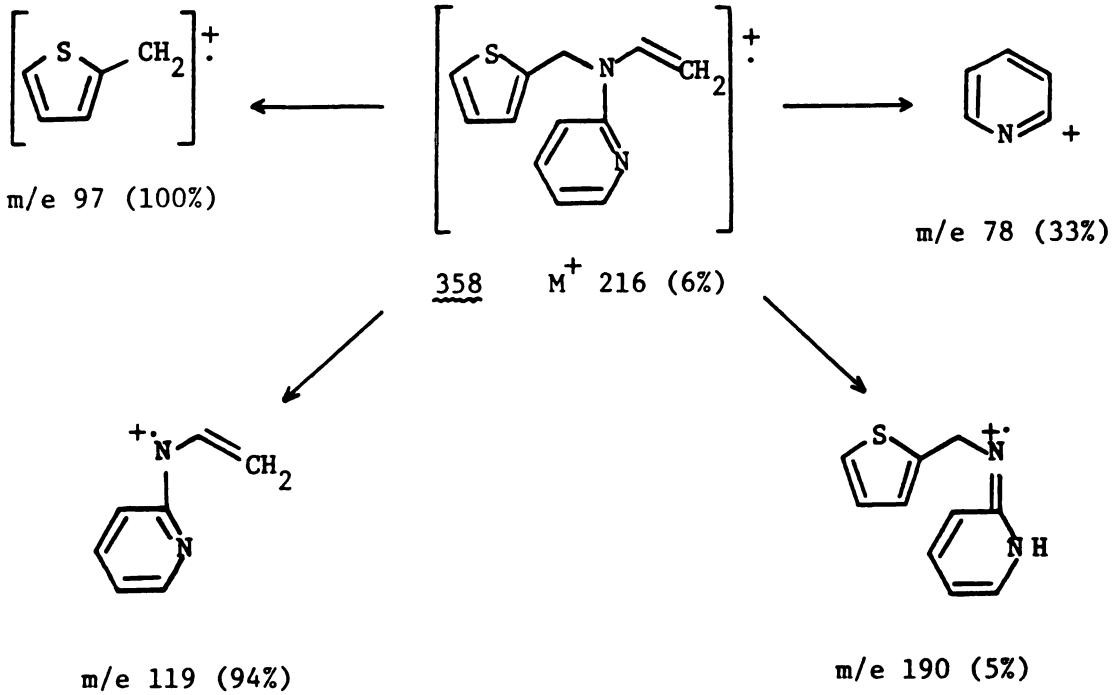


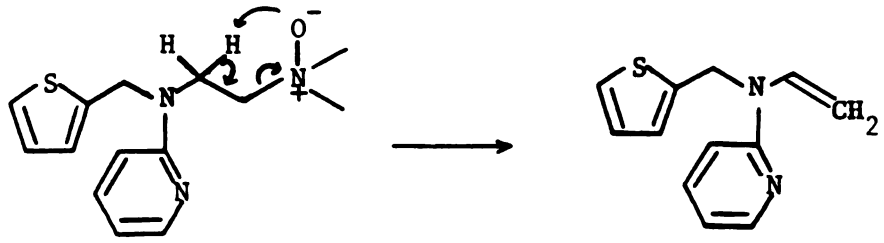
Figure 34. The gc-eims of 358 from the metabolism of 2.

f. Methapyrilene N-oxide (351)

A compound with a molecular ion at M^+ 216 was detected in the TIC trace of the methapyrilene postincubates (Figure 24). The nominal mass (216 amu) and the gc-eims (Figure 34) suggest that this compound has the N-vinyl structure 358. GC-EIMS analysis of the product isolated by tlc is consistent with this interpretation. The fragment ions at m/e 78, 97, and 190 indicate the intactness of the pyridyl and thienylmethyl moieties. The ion at m/e 119 is assigned to the fragment resulting from the cleavage of the thienylmethyl moiety.

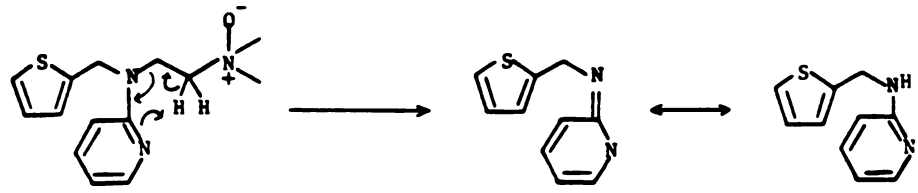


It is possible that the N-vinyl compound 358 arises from the ther-

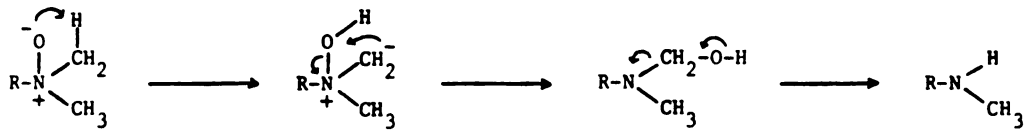


mal degradation of methapyrilene N-oxide (351) upon gc analysis. Indeed, gc-eims analysis of synthetic methapyrilene N-oxide (vide infra) did reveal the presence of 358.

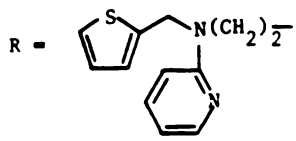
The gc-eims analysis of authentic methapyrilene N-oxide (obtained by the oxidation of 2 with H_2O_2) revealed the presence of 3 components in addition to 358; the tracing obtained from the gc analysis of authen-

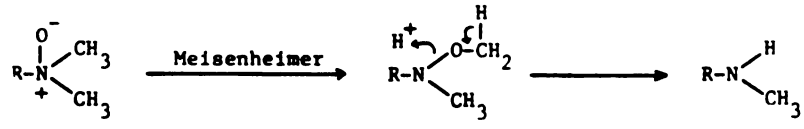


351 258



351 327





351 327

tic 351 is depicted in Figure 35. As expected, the N-oxide did not survive the thermal conditions of gc or gc-eims analysis.[171] One of the major components was the parent tertiary amine 2, the product of pyrolytic

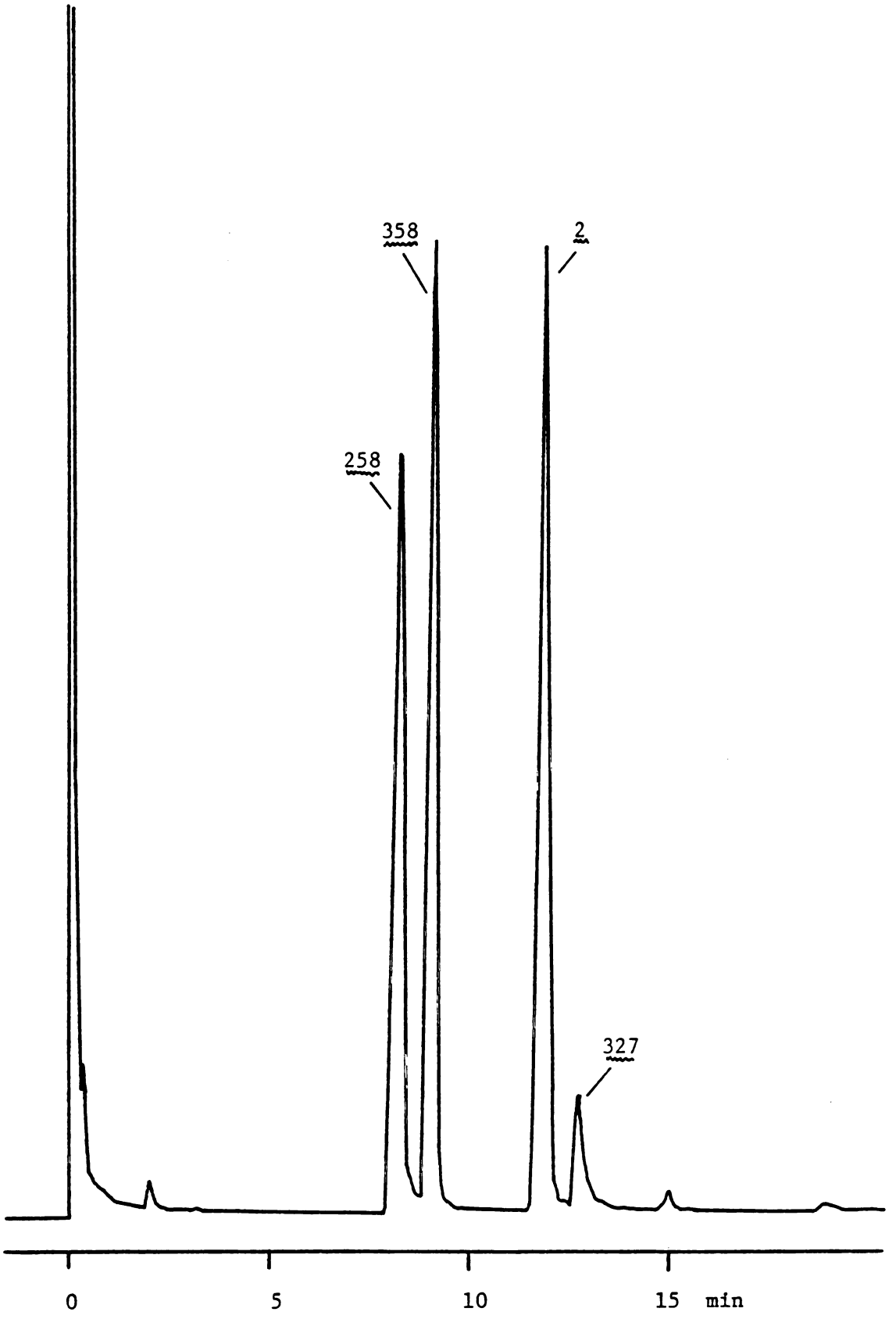


Figure 35. The gc tracing of synthetic methapyrilene N-oxide (351).

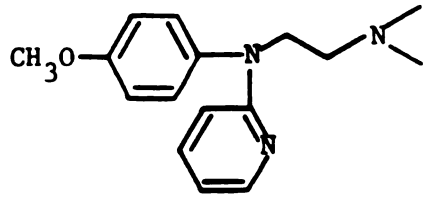
deoxygenation of the N-oxide 351. In addition, the secondary amine 258 and normethapyrilene (327) were detected upon gc-eims analysis of 351. The secondary amine 258 might arise from a retro-Michael type reaction. The formation of 327 might be rationalized by a mechanism which involves proton transfer from carbon to oxygen or by a Meisenheimer rearrangement.[171]

These data raise the question as to what portion of the large amount of methapyrilene observed upon gc and gc-eims of methapyrilene postincubate extracts originates from the pyrolysis of the N-oxide. Furthermore, it has been demonstrated that normethapyrilene might be an artifactual product of the N-oxide instead of an authentic methapyrilene metabolite. The former possibility seems rather unlikely when one considers the observation that 327 is a very minor decomposition product of synthetic 351 while 327 is a major metabolic product of methapyrilene. Nevertheless, the involvement of methapyrilene N-oxide and its decomposition products must be considered when examining the quantitative aspect of methapyrilene metabolism.

3. THE QUANTITATION OF METHAPYRILENE METABOLIC PRODUCTS

In order to properly assess the possible roles of the above metabolites in the toxicity of methapyrilene, it is important to quantitate the microsomal metabolism of methapyrilene. The quantitative description of the metabolism of methapyrilene will allow us to assess the efficiency of our metabolite isolation methods. If the amount of substrate metabolized can be accounted for in the metabolites described thus far, then it is probable that one or more of these metabolic products is responsible for the observed toxicity of methapyrilene. On the other hand, if the total amount of isolated products does not account for the metabolized

substrate then unknown products may be responsible.



359

Pyrilamine (359, gc retention time 14.8 min) was selected as a gc internal standard for methapyrilene (gc retention time 10.6 min). The typical gc tracing obtained from the base extracts of methapyrilene-sodium cyanide coincubations is shown in Figure 36 and the gc tracing of the corresponding control incubation (without NADPH) is depicted in Figure 37. Approximately 50% of the substrate was metabolized by rabbit liver microsomal preparations (20% in the rat) after one hour at 37°. The data (Table XII) also showed that 1.0 mM sodium cyanide does not inhibit the *in vitro* metabolism of methapyrilene.

Table XII. Quantitation of methapyrilene (2) metabolic products.

Expt.#	NaCN	Substrate Metabolized (as % of 2 incubated)	Normethapyrilene Found (as % of 2 incubated)	Metabolized Substrate not Accounted for (as % of 2 incubated)	Metabolized Substrate not Accounted for (as % of 2 metabolized)
1	+	33	9	24	73
2	+	46	10	36	78
	-	45	16	29	66
3	+	47	12	35	75
	-	44	14	30	68

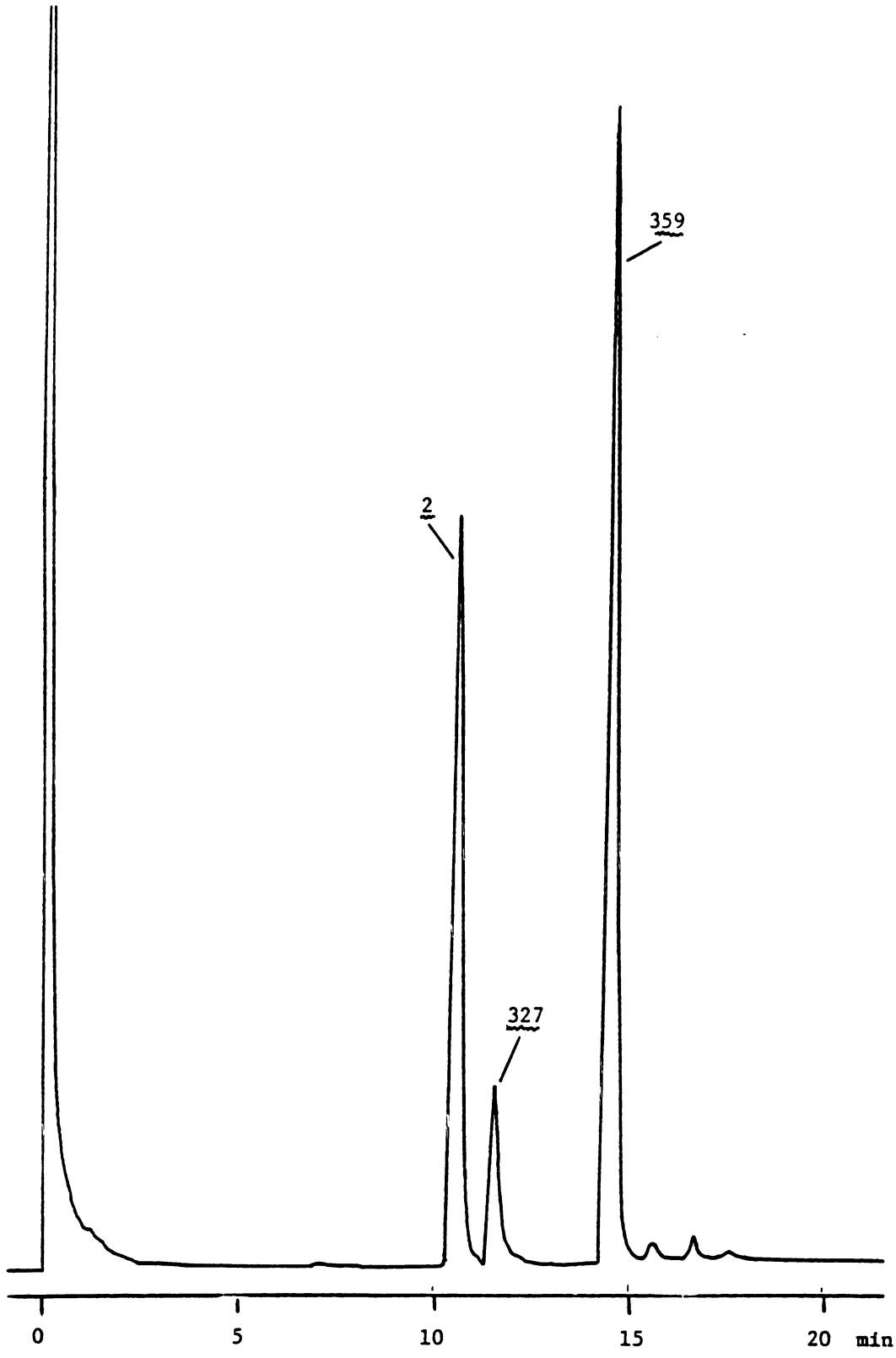


Figure 36. The typical gc tracing of the base extract obtained from the postincubates of methapyrilene with sodium cyanide (and gc internal standard 359).

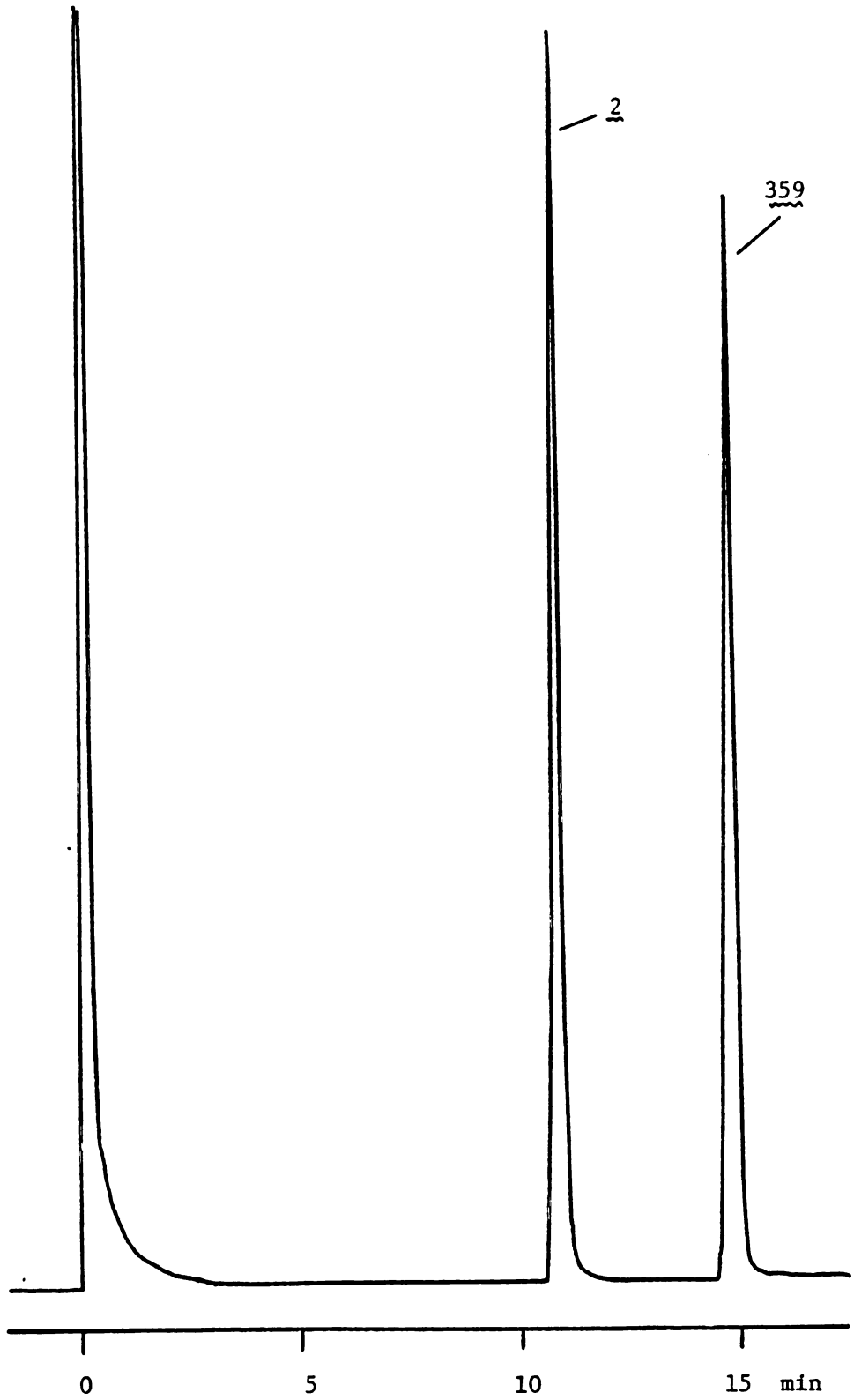


Figure 37. The typical gc tracing of the base extract obtained from control (-NADPH) incubations of 2 with sodium cyanide (with gc internal standard 359).

Assuming that the gc characteristics of the metabolic products are similar to those of the substrate and the internal standard and that gc peak heights approximates the amount of sample detected, we have made estimates of the quantities of metabolic products formed. GC analysis of the methapyrilene postincubates showed that normethapyrilene (327) was the only metabolic product obtained in measurable amounts. As depicted in Figure 36, the gc peaks corresponding to the metabolic products of methapyrilene are miniscule compared to the gc peaks resulting from 2 and the internal standard 359. As a result, normethapyrilene was the only metabolic product which could be quantitated with reasonable accuracy. Relative to 327, we must assume that the amounts of the other metabolic products detectable by gc analysis are small. Table XII summarizes the results of our attempts to quantitate the metabolism of methapyrilene and the formation of normethapyrilene (327) as the major metabolic product. These data indicate that the detected metabolic products (primarily 327) account for only 20-35% of the metabolized substrate; thus, the majority of the metabolized methapyrilene remains unaccounted for. These metabolic products are either not extracted into CH_2Cl_2 at pH 10 or can not be detected by gc methods. Because all incubations were conducted with the 100,000 x g microsomal fraction, the possibility of conjugate formation was discounted. The missing non-extractable polar metabolic products might be amino acids or phenolic compounds. Another possibility is the formation of nitrogen or sulfur oxidation products.

Attempts to isolate products of N- or S-oxidation by exhaustive extraction of the alkaline postincubates with CH_2Cl_2 were unsuccessful. It should be possible to isolate possible amino acid or phenolic products

by extraction of the postincubates at a neutral to acidic pH range. Extraction of the methapyrilene incubation mixtures with CH_2Cl_2 at pH 4, 4.5, 5, 6, 7, and 8 did not lead to metabolite identification by gc and gc-eims analysis. The extraction of methapyrilene postincubates at pH 4.0, 6.0, and 7.4 by continuous extraction methods with CH_2Cl_2 followed by treatment with diazomethane failed to produce detectable amounts of methyl ester or ether derivatives by gc and gc-eims analysis.

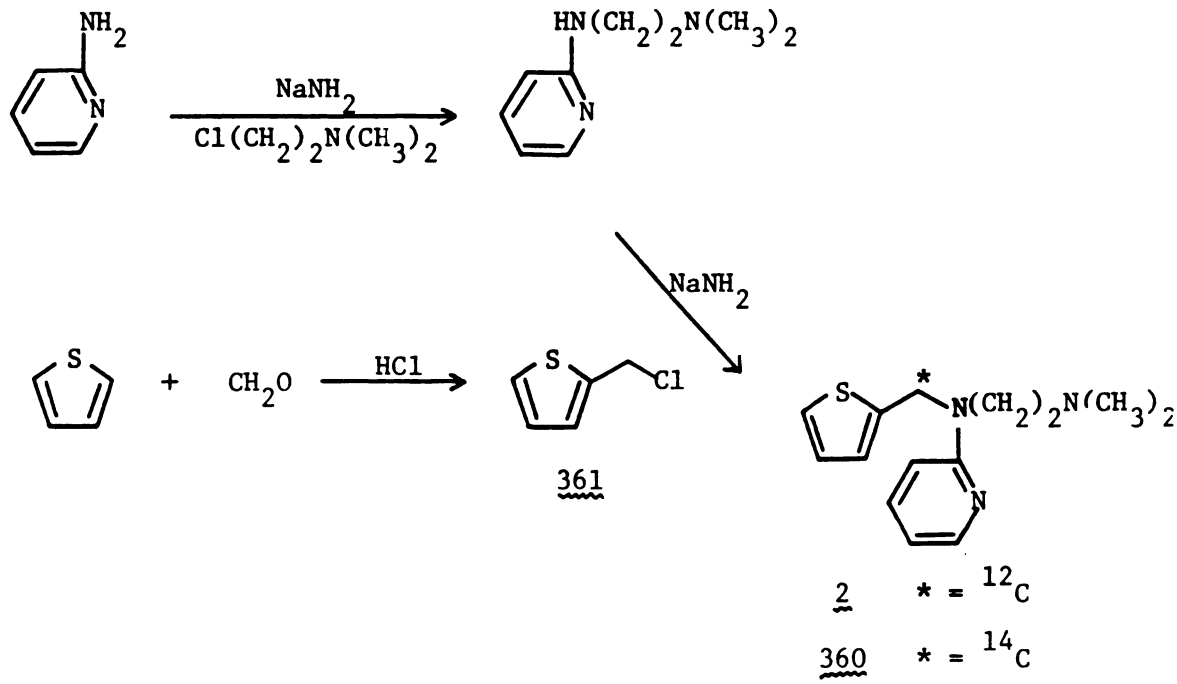
The use of an extraction technique not involving solvent partitioning might prove useful in isolating new methapyrilene metabolites. The use of Amberlite XAD-2 for extraction of biological materials has been described.[222,223] Postincubation mixtures of methapyrilene with ^{14}C-sodium cyanide were extracted on an Amberlite XAD-2 column. Analysis of the resulting methanol extracts by gc-eims revealed no new metabolic products; cims analysis of hplc (reverse phase) fractions of these extracts also proved to be unsuccessful in detecting new metabolic products of methapyrilene.

In the final analysis, it seems desirable to use radiolabeled methapyrilene to trace the metabolic fate of this drug *in vitro* and *in vivo*. Only in this way can one hope to elucidate the mechanism of toxicity and to characterize the metabolic pathway of methapyrilene. The apparent resistance of the thienylmethyl methylene carbon atom towards metabolic attack makes this position the ideal site for the radiolabel. The straightforward synthetic scheme for the synthesis of methapyrilene with ^{14}C-label at the thienylmethyl position (360) will be described in the next section.

C. CHEMISTRY OF THE METHAPYRILENE SYSTEM

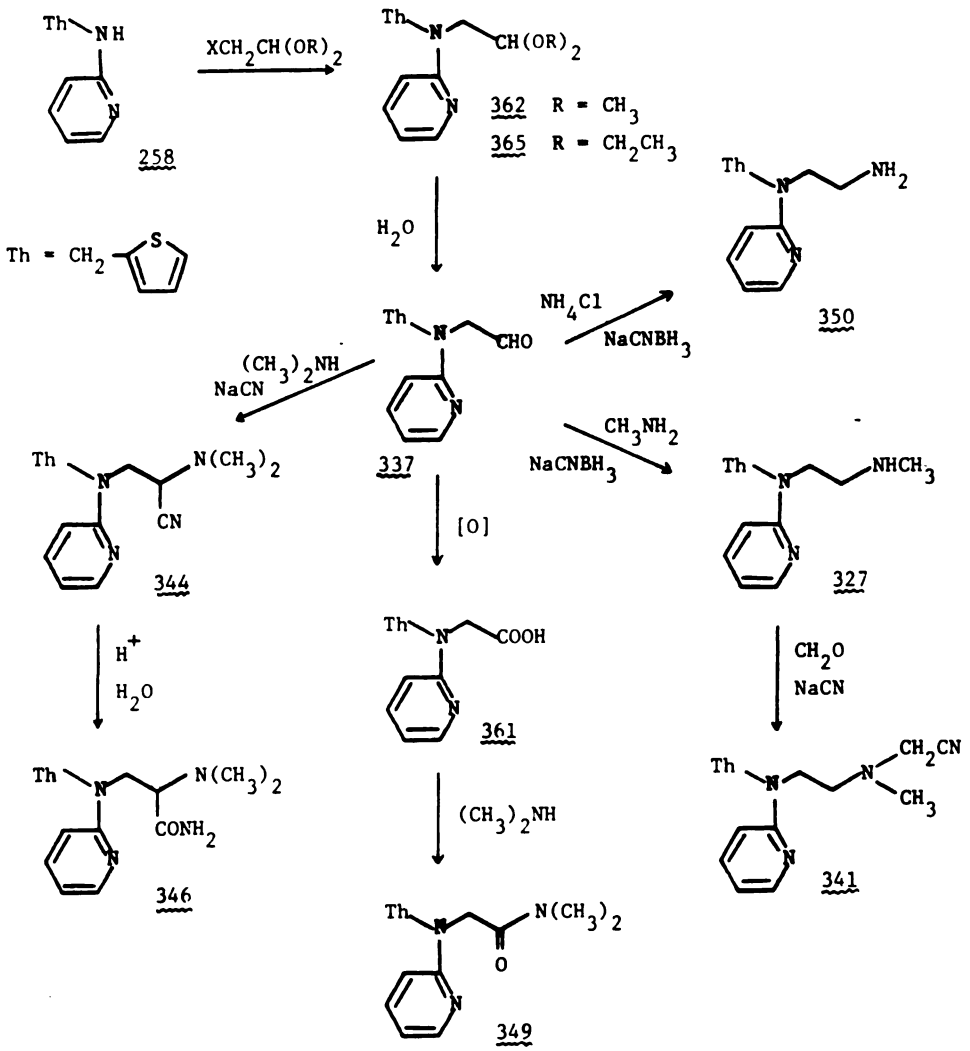
1. THE SYNTHESIS OF METHAPYRILENE METABOLIC PRODUCTS

Confirmation of the structures proposed for the methapyrilene metabolic products described above required the comparison of spectral characteristics with authentic compounds. This section describes our efforts concerning the synthesis and studies on the chemical behavior of these putative metabolic products.



Methapyrilene (2) was synthesized in 1947 according to the synthetic route described above.[224] Synthesis of the desired ^{14}C-labeled methapyrilene (360) can also be achieved by utilizing ^{14}C-labeled formaldehyde in the synthesis leading to 2-chloromethylthiophene (361).[225]

A synthetic scheme with a high degree of versatility was desirable for the synthesis of the observed metabolic products. The scheme leading to the synthesis of the desired compounds is outlined in Scheme 6.



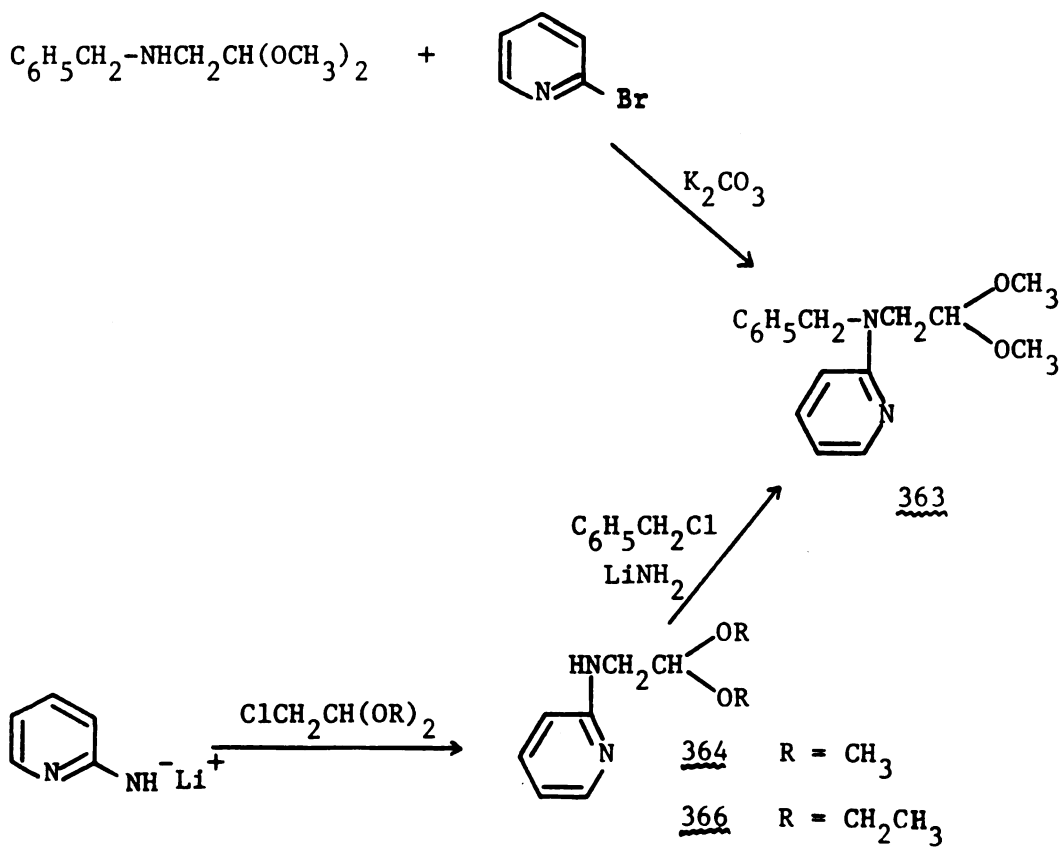
Scheme 6

The key intermediate in this scheme is the aldehyde, N-(2-pyridyl)-N-(2-thienylmethyl)aminoacetaldehyde (337). Reductive amination of this compound can give rise to the primary amine 350 or to 327 which can subsequently undergo cyanomethylation to 341. Oxidation of 337 by various means gives the amino acid 361 which can react with dimethylamine to give the amide product 349. Furthermore, 337 can be treated with NaCN and dimethylamine to produce the desired cyano adduct 344. Hydrolysis of this cyano product would give the amide 346.

2. THE CHEMISTRY OF N-(2-PYRIDYL)-N-(2-THIENYLMETHYL)AMINOACETALDEHYDE (337)

a. Synthesis

The synthesis of 337 via the hydrolysis of the corresponding dimethylacetal 362 has been reported in the literature.[226] The authors claim to have isolated the aldehyde 337 as its oxime derivative but the characterization of 362, 337, or the oxime of 337 was not reported and the experimental details were not described in this publication.[226] Nevertheless, this report does describe the synthesis of the related N-benzyl analog 363 by the condensation of N-benzylaminoacetaldehyde dimethyl acetal with 2-bromopyridine. Compound 363 can also be prepared by the condensation of 2-chloroacetaldehyde dimethyl acetal with the lithium salt of 2-aminopyridine and the subsequent treatment of the resultant secondary amine 364 with benzylchloride and lithamide.[226]

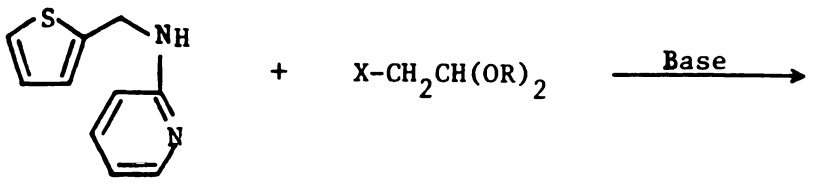


We believed that the desired acetal could be obtained by the straightforward alkylation of the secondary amine 258. The suspected metabolite, 2-(2-thienylmethylamino)pyridine (258), was synthesized by reductive amination of thiophene-2-carboxaldehyde with 2-aminopyridine.[227] Our attempts to determine optimal conditions for the alkylation of 258 with 2-halogenated acetaldehyde dialkyl acetals are summarized in Table XIII. Similar attempts at the direct synthesis of alkyl ester derivatives of the amino acid 361 are described in Table XIV. The apparent resistance of 258 towards alkylation is most probably due to the weak nucleophilic nature of the amino nitrogen atom (the pK_a of 2-aminopyridine is 6.7[217]) of 258 and its conjugate base. Other attempts to produce a 2-carbon chain alkylated derivative of 258 have failed. For example, treatment of 258 with 2-bromoethanol, ethylene oxide, or formaldehyde in the presence of sodium cyanide were all unsuccessful.

The desired diethyl acetal 365 was finally obtained in reasonable yield under rigorous reaction conditions. The alkylation of 258 with 2-bromoacetaldehyde diethyl acetal (Method A) required heating for 4 days in refluxing xylene and adding daily both the strong base $NaNH_2$ (1/4 molar equivalent) and the alkylating agent (1/2 molar equivalent). Further studies showed that 365 could be obtained in greater than 90% yield by the alkylation of the acetal 366 with 2-chloromethylthiophene (Method B). The acetal 366 was obtained after a procedure published by French workers.[228] The alkylation of 366 with 2-chloromethylthiophene required the daily addition of one-half equivalents of both $NaNH_2$ and the alkylating agent over 5 days at toluene reflux temperatures.

The formation of the desired N-(2-pyridyl)-N-(2-thienylmethyl)aminoacetaldehyde (337) required the acid-catalyzed hydrolysis of the acetal

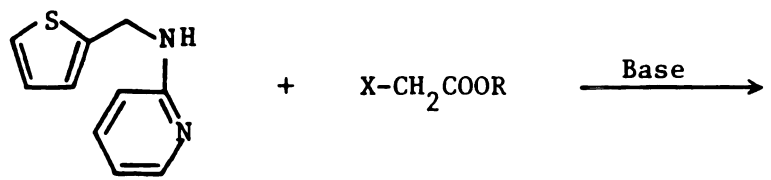
Table XIII. The alkylation of 258 with 2-haloacetaldehyde dialkylacetal



258

X	R	Base	Solvent	Reaction Conditions	Result
Cl	Me	$NaNH_2$	Toluene	Reflux, 48 hrs	Starting material by gc
		NaH	THF	Reflux, 96 hrs	" " " "
Cl	Et	NaH	THF	Reflux, 15 hrs	" " " "
		NaH	Toluene	Reflux, 12 hrs	" " " "
		K_2CO_3	Toluene	Reflux, 64 hrs	" " " "
		--	DMSO	150°, 64 hrs	Decomposition products by gc
		n-BuLi	THF	Reflux, 24 hrs	Starting material by gc
Br	Et	$NaNH_2$	Toluene	Reflux, 18 hrs	20% product by gc
		$NaNH_2$	Xylene	Reflux, 96 hrs Daily addition of base and acetal	> 80% product

Table XIV The alkylation of 258 with 2-haloethyl acetate derivatives



258

X	R	Base	Solvent	Reaction Conditions	Result
Cl	Me	--	Benzene	Reflux, 24 hrs	Starting material, no desired product by NMR
Br	Et	--	THF	55°, 12 hrs	Starting material by gc
		--	THF	RT, 24 hrs	" " " "
		Pyridine	THF	RT, 48 hrs	" " " "
		Pyridine	THF	RT, 96 hrs	" " " "

365. Studies on the optional conditions for the hydrolysis of 365 are summarized in Table XV.

As will be discussed in Section V.C.2.c., the aldehyde 337 has been characterized by nmr, ir, cims and eims. Attempts to isolate 337 in its pure form or as a derivative have failed.

Table XV. The hydrolysis of N-(2-pyridyl)-N-(2-thienylmethyl)aminoacetaldehyde diethyl acetal (365)

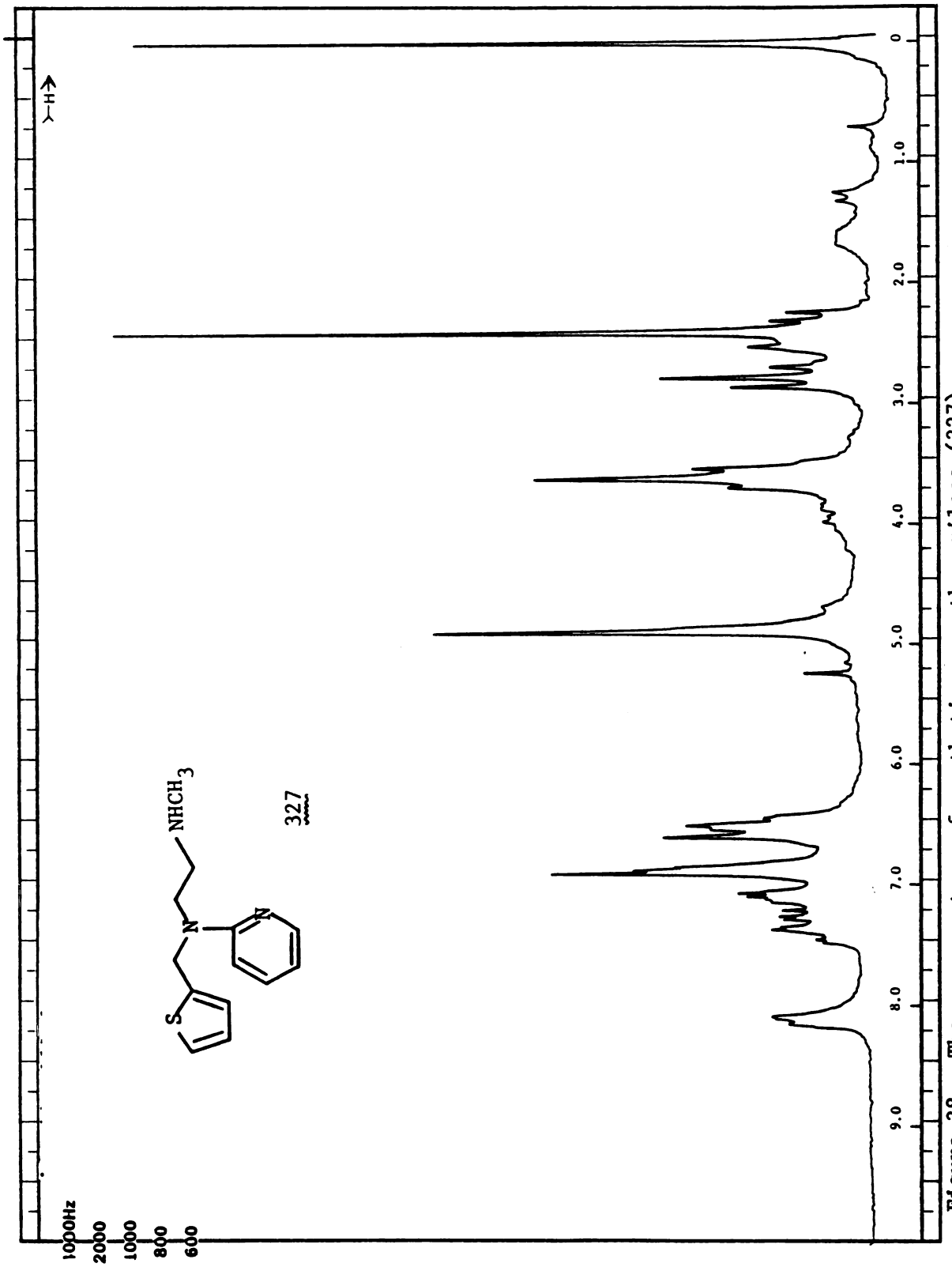
Acid	Solvent	Reaction Conditions	Results
8N HCl	H_2O	80°, 60 min	Mixture by gc, aldehyde present by NMR
2N HCl	THF	35°, 12 hrs	Mostly starting material by gc
10% CH_3COOH	--	45°, 15 hrs	" " " " "
90% CH_3COOH	--	90°, 12 hrs	" " " " "
Oxalic Acid	THF/H_2O	50°, 24 hrs	" " " " "
		100°, 50 hrs	" " " " "
6N HCl	--	80°, 45 min	Desired aldehyde (337) by NMR, IR, CIMS
2% H_3PO_4	--	85°, 48 hrs	Desired aldehyde
	--	80°, 2 hrs	" "
2N HCl	--	80°, 1 hr	Desired aldehyde, quantitative yield

Nevertheless, the aldehyde could be used as a precursor in the synthesis of other desired products.

b. Reactions

1) Formation of N-(2-pyridyl)-N-(2-thienylmethyl)-N'-methyl-1,2-ethanediamine (327) and N-(2-pyridyl)-N-(2-thienylmethyl)-N'-cyanomethyl-N'-methyl-1,2-ethanediamine (341)

Treatment of crude 337 (from the hydrolysis of 365) with methylamine and $NaCNBH_3$ provided a single compound with a gc retention time similar to the starting aldehyde and to the metabolically derived product assigned as 327 in Section V.B.2. The nmr spectrum of this synthetic product (Figure 38), as compared to the nmr spectrum of methapyrilene (Figure 39), is consistent with the expected normethapyrilene product 327; the signal (s, 2.42 ppm) assigned to the N-methyl group of 327 integrates as required for three protons. In addition, the nmr spectrum of this product showed no trace of the aldehydic proton of starting 337 (9.47 ppm, see Figure 40). Further evidence for the structure assignment of this product as 327 comes from the treatment of crude product with aqueous formaldehyde solution and NaCN. As expected, this process gave rise to the fully characterized cyanomethyl product 341 which is identical (gc, nmr, cims, eims) to the radioactive cyano adduct isolated from metabolic postincubates of methapyrilene. Attempts to purify crude 327 by column chromatography on neutral alumina resulted in the isolation of a pure product (eluted with 1% $MeOH/CH_2Cl_2$) with the expected gc characteristics of 327. However, the nmr spectrum of this material (Figure 41) displays a downfield shift of the N-methyl group from 2.42 ppm observed for the crude product 327 (Figure 38) to 3.62 ppm for the chromatographed product. This information allowed us to tentatively assign the N-hydroxylamine structure 367 to the chromatographed product. Treatment of this material with zinc in acetic acid resulted in the partial reduction of 367 to the amine



327

Figure 38. The nmr spectrum of synthetic normethapyrilene (327).

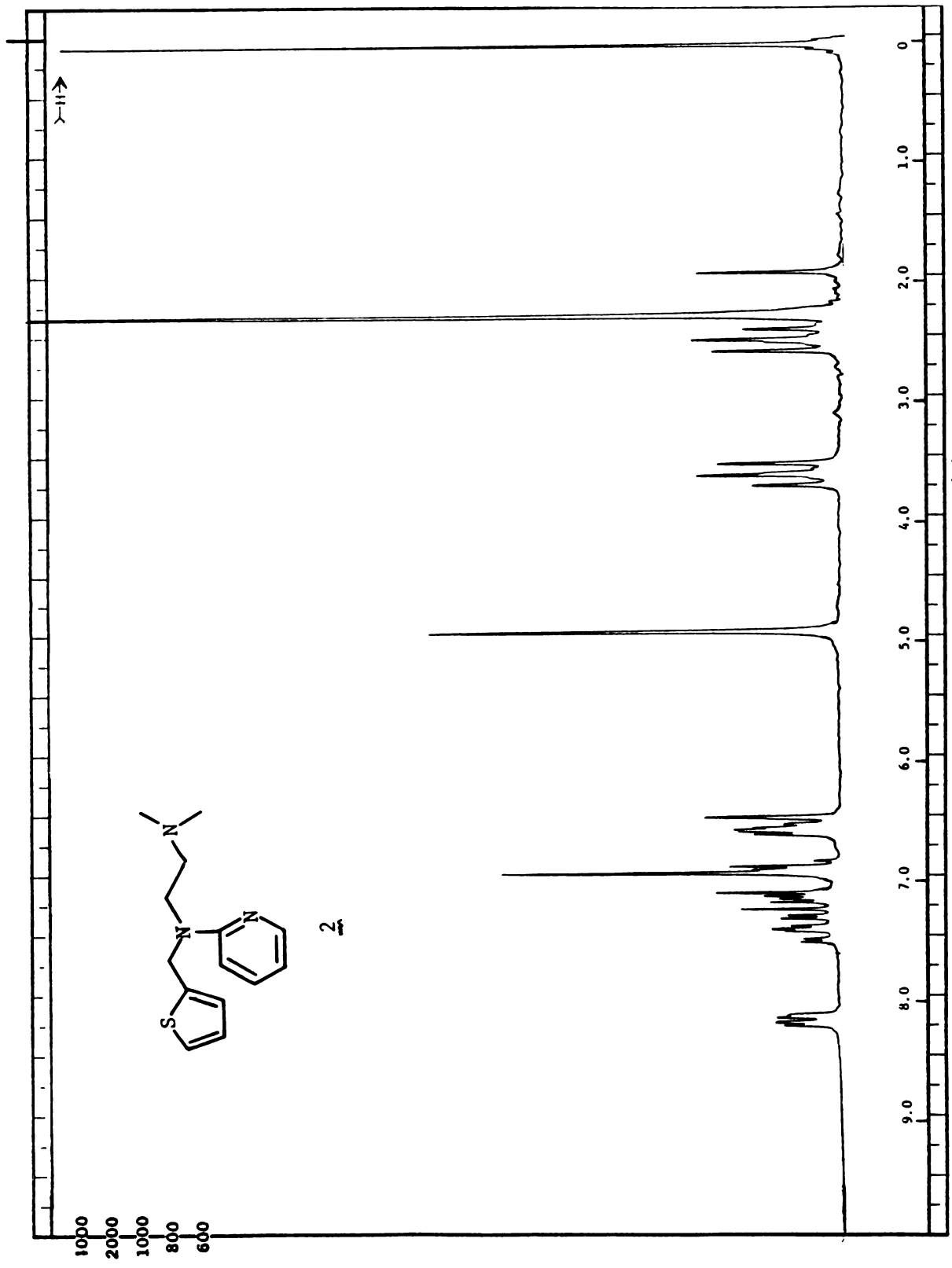


Figure 39. The nmr spectrum of authentic methapyrilene (2).

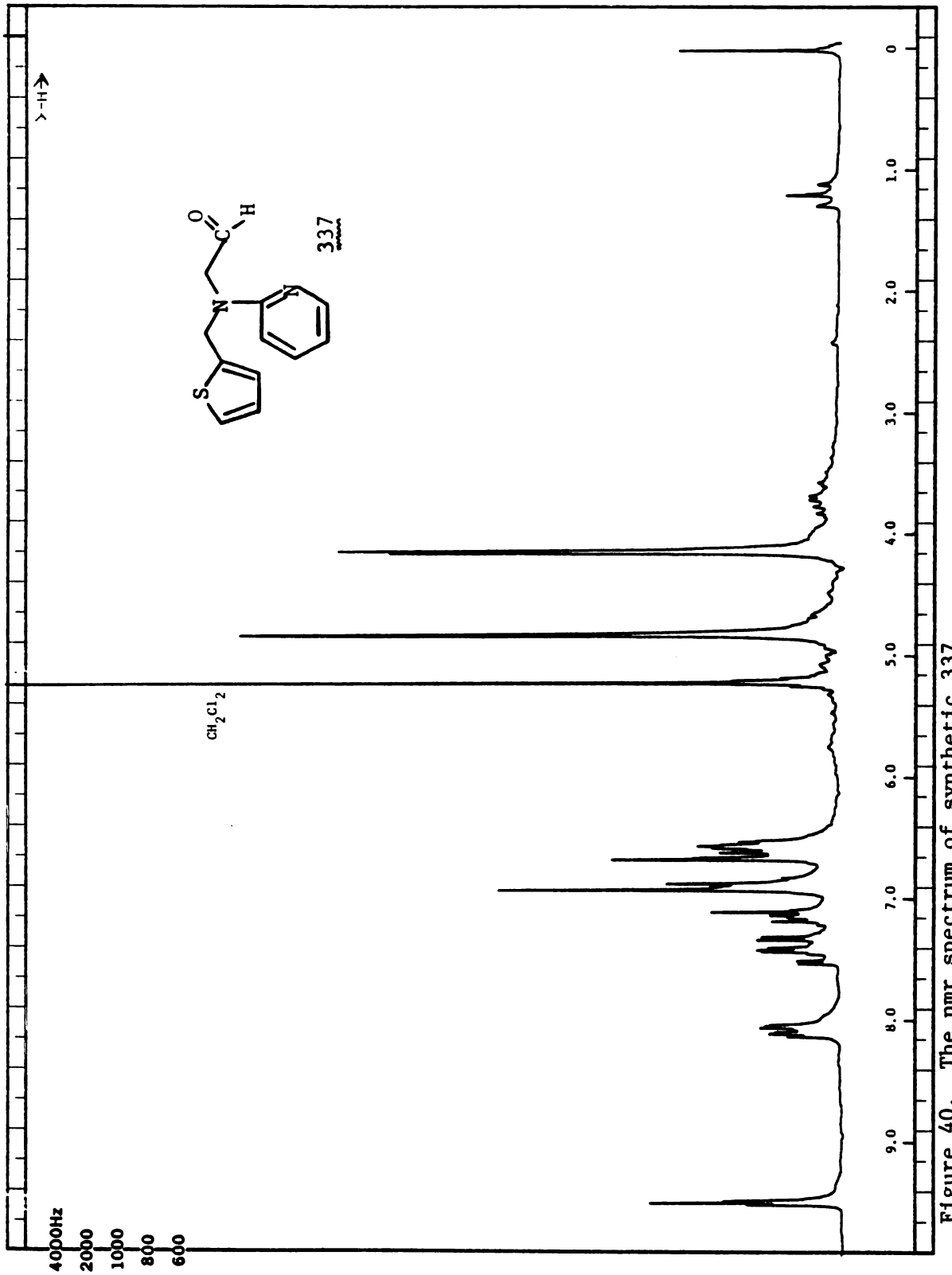
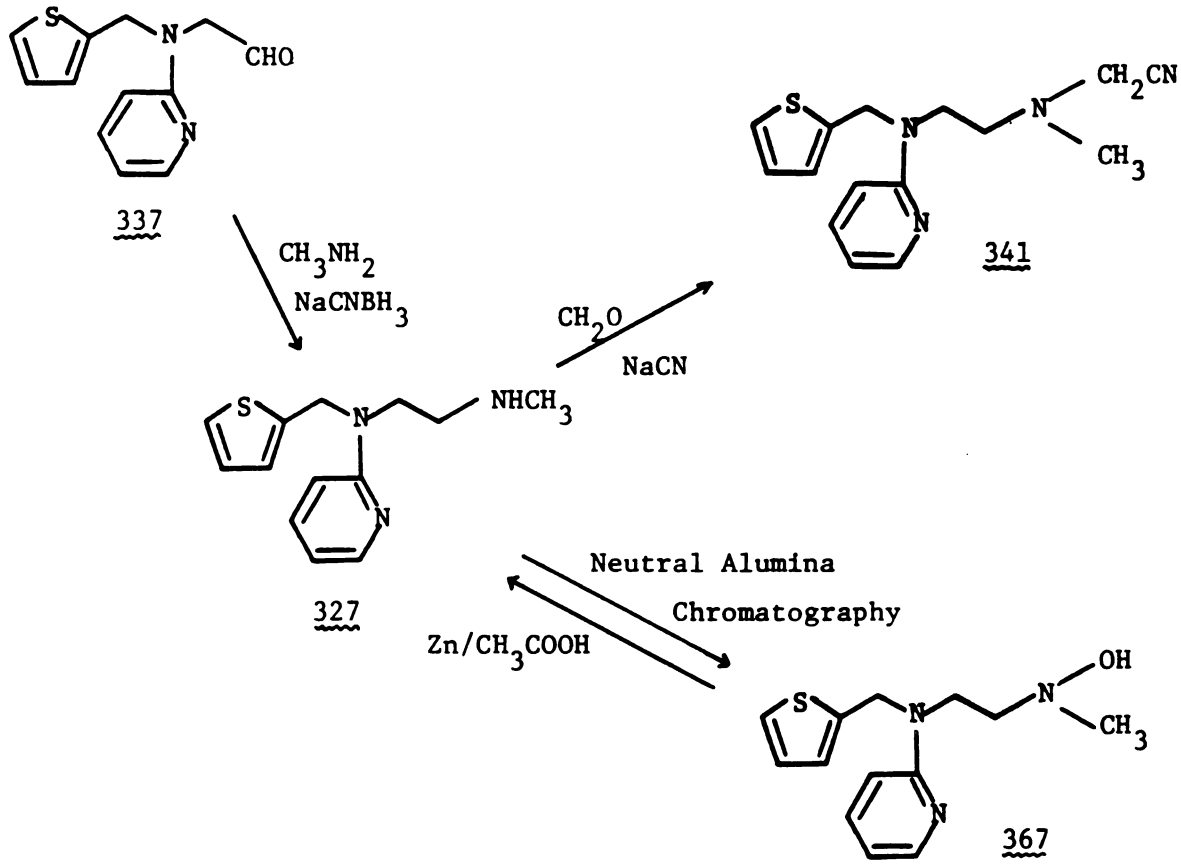


Figure 40. The nmr spectrum of synthetic 337.

327 as evidenced by nmr (Fig. 42). Presumably, trace transition metal ions in the alumina can catalyze the oxidation of the secondary amine 327 to the corresponding hydroxylamine 367.



2) Oxidation to N-(2-pyridyl)-N-(2-thienylmethyl)aminoacetic acid (361)

Attempts to oxidize the crude aldehyde 337 to the corresponding acid derivative 361 with Ag_2O failed. Only the secondary amine 258 and other decomposition products were detected in the crude reaction mixture. Treatment of the reaction mixture with diazomethane failed to produce any trace of the desired methyl ester of 361. When available, the dimethylamide 349 should be readily accessible from the acid 361. The use of other oxidizing agents, e.g. Jone's reagent, pyridinium

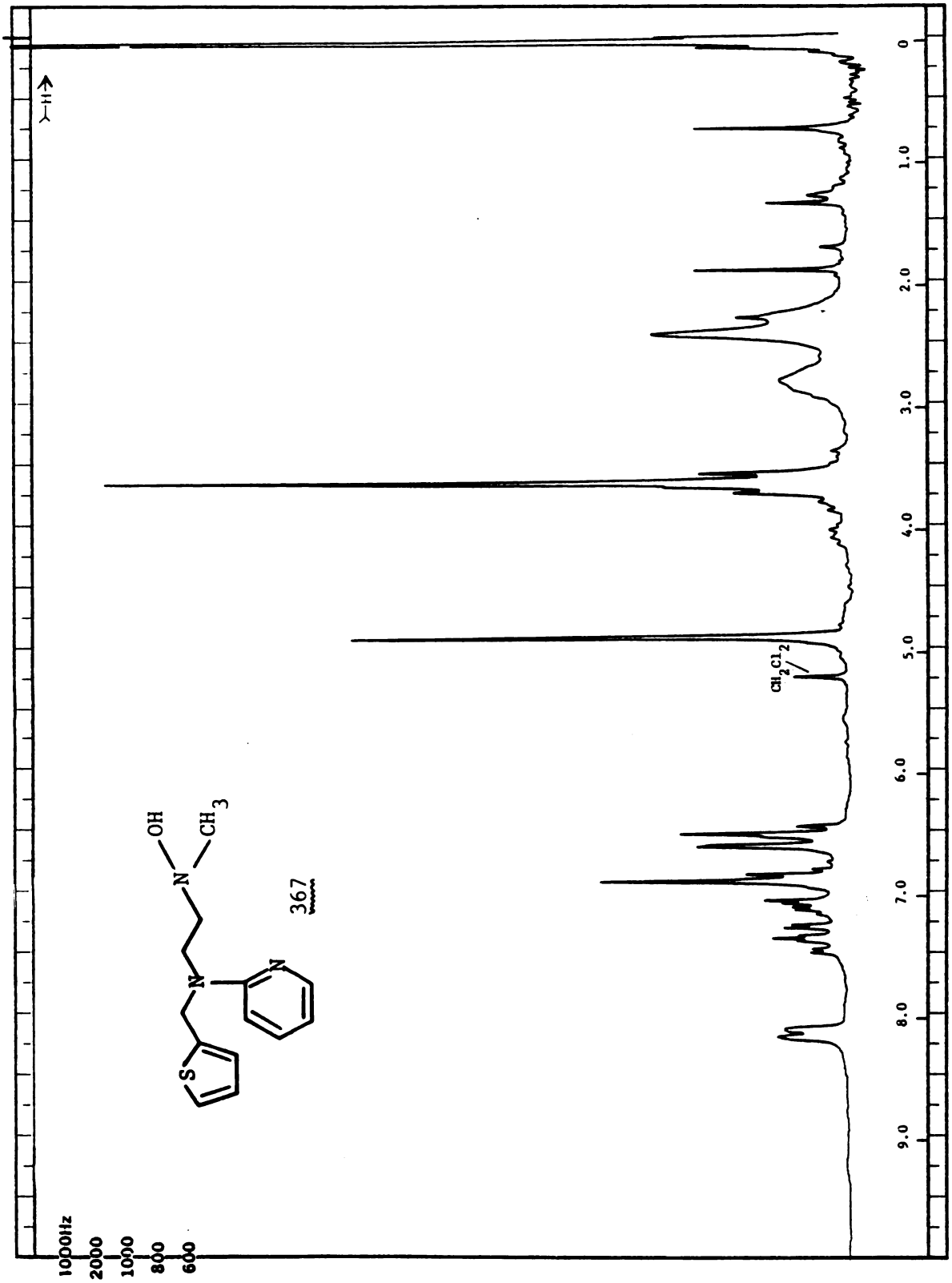


Figure 41. The nmr spectrum of N-hydroxy-normethapyrilene (367).

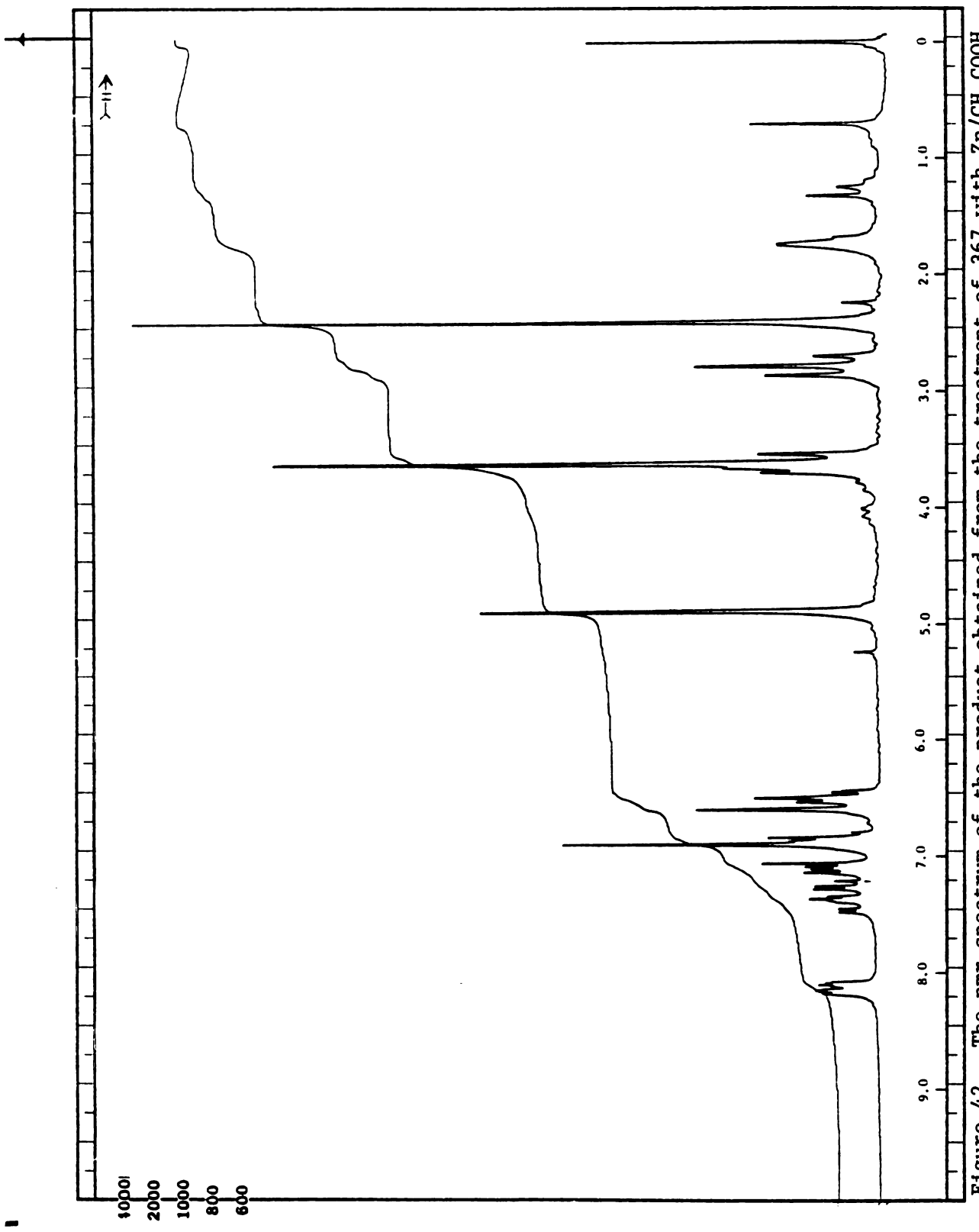


Figure 42. The nmr spectrum of the product obtained from the treatment of 367 with Zn/CH_3COOH.

chlorochromate or pyridinium dichromate might prove successful for the desired oxidation.

3) Synthesis of N-(2-pyridyl)-N-(2-thienylmethyl)-N',N'-dimethyl-1-cyano-1,2-ethanediamine (344)

The synthesis of the α-cyanoamine 344 was achieved by treating the crude aldehyde 337 with an aqueous solution of $NaHSO_3$ followed by $(CH_3)_2NH$ and NaCN. The product could be obtained in 22% yield. As we shall see in Section V.C.3., the low yield might be due to the labile nature of 344 or inappropriate reaction conditions. As discussed in Section V.B.1., attempts to analyze the synthetic 344 by probe eims, gc-eims, and high resolution eims resulted only in the characterization of the HCN elimination product 345. However, cims of the synthetic 344 displays a parent ion (MH^+ 287) which is consistent with a cyano substituted methapyrilene structure. In addition, the ir spectrum displays the required C≡N band at 2200 cm^{-1} and the nmr spectrum of synthetic 344 (Figure 43) supports the structure assignment of this product as the α-cyanoamine 344.

c. Chemical Behavior

The crude product isolated from the acid hydrolysis of the acetal 365 displayed spectral properties consistent with the assigned aldehyde structure 337. The nmr spectrum of the crude product (Figure 40) displays a signal at 9.47 ppm characteristic for aldehyde protons. IR analysis reveals a fairly strong carbonyl band at 1750 cm^{-1}. GC analysis of the crude product mixture indicates the presence of largely one component with a retention time similar to the acetal 365 and the α-cyanoamine 344. The gc-ei mass spectrum of this product (Figure 44) displays the required

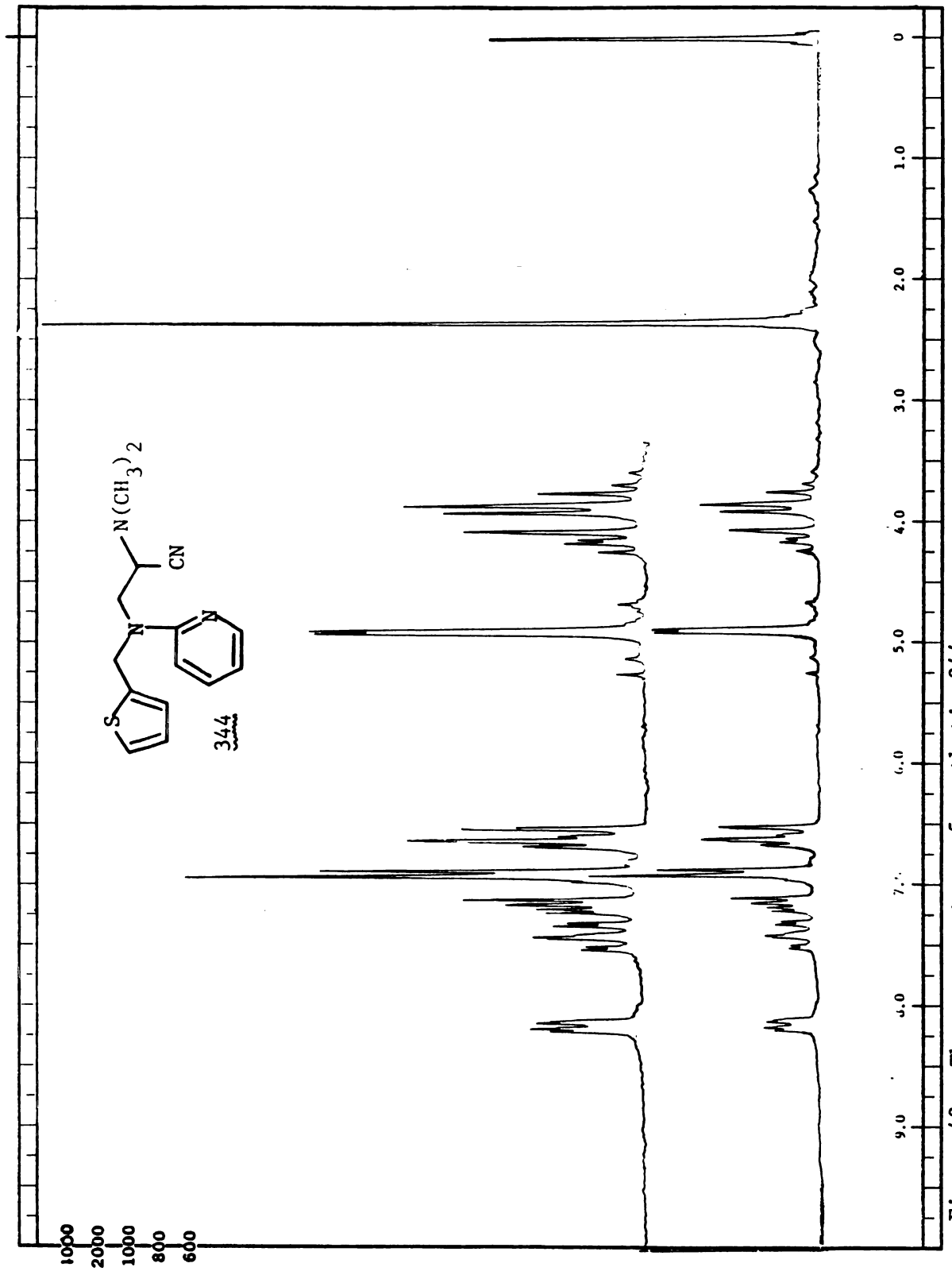


Figure 43. The nmr spectrum of synthetic 344.

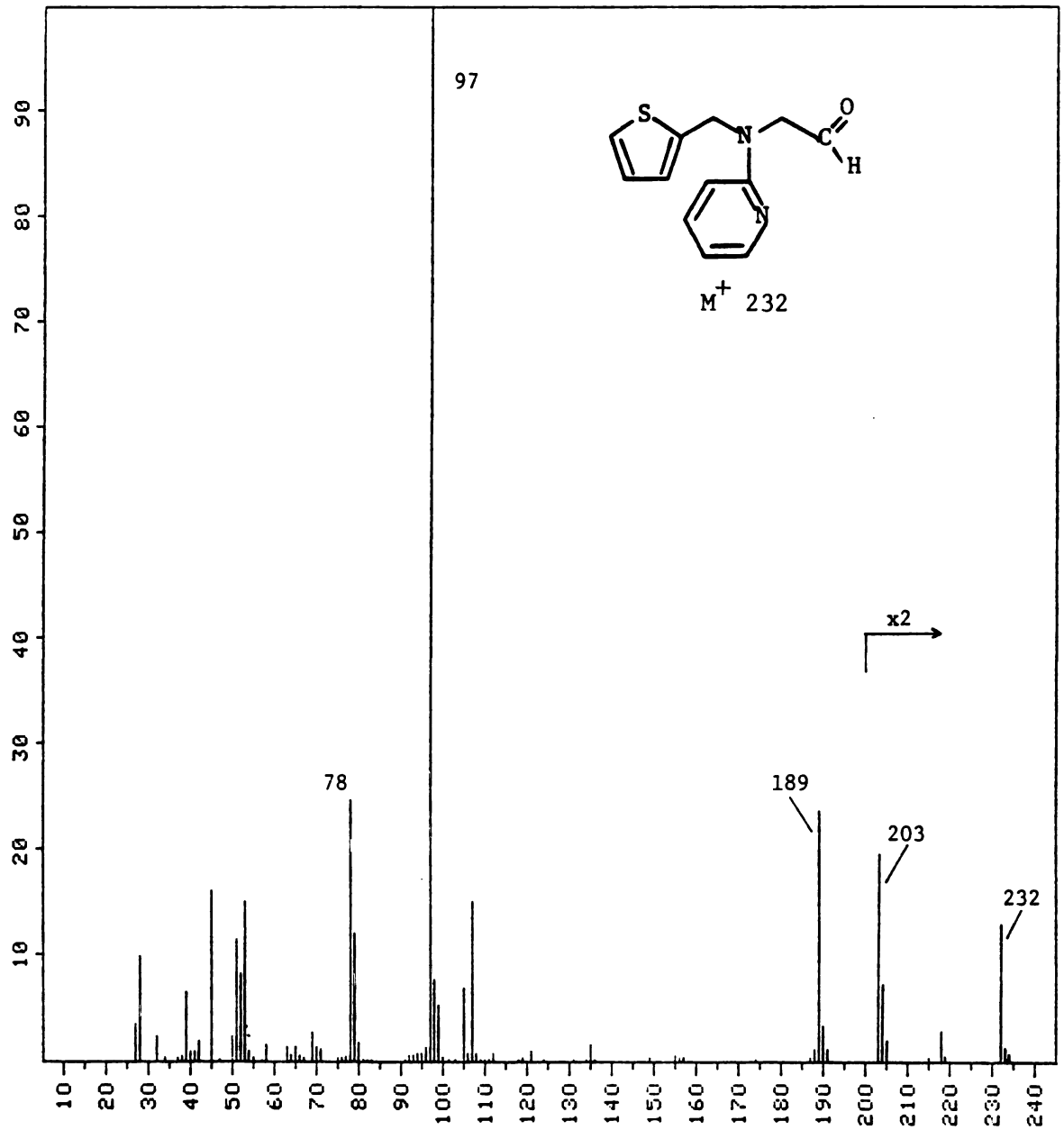
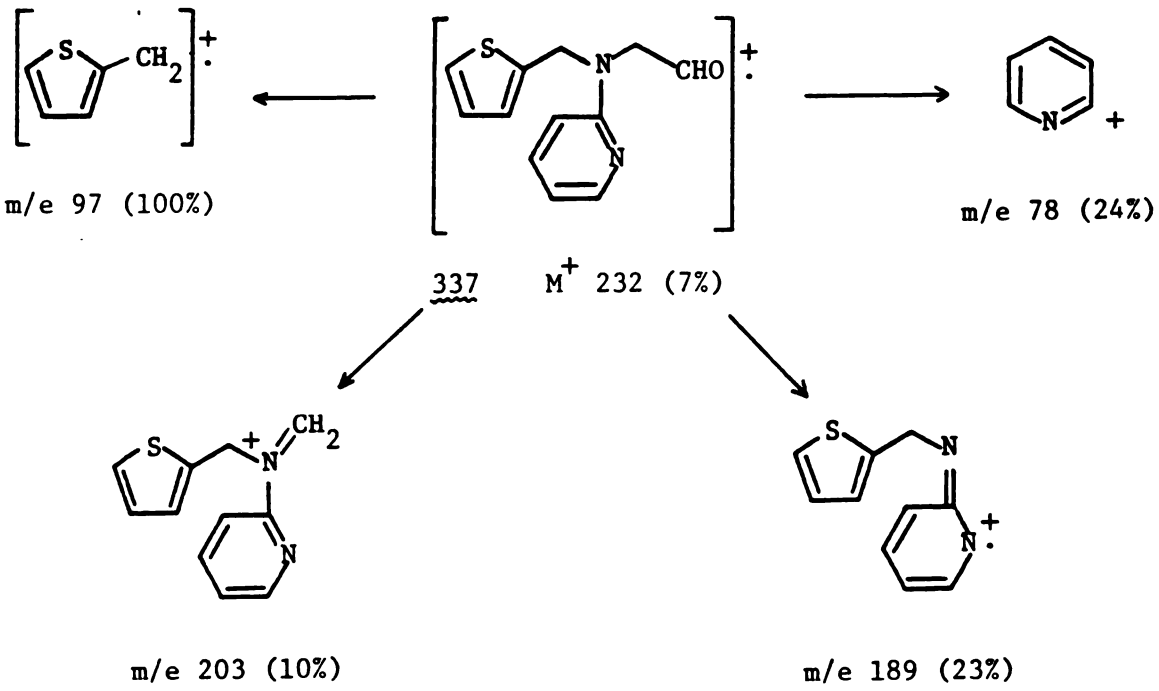


Figure 44. The gc-eims of synthetic 337.

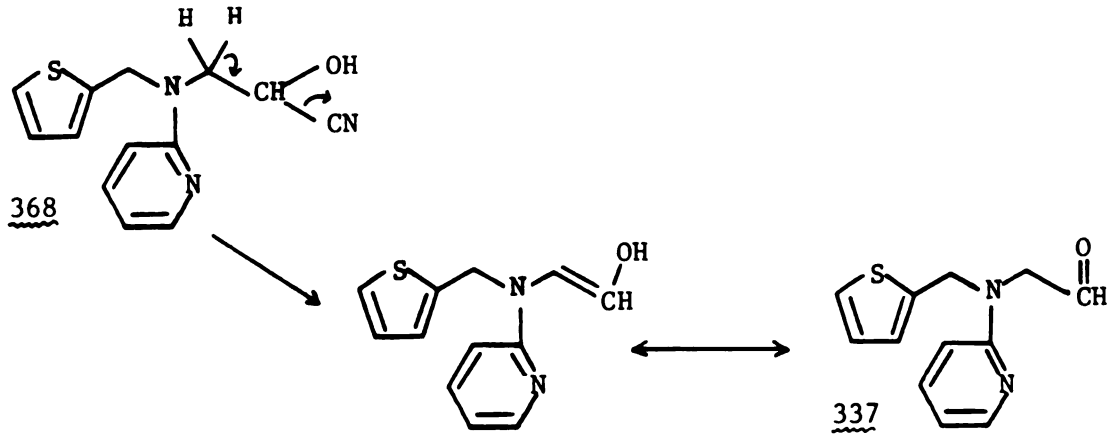
parent ion M^+ 232 and fragment ions at m/e 203, 189, 97 (100%), and 78 all of which are consistent with structure 337. Furthermore, cims (MH^+ 233) and high resolution-eims analysis of this product agrees with the postulated structure. Thus, it seems fairly certain that the acid hydrolysis of 365 yields the aldehyde 337.



However, all attempts to isolate 337 in its pure form or as a derivative have failed. The distillation of crude 337 *in vacuo* gave rise only to a mixture of unidentified decomposition products. Attempts to form the picrate salt of 337 were unsuccessful. Cation exchange chromatography of crude 337 led to a mixture of products including the secondary amine 258. Attempts to form a 2,4-DNP or oxime derivative of 337 have been unsuccessful. Finally, it has been demonstrated that crude 337 does not migrate off the origin of tlc plates (silica or neutral alumina) even when CH_3CN (1% Et_3N) was used as the developing solvent.

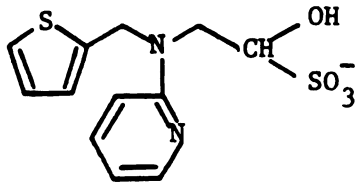
The tlc behavior of this material is indicative of the polar nature of this compound.

In the reaction leading to the α-cyanoamine 344, 337 was treated with $NaHSO_3$, $(CH_3)_2NH$, and NaCN in pH 5.3 acetate buffer. In addition to 344, silica gel column chromatography of the crude reaction mixture yielded two components both with gc retention times identical to that of 337. The first, less polar compound (eluting with ∿5% EtoAc/CH_2Cl_2) displays the nmr spectrum depicted in Figure 45. The absence of the aldehydic proton signal at ∿ 9.5 ppm, the proton quartet centered at 4.71 ppm, and the D_2O-exchangeable signal at 8.34 ppm are consistent with the postulated cyanohydrin structure 368. The cims of this compound displays a parent ion (MH^+ 233) consistent with the aldehyde structure 337. This can be rationalized by postulating that the cyanohydrin 368 eliminates HCN upon thermolysis to give the enol form of 337.



The nmr spectrum of the second polar component (eluting with 1:1 acetone/MeOH) is depicted in Figure 46. Comparison of this nmr spectrum with that of starting 337 (Figure 40) shows that the compounds are structurally related except the singlet aldehydic proton signal of 337

is replaced by a triplet centered at 8.74 ppm. The polar nature of this product suggests three possible structures. The bisulfite addition product 369 is more or less consistent with the nmr spectrum. The aldehyde hydrate 370 probably can be ruled out since it would be quite unstable. Additionally, the nmr spectrum of this hydrate would be expected to be similar to that of the acetal 365 (Figure 47). The methine triplet (4.72 ppm) of 365 appears at a much higher field than the corresponding triplet (8.74 ppm) of the polar product. The third possible structure consistent with the nmr spectrum is the zwitterionic compound 371. This zwitterion is a tautomeric form of 337 which may be preferred in a polar medium like silica gel. It should be noted that the nmr spectrum (Figure 40) which depicts an aldehydic proton was recorded in unpurified $CDCl_3$ which is likely to contain amounts of hydrochloric acid. It is possible that in this case protonation of the pyridine nitrogen by trace HCl will hamper the formation of the zwitterion 371. On the other hand, nmr spectrum depicting the putative zwitterionic structure (Figure 46) was recorded in purified $CDCl_3$ stored over K_2CO_3. Later attempts to repeat the isolation of this polar product were unsuccessful and led only to the aldehyde 337. Further work will be necessary to determine the structure of this compound unambiguously.



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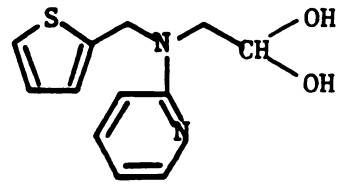

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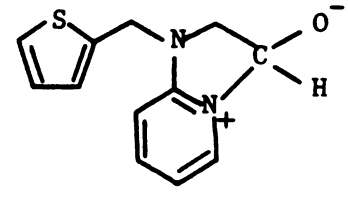

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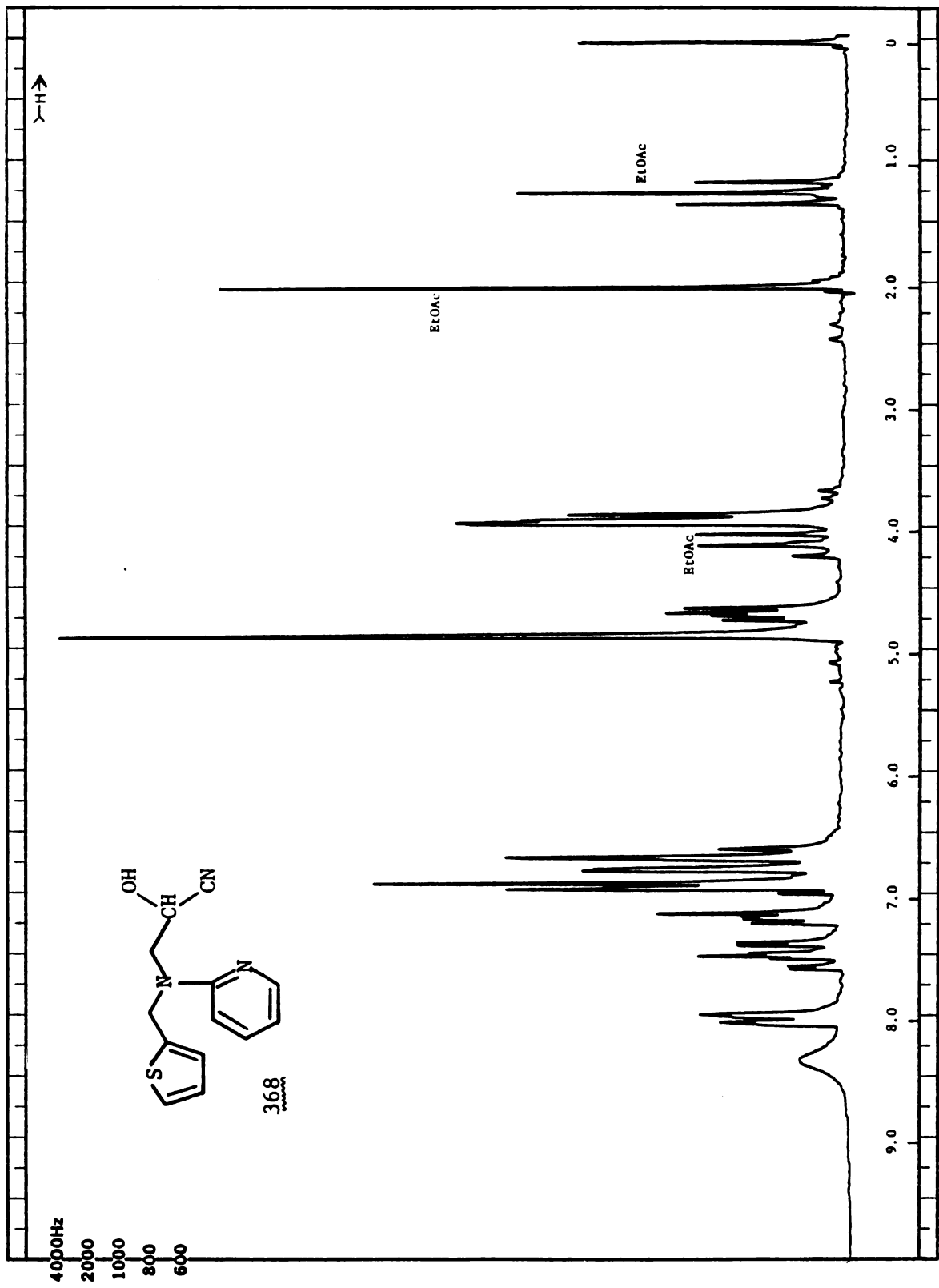


Figure 45. The nmr spectrum of 368 from silica gel column chromatography of the 344 reaction mixture.

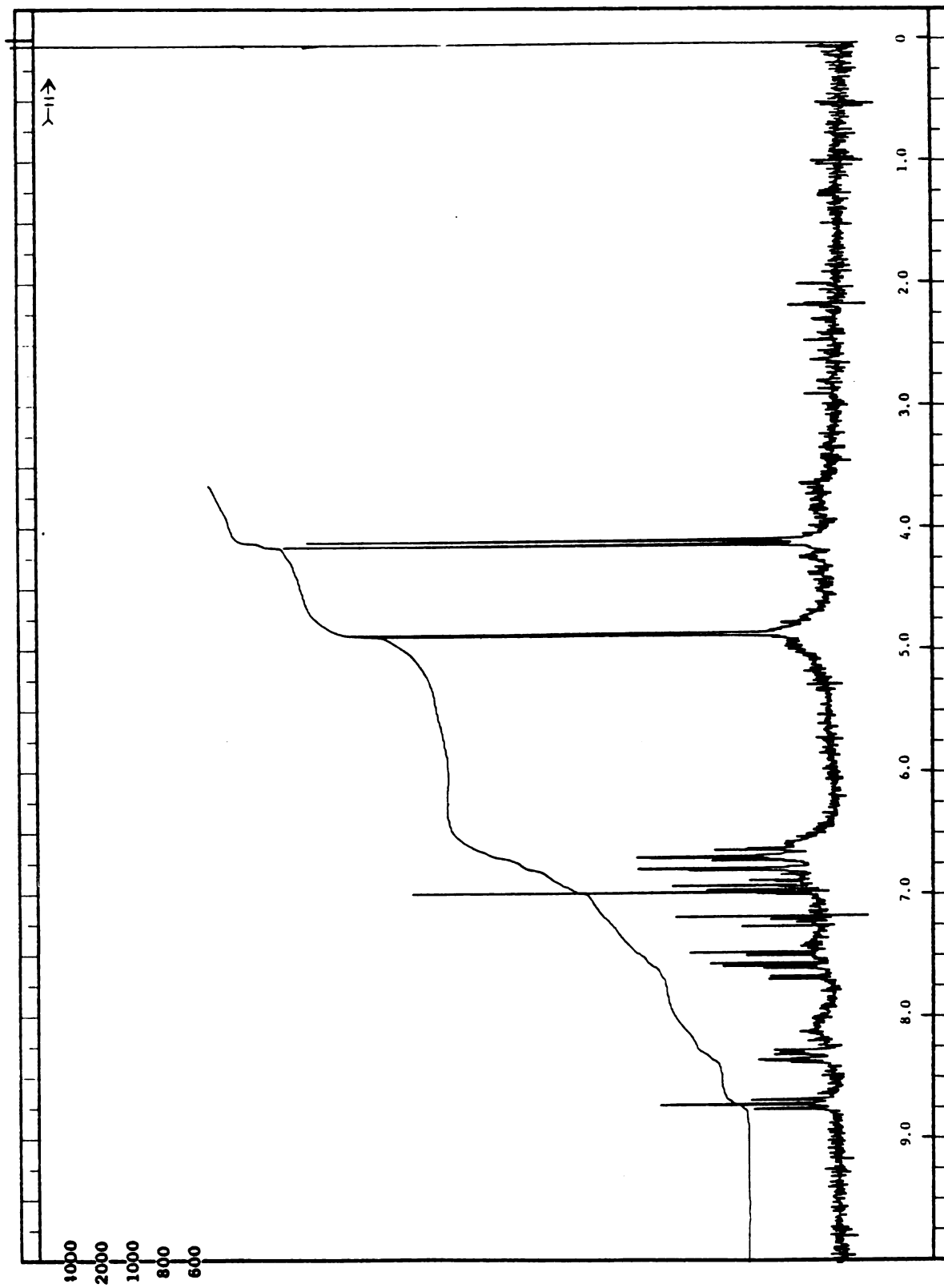


Figure 46. The nmr spectrum of the polar material from silica gel chromatography of the 344 reaction mixture.

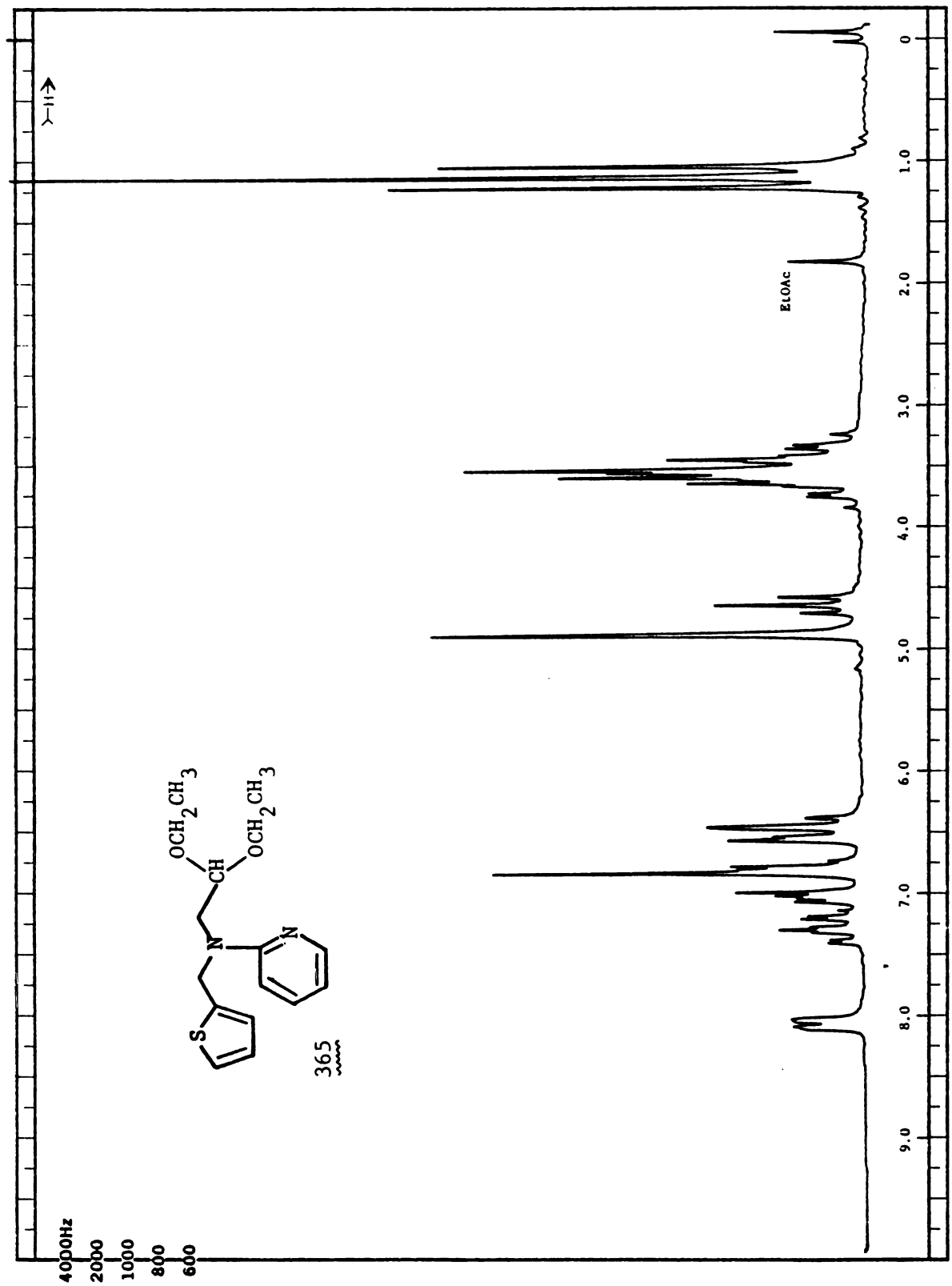
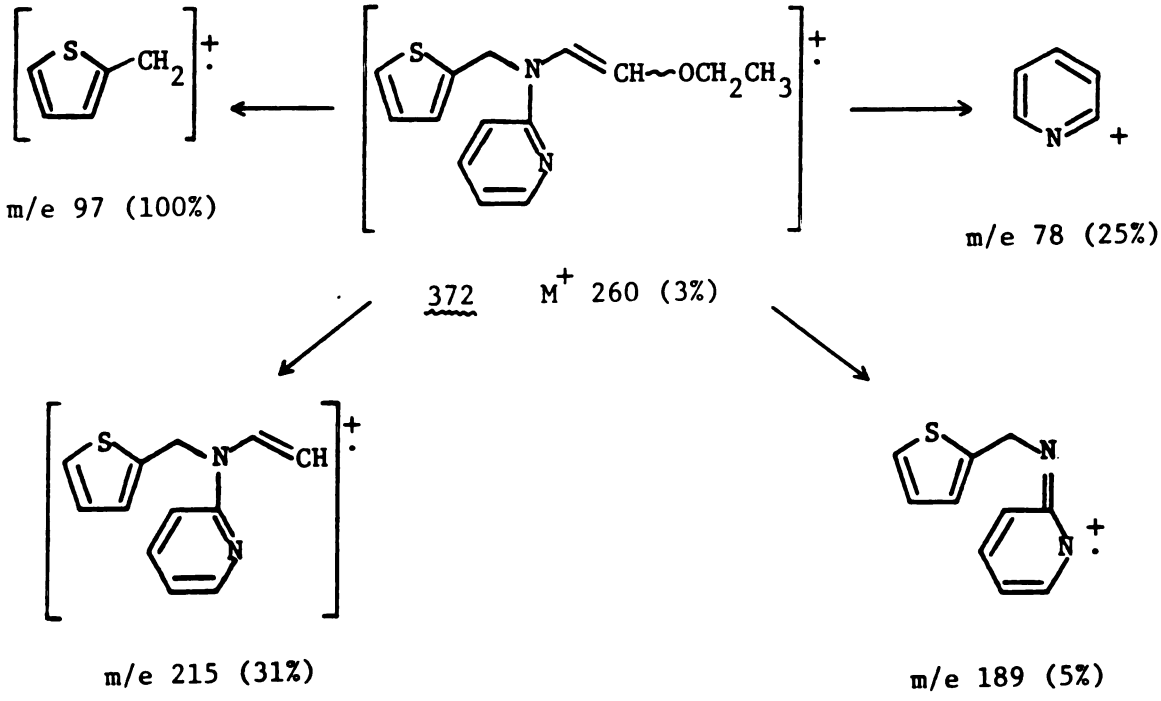


Figure 47. The nmr spectrum of the synthetic acetal 365.

The hydrolysis of the acetal 365 in 2N HCl at 80° gives a quantitative yield (as evidenced by gc analysis) of the aldehyde 337 after one hour. This hydrolysis reaction is much slower when 2% H_3PO_4 is used and the formation of intermediates and by-products are observed. The gc-eims analysis of the H_3PO_4 hydrolysis mixture after 4 hours at 80° showed the major product to be the secondary amine 258. In addition to the starting acetal 365 and the aldehyde 337, three other components were present in the reaction mixture. The gc-eims of the first component is shown in Figure 48; this product is identical to the N-formyl compound 348 described in Section V.B.2. The other two components with different gc retention times displayed the same gc-ei mass spectra (Figure 49) which suggests they are isomeric. The nominal mass (260 amu) and ei-induced fragmentation pattern of these products suggest their structures correspond to E- and Z-isomers of the vinyl ether 372. The fragmentation pattern of 372 may be assigned as follows.



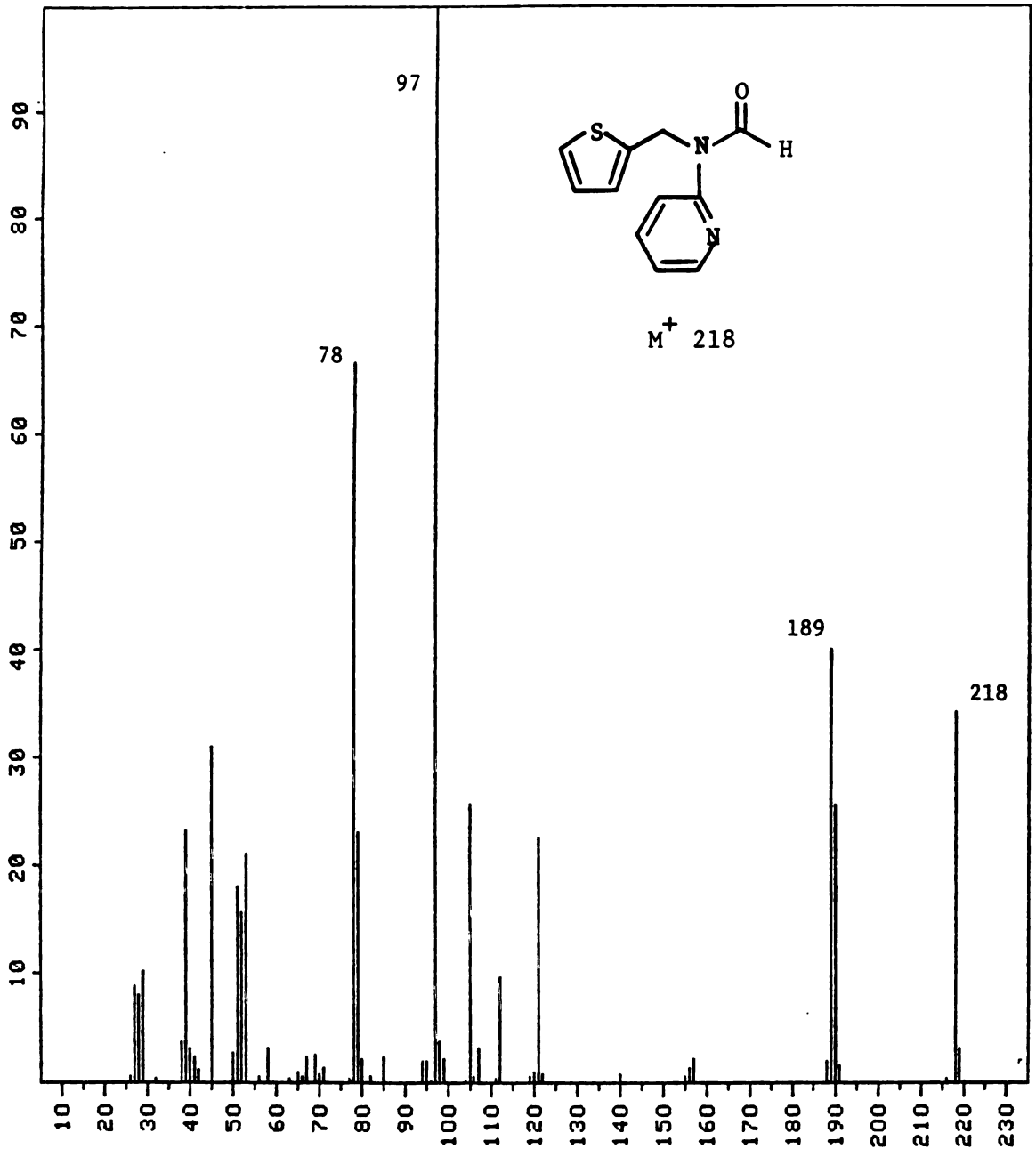


Figure 48. The gc-eims of 348 derived from the acid hydrolysis of the aldehyde 337.

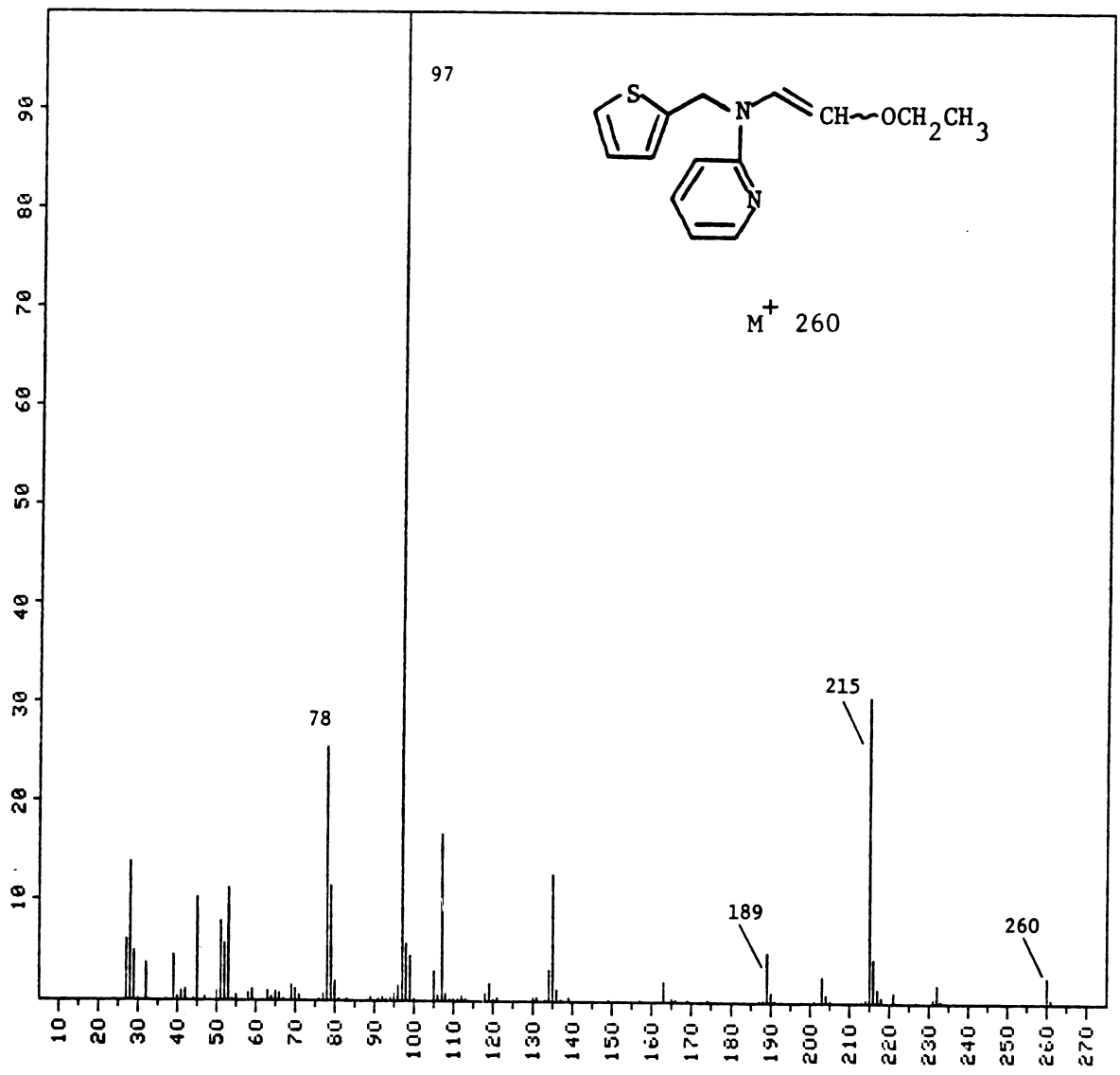
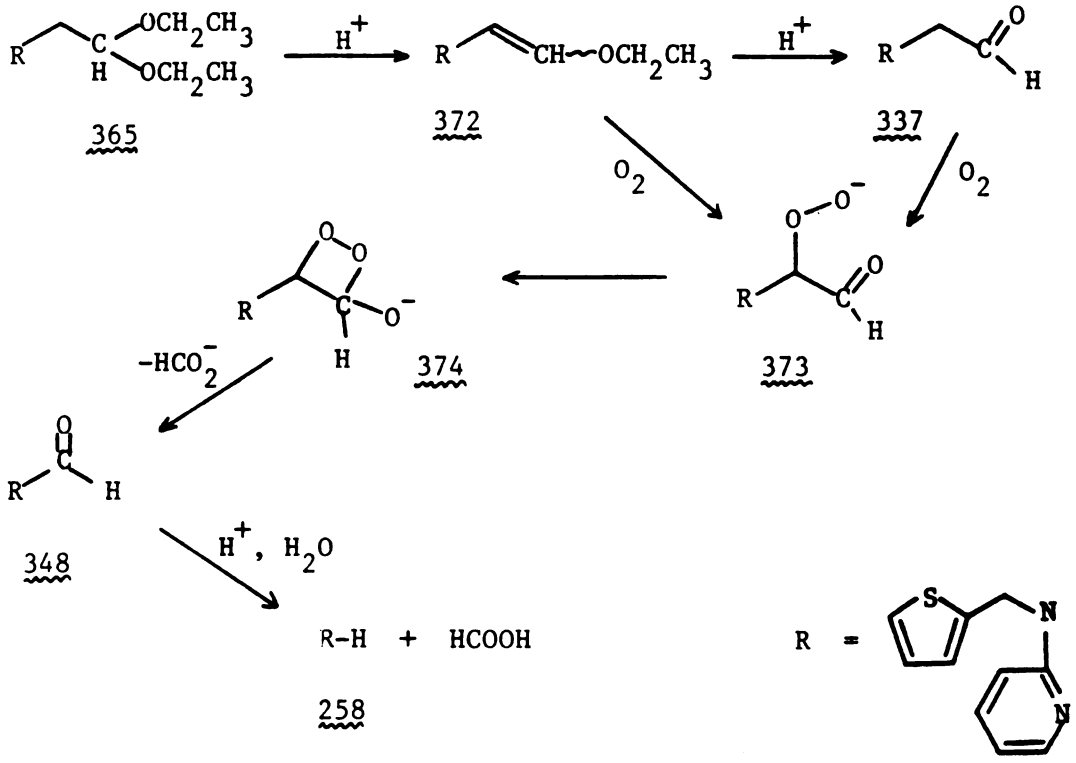


Figure 49. The gc-eims of 372 derived from the acid hydrolysis of 337.

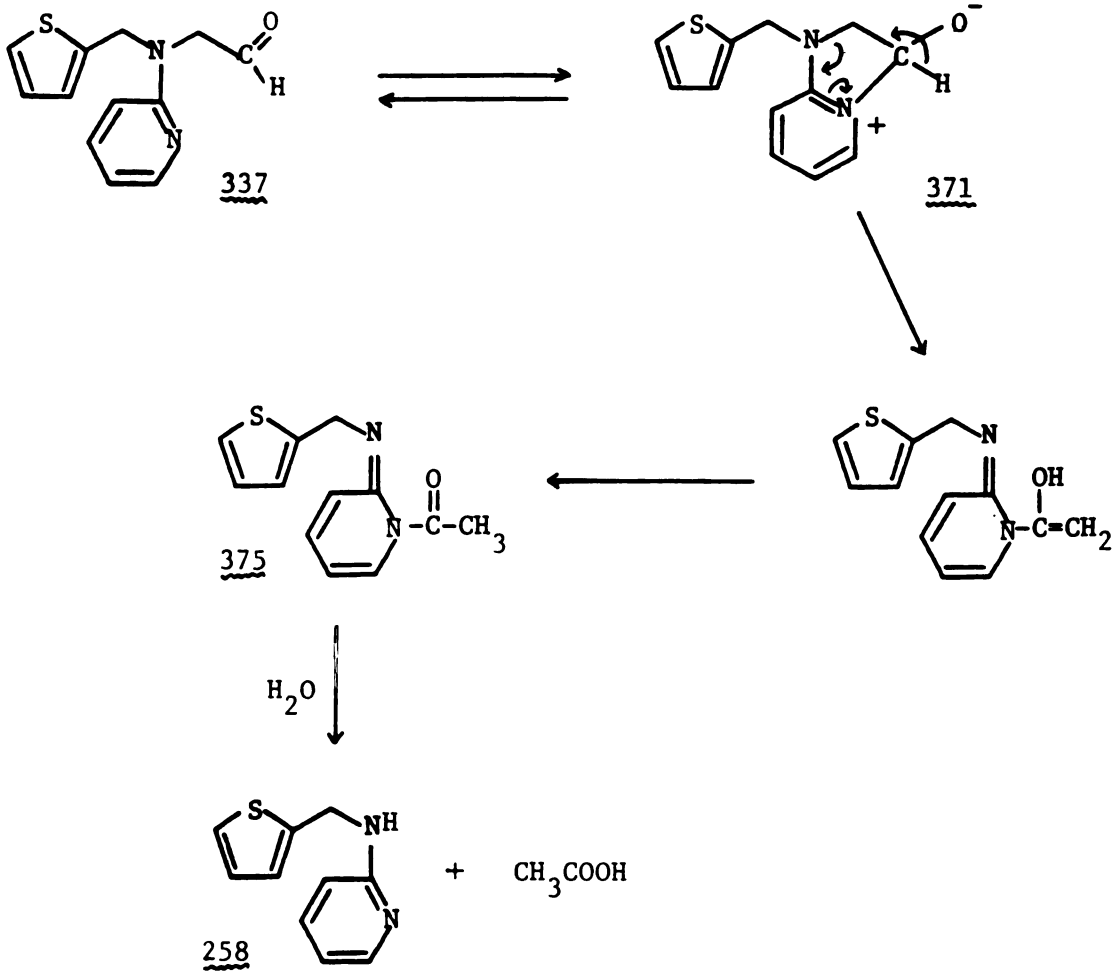
Detection of 372, 337, and 348 in the hydrolysis of 365 allows us to postulate the following mechanism for the formation of the N-formyl product.



In this scheme, both the vinyl ether 372 and the aldehyde 337 may react with molecular oxygen to form the N-formyl product 348 via the intermediates 373 and 374. This mechanism is supported by the observation that the addition of hydroquinone to or the exclusion of oxygen from the acetal hydrolysis reaction eliminates the formation of 348. The subsequent acid catalyzed hydrolysis of 348 may lead to the observed major product 258 and formic acid.

The neat aldehyde 337 has been observed to decompose in air to the secondary amine 258 (major product) and the N-formyl compound 348 (minor). A CH_2Cl_2 solution of the aldehyde 337 exposed to air shows (by gc analysis)

the gradual formation of the secondary amine 258 and disappearance of 337. After 96 hours, the solution was comprised mainly (> 80%) of the secondary amine. Likewise, a CH_2Cl_2 solution of 337 under nitrogen showed the gradual formation of 258. In this case 258 was formed more slowly. After 4 days, a 1:1 mixture of 258 and 337 was observed. Under the conditions of these studies, one would not expect the secondary amine 258 to be formed *via* a hydrolytic pathway as proposed above. One could propose the following alternative mechanism involving the intermediacy of the N-acetyl compound 375.

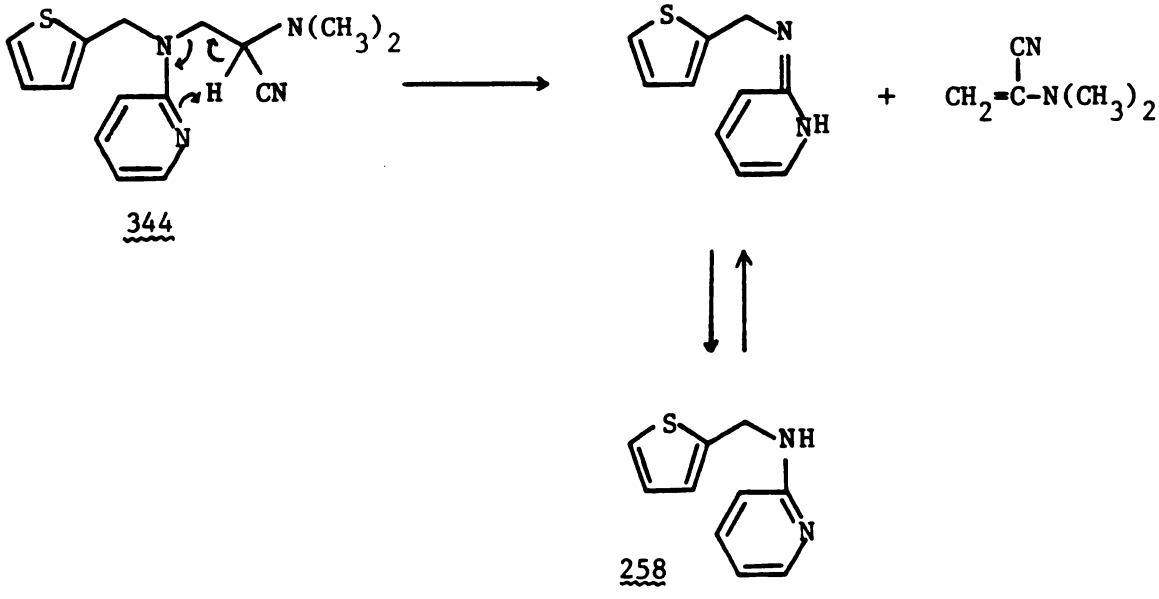


The N-acetyl intermediate which would result from the rearrangement of the zwitterionic tautomer of 337 is hydrolyzed to give 258 and acetic

acid. Consistent with this mechanism is the presence of a strong signal (singlet) at 2.15 ppm (CH_3COOH) in the nmr spectra of the residues of both CH_2Cl_2 solutions (air and nitrogen after 4 days) discussed above. This result clearly indicates the presence of acetic acid in these solutions. The possible formation of the N-acetyl derivative 375 may be of toxicological significance because this product may prove to be a potentially toxic acylating agent.

3. CHEMISTRY OF N-(2-PYRIDYL)-N-(2-THIENYLMETHYL)-N',N'-DIMETHYL-1-CYANO-1, 2-ETHANEDIAMINE (344)

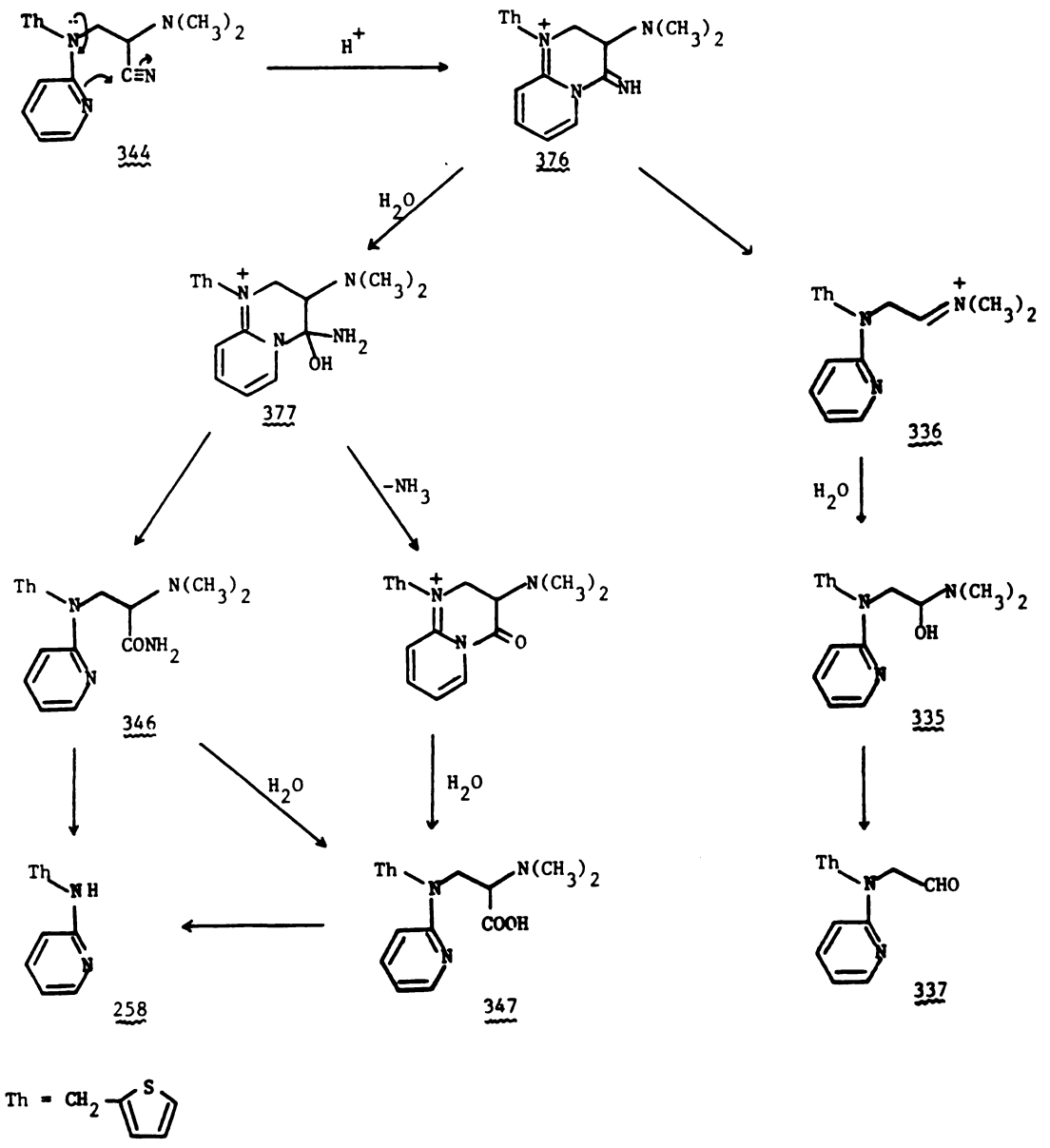
In Section V.B.1., we alluded to the chemical instability of the cyanoamine 344 as a possible reason for our inability to isolate 344 from microsomal incubations of methapyrilene with NaCN. Our studies have shown that 344 is unstable and decomposes to several products under incubation conditions (37° for 1 hr at pH 7.4) and workup conditions (CH_2Cl_2 extraction of the alkaline microsomal mixture). The tlc charac-



teristics of one of these decomposition products is identical to that of synthetic 258. When a sample of 344 was exposed to air for three weeks, the major product isolated by tlc of the exposed sample was identified as 258 by gc-eims analysis. In order to rationalize the formation of 258 as a decomposition product of 344, the above mechanism in which 344 undergoes a retro-Michael reaction is suggested.

Another polar product resulting from the decomposition of 344 in air was isolated by tlc. The cims of the isolated material reveals a protonated molecular ion at MH^+ 305. The nominal mass (304 amu) and the nmr spectrum (Figure 50) of this compound are consistent with the amide structure 346. The mechanism depicted in Scheme 7 attempts to rationalize the formation of 346. This mechanism postulates the intermediacy of the intramolecularly stabilized imine species 376 which can subsequently undergo hydrolysis to the carbinolamine 377. Compound 377 can then cleave to the observed amide product 346. The carbinolamine may also lose NH_3 and undergo hydrolysis to the acid product 347. This amino acid may also be formed from the direct hydrolysis of 346. Both the amide 346 and the amino acid 347 may undergo the retro-Michael reaction described for 344 to give the observed secondary amine decomposition product 258. It should be noted that neither 346 nor 347 have been detected in methapyrilene metabolic incubations.

The gc-eims analysis of the basic material isolated from the treatment of synthetic 344 with an aqueous solution of methanolic HCl revealed the presence of five products. The gc-ei mass spectrum of the first component was identical to the spectrum of authentic 258. The gc-eims of the component with the longest gc retention time is depicted in Figure 51. The mass fragmentation pattern and the high resolution eims analysis


Th
N(CH3)2
C≡N
H+
NH
344
376
H2O
NH2
OH
377
-NH3
336
CONH2
346
O
335
CHO
337
COOH
347
NH
258
Th = CH2 S

Scheme 7

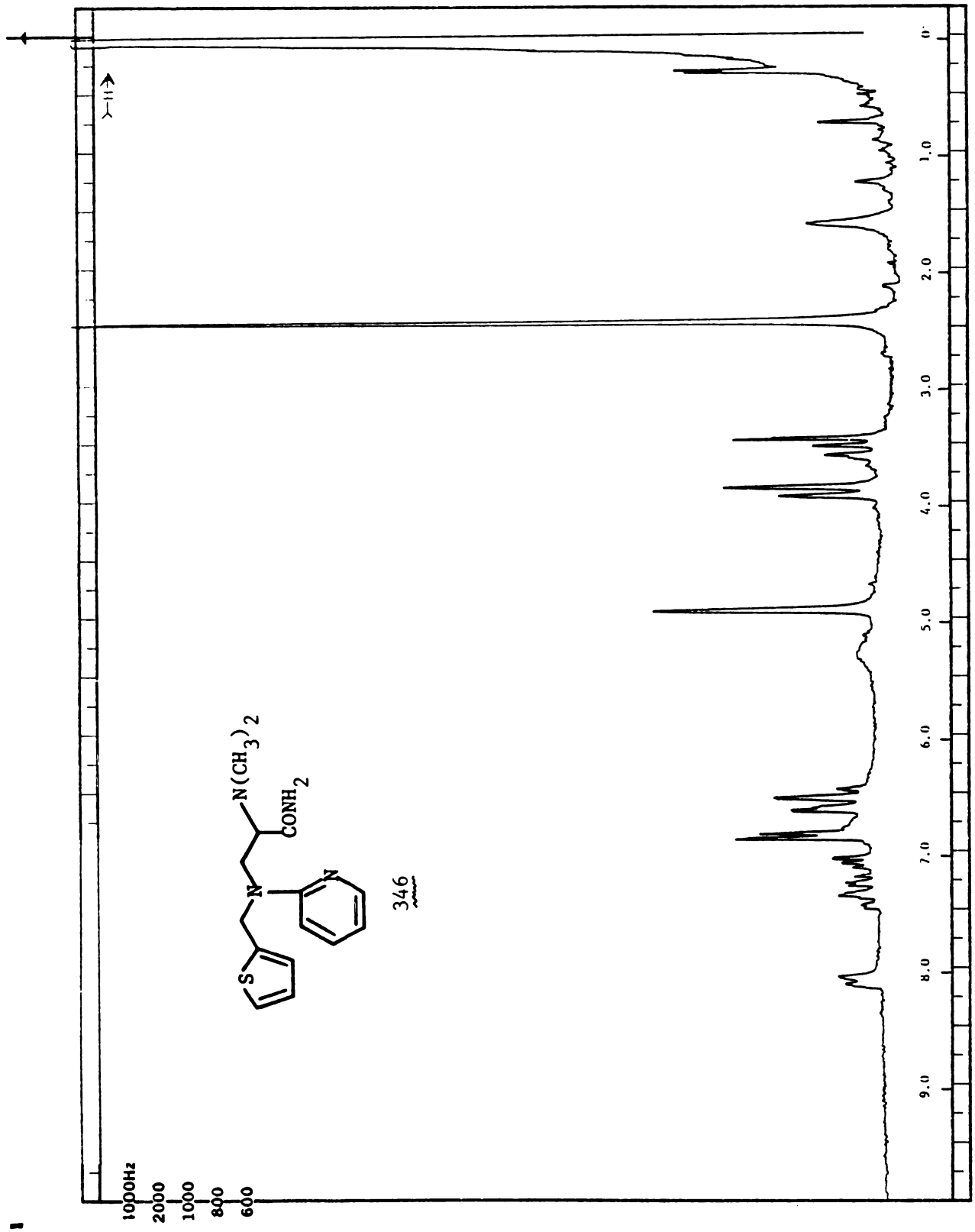


Figure 50. The nmr spectrum of 346 from the decompositon of 344 in air.

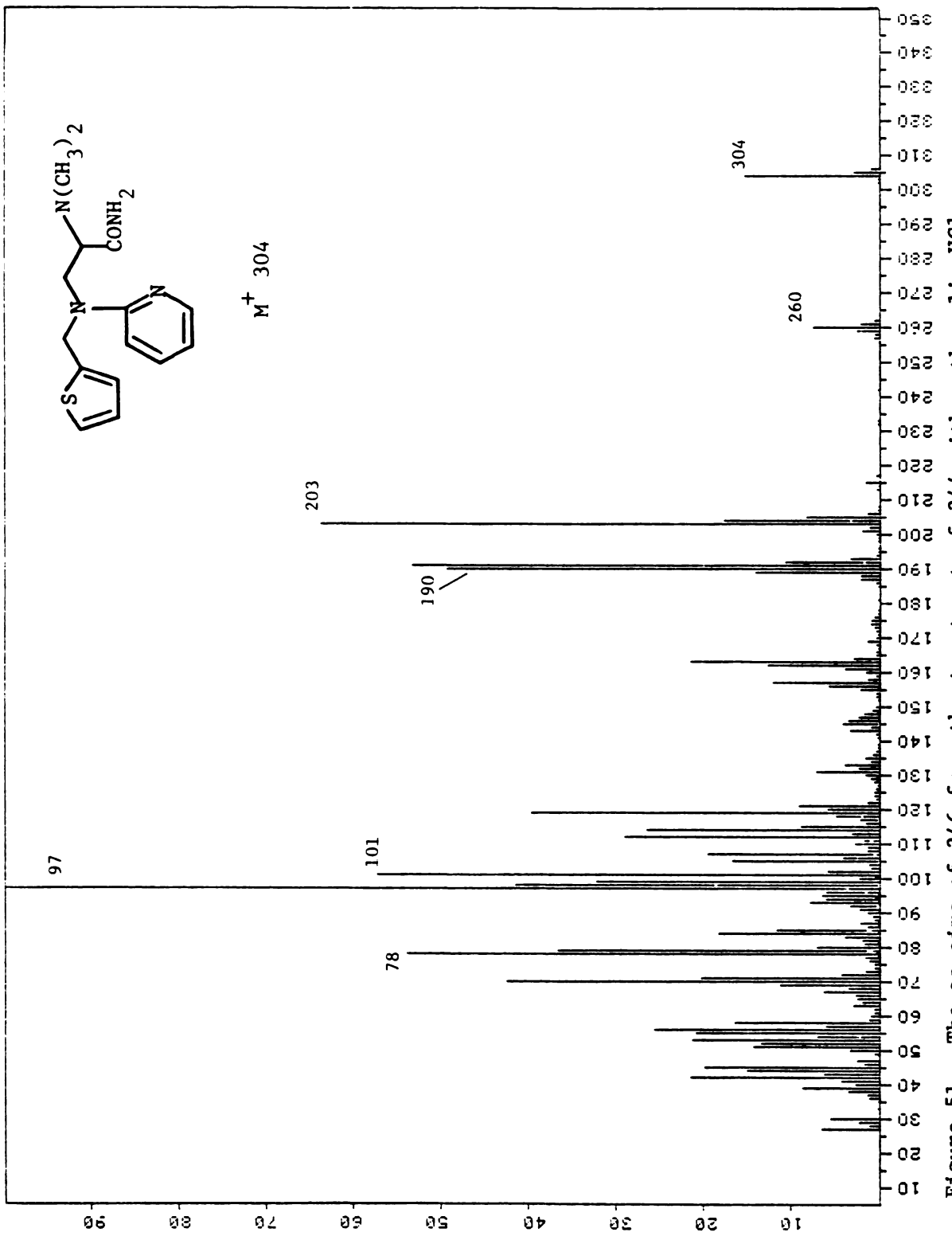


Figure 51. The gc-eims of 346 from the treatment of 344 with methanolic HCl.

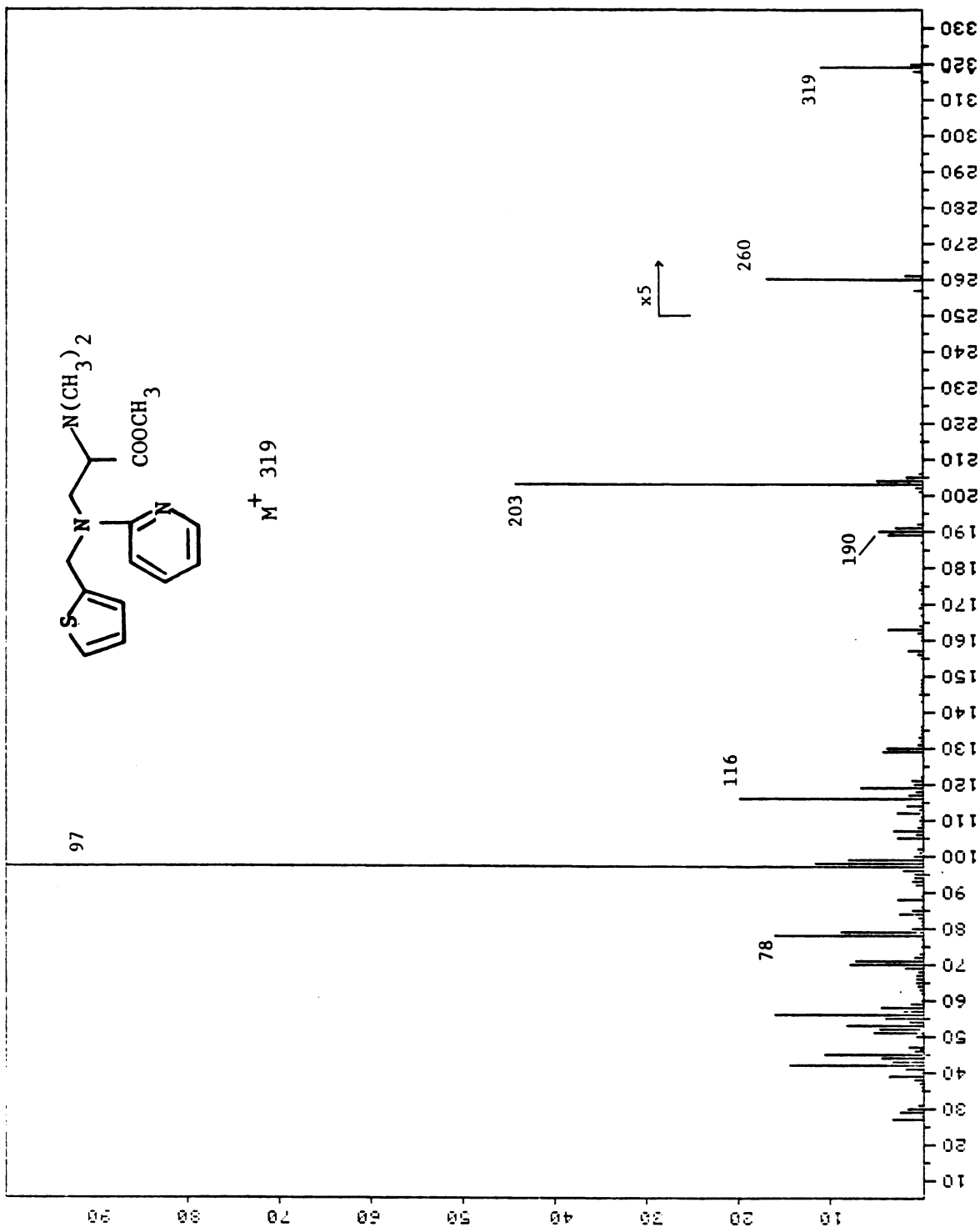
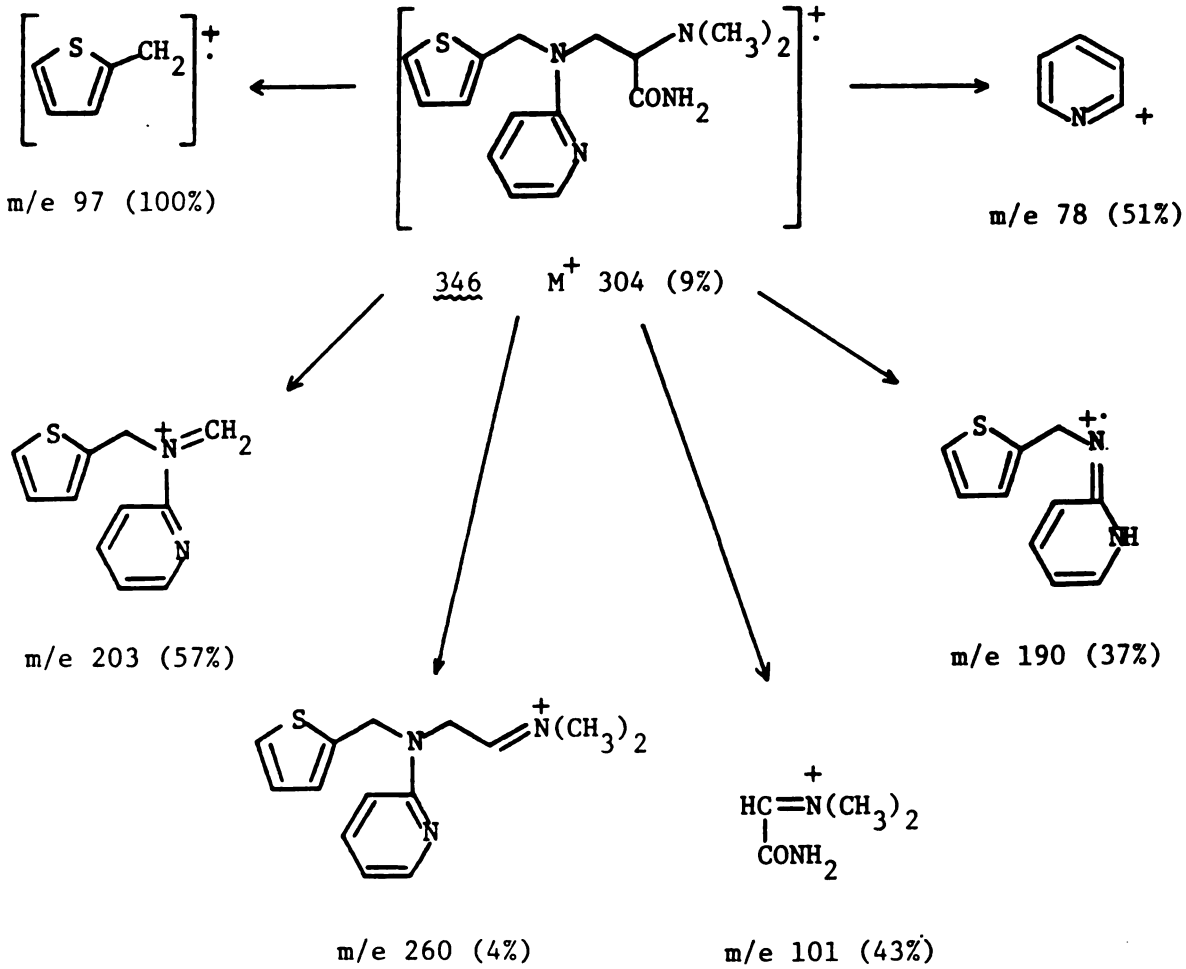
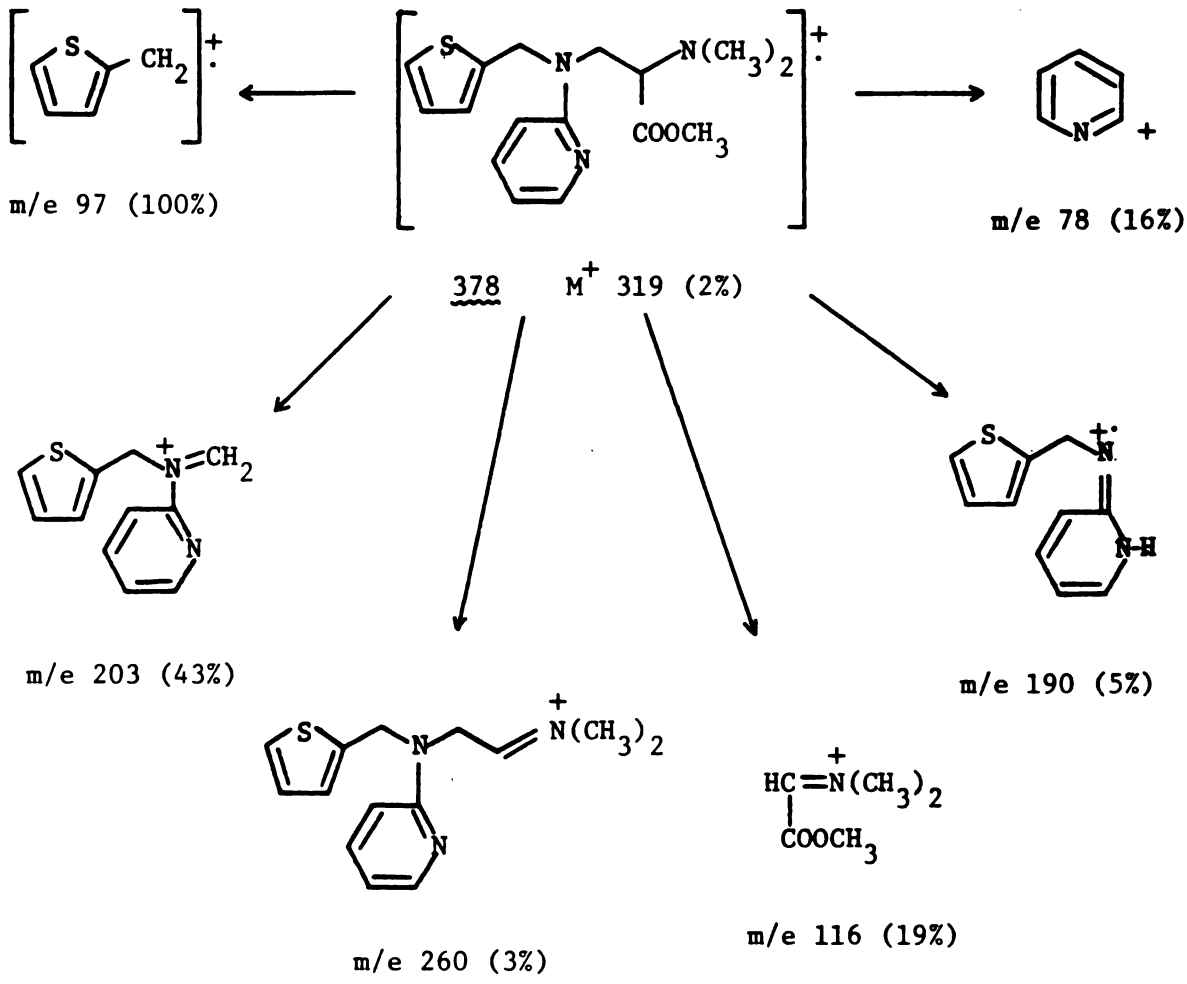


Figure 52. The gc-ems of 378 from the treatment of 344 with methanolic HCl.

(Calcd for $C_{15}H_{20}N_4OS$: M^+ 304. 1354; Found: M^+ 304.1330) of this component are consistent with the amide structure 346. The origins of the principal ions of 346 are rationalized as follows.



In addition to 258 and 346, another large component with a molecular ion at M^+ 319 was detected. The nominal mass (319 amu) and ei-induced fragmentation pattern (Figure 52) of this compound are consistent with the methyl ester structure 378. This methyl ester would result from the methanolysis of the amide 346 or acid 347. The fragment ion assignments for 378 are as follows:



The gc-eims of the last two components identified these compounds as the aldehyde 337 and the N-formyl compound 348. If these two compounds arise directly from the decomposition of the cyano compound 344, the formation of these products would be hard to rationalize. A possible mechanism shown above in Scheme 7 invokes the intermediary of the cyclic imine intermediate 376 which may rearrange to the iminium species 336. Hydrolysis of 336 and subsequent cleavage of the resultant carbinolamine 335 would give rise to the observed aldehyde 337. Formation of the N-formyl product 348 might result from the oxidation of 337 as described in Section V.C.2.

The chemistry of the α-cyanoamine 344 is obviously complex. Thus our failure to find definitive evidence for its presence in microsomal incubations of methapyrilene with sodium cyanide should be viewed in light of this fact. In summary, our studies on the *in vitro* metabolism of methapyrilene provides preliminary evidence as to the complexity of the methapyrilene system. We have identified, at least tentatively, several metabolic products of this compound but large amounts of metabolites remain unaccounted for. In view of the chemical complexity of the system, it remains to be seen which species actually may be responsible for the observed toxicity of methapyrilene.

CHAPTER VI

EXPERIMENTAL

A. CHEMISTRY

All reactions were carried out under a nitrogen atmosphere. Solvents for reactions were dried and distilled prior to use. Proton nmr spectra were recorded on a Perkin-Elmer R12B (60 MHz) or a Varian FT-80 (80 MHz) instrument; all spectra were recorded in $CDCl_3$. Chemical shifts are reported in parts per million (ppm) relative to TMS as an internal standard; s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. Infrared spectra were recorded on a Perkin Elmer 337 grating IR spectrophotometer. Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory of the University of California, Berkeley.

Analytical gas chromatography (gc) was performed on a Varian Aerograph Series 2100 gas chromatograph with an H_2 flame ionization detector utilizing an U-shape 2 m x 2 mm i.d. glass column packed with 3% OV-25 on acid washed DMCS Chromosorb W (mesh 100/120). High pressure liquid chromatography (hplc) was carried out using an Altex Model 110 pump equipped with a Hitachi Model 100-10 spectrophotometer as a detector (mainly at 240 nm). The hplc columns used were either Lichrosorb reverse phase C-8 or Lichrosorb SI-60 silica gel purchased from Altex Scientific Inc., Berkeley, California. Chemical ionization mass spectra (cims) were obtained on an AEI MS-9 instrument modified for chemical ionization. Gas chromatography electron impact mass spectrometry (gc-eims) was performed either on an Infotronics 2400 gas chromatograph interfaced to an AEI

MS-12 mass spectrometer or on a 10 m x 0.325 mm i.d. glass capillary column coated with either 0.1% SE-52 or 0.1% OV-1 coupled directly to a Hitachi M-52 GC-MS. The multichannel averaged narrow scans were performed on a 10 m x 0.325 mm i.d. glass capillary column coated with 0.1% SE-52 coupled directly to a Hitachi M-52 GC-MS. High resolution electron impact mass spectra and high resolution gc-ei mass spectra were obtained from the Bio-organic, Biomedical Mass Spectrometry Resource, Space Sciences Laboratory, University of California, Berkeley. Scintillation counting was performed on a Packard Tri-Carb Model 3375 liquid scintillation spectrophotometer using Aquasol or Aquasol-2 (New England Nuclear) as the scintillation cocktail. Radioscans of the TLC plates were carried out on a Varian Aerograph Radio Scanner Model LB 2722. Analytical (250μ) and preparative (2000 μ) silica gel TLC plates were purchased from Analtech.

1. 1-BENZYLPYRROLIDINE

a. 1-Benzylpyrrolidine-2,2-d_2 (1-2,2-d_2)

To a stirred solution of $LiAlD_4$ (0.01 mol) in 15 ml freshly distilled ether at 0° was added dropwise 1-benzyl-2-pyrrolidinone (220, 0.01 mol, Aldrich) in ether. The reaction mixture was heated under reflux for 8 hours and stirred overnight at room temperature. After cooling to 0°, 0.4 ml H_2O, 0.4 ml 15% NaOH, and 2 ml H_2O were added sequentially and the resulting mixture was stirred for 30 min. The white precipitate was filtered and washed with ether. The combined ether washes and filtrate were dried over Na_2SO_4 and distilled (bulb to bulb) to give a clear liquid in 40% yield: bp 50° (0.15 mm); NMR ($CDCl_3$) δ 1.6-1.9 (m, 4H), 2.3-2.7 (m, 2H), 3.5 (s, 2H), 7-7.5 (m, 5H); EIMS 163 (M^+, 39%), 91 (100%), 86

(69%), 72 (35%); (m/e 86/Σm/e 84-86) x 100 = 97% deuteration ($d_2/d_1/d_o$, 97:1:1).

b. 1-Benzylpyrrolidine-3,3,4,4,-d_4 (1-3,3,4,4-d_4)

Benzylamine (0.1 mol) was added to a stirred solution of succinic anhydride (0.1 mol) in toluene. A white precipitate appeared after a few minutes. The solution was heated under reflux for 4 days with a Dean-Stark trap. The clear solution upon cooling yielded a white precipitate of N-benzylsuccinamic acid melting at 138° (lit.[229] mp 138°). The precipitate was filtered and the filtrate evaporated to dryness leaving a yellow residue which was crystallized from hot $CHCl_3$/hexane to give a white solid in 40% yield: mp 99-102° (lit.[230] mp 103°).

The 1-benzylsuccinimide (0.03 mol) was dissolved in anhydrous pyridine and 20 ml 99.8% D_2O (1 mol) was added and the solution heated under reflux. The exchange reaction was followed by nmr with fresh D_2O added repeatedly. After 30 days the reaction mixture was evaporated to dryness to provide a yellow solid which was recrystallized repeatedly from hot $CHCl_3$/hexane to provide a product melting sharply at 103°; CIMS MH^+ 194.

To a stirred solution of $LiAlH_4$ (6 mmol) in dry THF at 0° was added slowly 100% H_2SO_4 (3 mmol). To this solution 1-benzylsuccinimide-d_4 (1.5 mmol) was then added slowly with stirring at 0° and the mixture was heated under reflux overnight. To the cool reaction mixture was added 0.25 ml H_2O, 0.25 ml 15% NaOH, and 0.75 ml H_2O. The solution was stirred at 0° for 15 min. The resulting precipitate was filtered and washed with THF and the combined THF washes and filtrate were dried over Na_2SO_4 and evaporated to a clear oil. The oil was distilled to yield 50 mg of a clear liquid: bp 70° (0.25 mm); EIMS 165 (M^+, 38%), 91 (100%), 88 (57%), 74

(39%); (m/e 165/Σm/e 161-165) x 100 = 90% deuteration ($d_4/d_3/d_2/d_1/d_o$, 89:3:6:2:1).

c. 1-Benzylpyrrolidine-α,α-d_2 (1-α,α-d_2)

To a stirred solution of $LiAlD_4$ (12.5 mmol) in 25 ml anhydrous ethyl ether was added 1-benzoylpyrrolidine (227, 10 mmol) at 0°. The solution was allowed to warm to room temperature, heated under reflux for 4 hours, and then stirred at room temperature overnight. The reaction mixture was cooled to 0° and 0.5 ml H_2O, 0.5 ml 15% NaOH, and 1.5 ml H_2O were slowly added sequentially. The resulting precipitate was filtered and washed with ether and the combined washes and filtrate were dried over Na_2SO_4 and rotary-evaporated to a clear oil. The oil was distilled to give a clear liquid in 81% yield: bp 70° (0.25 mm); NMR ($CDCl_3$) δ 1.5-2.0 (m, 4H), 2.2-2.7 (m, 4H), 7.0-7.5 (s, 5H); EIMS 163 (M^+, 36%), 93 (100%), 86 (54%), 70 (40%); (m/e 86/ m/e 85-86) x 100 = 93% deuteration ($d_2/d_1/d_o$, 93:3:4).

d. 1-Benzylpyrrolidine-α,α-$^3H_2$ (1-α,α-$^3H_2$)

To a stirred solution of $LiAlH_4$ (0.2 g, 0.5 mmol) in 15 ml ether at 0° was added 1-benzoylpyrrolidine (0.875 g, 5 mmol) in 5 ml ether. The solution was stirred at room temperature for 2 hours followed by the addition of $LiAl^3H_4$ (0.8 mg, 240 mCi/mmol, New England Nuclear) at 0°. The solution was stirred with heating for 2 hours and allowed to stir overnight at room temperature. More $LiAlH_4$ (0.18 g, 4.5 mmol) was added to the reaction mixture and the solution was allowed to stir for 3 hours at room temperature. After cooling, 0.2 ml H_2O, 0.2 ml 15% NaOH, and 0.6 ml H_2O were added sequentially and the resulting white precipitate was fil-

tered and washed with ether. The combined ether extracts and washes were dried over Na_2SO_4 and rotary-evaporated to a clear oil which was distilled (bulb to bulb) to give the product in 36% yield. The specific activity (scintillation counting) was 1.97 mCi/mmol.

e. 1-Benzyl-2-cyanopyrrolidine (223)

To a solution of mercuric acetate (0.2 mol) dissolved in 150 ml of 5% aqueous CH_3COOH was added 0.05 mol of 1-benzylpyrrolidine (Aldirch). The reaction mixture was stirred at 50° for 7 hours and the resulting mercurous acetate precipitate was filtered and washed with 5% aqueous CH_3COOH. The combined washes and filtrate were saturated with H_2S at 0° and the resulting black precipitate filtered. The pH of the filtrate was adjusted to 4-5 with saturated $KHCO_3$ solution. NaCN (0.25 mol) was then added and the reaction mixture stirred overnight at room temperature. The solution was then made alkaline (pH 8) with saturated $KHCO_3$ solution and extracted with ether. The ether extracts were dried over Na_2SO_4 and rotary-evaporated to give a gold colored oil. The oil was column chromatographed on 53 grams of silica gel (40-140 mesh) with acetone as the developing solvent. The desired fraction (by gc) was dried over Na_2SO_4 and evaporated to a gold colored oil which upon distillation (bulb to bulb) gave a colorless liquid in 27% yield: bp 75-80° (0.25 mm); NMR ($CDCl_3$) δ 1.6-2.3 (m, 4H), 2.3-3.2 (m, 2H), 3.5-3.9 (m, 3H), 7.1-8.5 (m, 5H); IR (neat) 2240 cm^{-1} (C≡N); EIMS 186 (M^+, 4%), 159 (28%), 91 (100%).

Anal. Calcd for $C_{12}H_{14}N_2$: C, 77.42; H, 7.53; N, 15.05. Found: C, 77.25; H, 7.48; N, 14.90.

f. 1-(4-Acetoxybenzoyl)pyrrolidine (249)

Pyrrolidine (80 mmol) and 4-acetoxybenzoylchloride (250, 40 mmol) were stirred overnight in 25 ml $CHCl_3$ at room temperature. The reaction mixture was washed twice with 0.1N HCl, dried over K_2CO_3, and evaporated to a yellow oil. The oil was distilled (bulb to bulb) at 155° (0.025 mm) to give a clear oil. The oil later crystallized to a white solid which was recrystallized from benzene/hexane: mp 90-95°; NMR ($CDCl_3$) δ 1.9 (m, 4H), 2.3 (s, 3H), 3.4-3.7 (m, 4H), 7.0-7.6 (m, 4H); EIMS 233 (M^+, 49%), 191 (43%), 121 (100%).

Anal. Calcd for $C_{13}H_{15}NO_3$: C, 66.95; H, 6.44; N, 6.01. Found: C, 66.89; H, 6.45; N, 5.87.

g. 1-(4-Hydroxybenzyl)pyrrolidine (248)

To a stirred solution of $LiAlH_4$ (15 mmol) in dry THF at 0° was added slowly 100% H_2SO_4 (7.5 mmol). The amide 249 (5 mmmol) was then slowly added to the stirred AlH_3 solution at 0°. The reaction mixture was stirred under reflux for 24 hours and at room temperature for an additional 18 hours. The reaction mixture was treated with 0.6 ml H_2O and acidified with 1N HCl. The resulting precipitate was filtered and the filtrate was evaporated to a gel. The gel was dissolved in H_2O and the pH of the solution was adjusted to 9 with 0.1N NaOH. The aqueous solution was extracted with $CHCl_3$ and the $CHCl_3$ extracts were dried over K_2CO_3 and evaporated to a yellow oil which later crystallized. The crystals were sublimed at 80° (0.01 mm) to give a white crystalline product: mp 107-110; NMR ($CDCl_3$) δ 1.75 (m, 4H), 2.5-2.7 (m, 4H), 3.5 (s, 2H), 6.6-7.3 (m, 4H); CIMS MH^+ 178.

Anal. Calcd for $C_{11}H_{15}NO$: C, 74.58; H, 8.47; N, 7.91. Found:

C, 74.49; H, 8.56; N, 7.88.

2. METHAPYRILENE

a. 2-(2-Thienylmethylamino)pyridine (258)

This compound was synthesized according to ref. 227 (45% yield): m.p. 77-79° (lit.[227] 78-80°): NMR ($CDCl_3$) δ 4.72 (s, 2H), 6.35-7.43 (m, 6H, aromatic), 8.13 (d, 1H, aromatic).

b. N-(2-pyridyl)aminoacetaldehyde diethyl acetal (366)

The compound was prepared according to ref. 228 (47% yield): NMR ($CDCl_3$) δ 1.20 (t, 6H, 2 x CH_3), 3.35-3.84 (m, 6H, 3 x CH_2), 4.46 (t, 1H, $CH_2\underline{CH}(OR)_2$), 4.84 (br, 1H, N-H), 6.34-6.61 (m, 2H, aromatic), 7.36 (t, 1H, aromatic), 8.07 (d, 1H, aromatic).

c. 2-Chloromethylthiophene (361)

This compound was prepared in 47% yield according to ref. 225. Care was taken not to heat the oil bath over 120° during distillation of the product (b.p. 78-80°, 20 mm Hg): NMR ($CDCl_3$) δ 4.75 (s, 2H, CH_2), 6.85-7.30 (m, 3H, aromatic).

d. N-(2-pyridyl)-N-(2-thienylmethyl)aminoacetaldehyde diethyl acetal (365)

Method A

$NaNH_2$ (0.98 g, 25 mmol) was added to a mixture of the secondary amine 258 (3.8 g, 20 mmol) and bromoacetaldehyde diethyl acetal (3.94 g, 20 mmol) in 30 ml anhydrous xylene. The reaction mixture was stirred under reflux for 24 hours. At this time, additional $NaNH_2$ (1/4 equi-

valent) and bromoacetaldehyde diethyl acetal (1/2 equivalent) were added. The reaction mixture was kept under reflux while this procedure was repeated at 48 and 72 hours. After 96 hours, the reaction mixture was cooled to room temperature, treated with H_2O, and the organic layer was separated. The aqueous layer was extracted twice with CH_2Cl_2. The organic material was combined, dried over K_2CO_3, and evaporated (in vacuo) to a dark-brown oil. The oil was column chromatographed on silica gel (40-140 mesh) with CH_2Cl_2 (ethyl acetate gradient) as the developing solvent. Distillation of the residue obtained from the desired fractions (2-5% EtOAc/CH_2Cl_2, analyzed by gc) gave a colorless oil in 77% yield (bp 120-125°, 0.25 mm Hg): NMR ($CDCl_3$, Figure 47) δ 1.18 (t, 6H, 2 x CH_3), 3.40-3.84 (m, 6H, 3 x CH_2), 4.72 (t, 1H, $CH_2\underline{CH}OR_2$), 4.96 (s, 2H, thienylmethyl CH_2), 6.47-7.60 (m, 6H, aromatic), 8.17 (d, 1H, aromatic); GC-EIMS 306 (M^+, 18%), 261 (9%), 203 (9%), 189 (6%), 103 (89%), 97(100%), 78 (35%), 75 (78%).

Anal. Calcd for $C_{16}H_{22}N_2O_2$: C, 62.75; H, 7.19; N, 9.15. Found: C, 62.60; H, 7.29; N, 8.99.

Method B

$NaNH_2$ (2.23 g, 51.1 mmol) was added to a mixture of the acetal 366 (20 g, 47.6 mmol) and 2-chloromethylthiophene (6.31 g, 47.6 mmol) in 200 ml anhydrous toluene and the reaction mixture was then stirred under reflux. $NaNH_2$ (1.1g, 28.2 mmol) and 2-chloromethylthiophene (3.1 g, 23.4 mmol) were added to the reaction mixture after 45 hours. After stirring under reflux for 68 hours, another 14.1 mmol of $NaNH_2$ and 11.7 mmol of 2-chloromethylthiophene was added. After 80 hours, the reaction mixture was cooled, quenched with H_2O, and worked up as in Method A. A pure (by gc) yellow oil with a strong sulphur odor was

obtained in 96% yield after distillation. This oil was repeatedly partitioned into acid and base (treated with 2 N HCl, extracted with $CHCl_2$, aqueous fraction made basic with Na_2CO_3, and re-extracted with CH_2Cl_2) and then distilled (bp 135-140°, 0.15 mm) to give a clear, odorless oil (pure by gc). NMR spectrum of this product was identical to that of the product obtained by Method A.

e. N-(2-pyridyl)-N-(2-thienylmethyl)aminoacetaldehyde (337)

Crystals of hydroquinone were added to a solution of the acetal 365 (3.7 g, 12 mmol) in 50 ml 2N HCl. The reaction mixture was then heated at 80° for 1 hour. The initially clear solution turned milky after ca. 30 min. The reaction mixture was cooled to 0° and extracted with 50 ml CH_2Cl_2. $Na_2CO_{3(s)}$ was slowly added to the aqueous layer at 0° until the pH of the solution was 10. The basic solution was extracted (5 x 50 ml) with CH_2Cl_2 and the combined CH_2Cl_2 extracts were dried over K_2CO_3 and evaporated to give the desired product as a yellow oil (100% yield). Distillation of the oil (>180°, 0.4 mm) gave only the secondary amine 258 and other unidentified products as analyzed by gc. Cation exchange chromatography of the oil (dissolved in HCl) on Dowex W-X4 (eluted with HCl gradient) gave rise to a mixture of products including compound 258. Attempts to obtain a solid derivative by treating the crude product in EtOH with 2,4-dinitrophenylhydrazine (in EtOH) failed. The crude product when treated with NH_2OH in H_2O, 10% NaOH, and EtOH, gave rise to a mixture of products including 258 as analyzed by gc-eims. Attempts to isolate a solid derivative by treating the crude product (in EtOH) with a saturated ethanolic solution of picric acid failed: NMR ($CDCl_3$, Figure 40) δ 4.18 (d, 2H, $C\underline{H}_2CHO$), 4.88 (s, 2H, thienylmethyl

CH_2), 6.62-7.60 (m, 6H, aromatic), 8.16 (m, 1H, aromatic), 9.47 (s, 1H, CHO); IR (neat) 1750 cm^{-1} (C=O); CIMS MH^+ 233; GC-EIMS (Figure 44) 232 (M^+, 7%), 203 (10%), 189 (23%), 97 (100%), 78 (24%).

Exact Mass (HR-EIMS). Calcd for $C_{12}H_{12}N_2OS$: M^+ 232.0668. Found: M^+ 232.0644.

f. N-(2-pyridyl)-N-(2-thienylmethyl)-N'-methyl-1,2-ethanediamine (327)

To a solution of the crude aldehyde 337 (0.57 g, 2.47 mmol) in 7 ml anhydrous MeOH was added 8.6 ml of a 5.4% methanolic solution of methylamine (14.8 mmol). The reaction mixture was cooled to 0°. Methanolic HCl (2N) was added to adjust the pH to 7, followed by the addition of $NaCNBH_3$ (0.32 g, 5.0 mmol). The reaction mixture was stirred for 24 hours at room temperature. The mixture was then treated with water, made basic with $K_2CO_{3(s)}$, and extracted with CH_2Cl_2. The CH_2Cl_2 extracts were dried over K_2CO_3 and evaporated to a brown oil (one peak by gc): NMR ($CDCl_3$, Figure 38) 2.42 (s, 3H, CH_3), 2.80 (t, 2H, CH_2), 3.64 (br t, 2H, CH_2), 4.91 (s, 2H, thienylmethyl CH_2), 6.47-7.41 (m, 6H, aromatic), 8.17 (br, s, 1H, aromatic).

Neutral alumina column chromatography of crude 327 with CH_2Cl_2 (MeOH gradient) as the developing solvent was attempted. The material eluting with 1% MeOH/CH_2Cl_2 showed one peak on gc analysis (retention time corresponding to the desired 327) and one spot on neutral alumina tlc (R_f = 0.33). The nmr spectrum (Figure 41) of the isolated product is consistent with the N-hydroxy compound 367. The treatment of the crude 367 with Zn dust in 50% CH_3COOH at room temperature for 3 days gave a product which showed one peak on gc analysis (retention time same as 327) and is consistent with a mixture of 327 and 367 on nmr analysis (Figure 42): NMR

($CDCl_3$, Figure 41) δ 2.78 (br m, 2H, CH_2), 3.62 (br s, 5H, CH_3 and $NCH_2CH_2N(OH)CH_3$), 4.89 (s, 2H, thienylmethyl CH_2), 6.52-7.45 (m, 6H, aromatic), 8.17 (br d, 1H, aromatic).

g. N-(2-pyridyl)-N-(2-thienylmethyl)-N'-cyanomethyl-N'-methyl-1,2-ethanediamine (341)

To a solution of the crude amine 327 (180 mg, 0.73 mmol) in 3.0 ml MeOH and 1.0 ml H_2O was added a solution of NaCN (73.5 mg, 1.5 mmol) in 2.0 ml H_2O followed by 0.6 ml of a 37.6% aqueous solution of formaldehyde (220 mg, 7.3 mmol). After stirring for one hour at room temperature, H_2O (30 ml) was added and the reaction mixture was extracted with CH_2Cl_2 (4 x 25 ml). The CH_2Cl_2 extracts were dried over K_2CO_3 and evaporated to an oil. The crude oil was then purified by preparative tlc (silica gel) with 1% CH_2CN/CH_2Cl_2 as the developing solvent to yield the product as a yellow oil: NMR ($CDCl_3$, Figure 19) δ 2.39 (s, 3H, CH_3), 2.67 (t, 2H, $CH_2N(CH_2CN)CH_3$), 4.86 (s, 2H, thienylmethyl CH_2), 6.49-7.42 (m, 6H, aromatic), 8.14 (br d, 1H, aromatic); EIMS (Figure 18) 286 (M^+, 3%), 203 (11%), 190 (8%), 97 (100%), 78 (14%).

Exact Mass (HR-EIMS). Calc'd for $C_{15}H_{18}N_4S$: M^+ 286.1252. Found: M^+ 286.1276.

h. N-(2-pyridyl)-N-(2-thienylmethyl)-N',N'-dimethyl-1-cyano-1,2-ethanediamine (344)

The crude aldehyde 337 (ca. 3.67 g, 12 mmol) from the hydrolysis of 365 was dissolved in a mixture of 5 ml H_2O, 10 ml pH 5.3 acetate buffer, 3 ml of a 42% $NaHSO_3$ solution (1.25 g, 12 mmol), and 35 ml MeOH and warmed to 40°. To the stirred reaction mixture was added 3.2 ml of a 20.15%

methanolic solution of dimethylamine (0.65 g, 14.4 mmol) followed by an aqueous solution of NaCN (0.59 g, 12 mmol). After 15 min, the reaction mixture was cooled to room temperature and extracted with CH_2Cl_2 (3 x 30 ml). The combined CH_2Cl_2 extracts were dried over K_2CO_3 and the solvent was removed in vacuo. The crude oil (3.22 g) was column chromatographed on 360 g of silica gel (40-140 mesh) with CH_2Cl_2 (EtOAc gradient) as the developing solvent. The desired (by gc) fractions eluting with 7% EtOAC/CH_2Cl_2 were combined and evaporated to give the pure (one peak by gc) α-cyanoamine as a colorless oil in 9% yield. The nmr, ir, and cims data are consistent with the α-cyanoamine, 344. GC-EIMS, eims, and hr-eims analysis resulted in the characterization of the expected HCN elimination product, the enamine 345: NMR ($CDCl_3$, Figure 43) δ 2.36 (s, 6H, CH_3), 3.71-4.26 (m, 3H, NCH_2CHCN), 4.93 (d, 2H, thienylmethyl CH_2), 6.55-7.56 (m, 6H, aromatic), 8.19 (br d, 1H, aromatic); IR ($CHCl_2$) 2200 cm^{-1} (C≡N); CIMS MH^+ 287; GC-EIMS (Figure 22) 259 (M^+, 20%), 162 (74%), 97 (100%), 92 (55%).

Exact Mass (HR-EIMS). Calc'd for $C_{14}H_{17}N_3S$: M^+ 259.1139. Found: M^+ 259.1139.

The fractions eluting with 5% EtOAC/CH_2Cl_2 from the silica gel column were combined and evaporated to a colorless oil with a gc retention time identical to that of the aldehyde 337. The nmr spectrum (Figure 45) of this product is consistent with the cyanohydrin 368: NMR ($CDCl_3$, Figure 45) δ 3.94 (m, 2H, CH_2), 4.71 (m, 1H, CH(CN)OH), 4.90 (s, 2H, thienylmethyl CH_2), 6.64-7.65 (m, 6H, aromatic), 8.06 (m, 1H, aromatic, 8.34 (b, 1H, OH); CIMS MH^+ 233 (MH^+ - HCN).

The polar material eluting with 50% acetone/MeOH also had a gc retention time identical to the aldehyde 337. The nmr spectrum of the

isolated material assigned as 371 is depicted in Figure 46. Attempts to repeat the isolation of this material have resulted only in the isolation of the aldehyde 337 as evidenced by nmr: NMR ($CDCl_3$, Figure 46) δ 4.12 (d, 2H, $NCH_2CH(O^-)R$), 4.86 (s, 2H, thienylmethyl CH_2), 6.63-7.62 (m, 6H, aromatic), 8.33 (m, 1H, aromatic), 8.74 (t, 1H, $CH(O^-)R$).

i. Methapyrilene N-oxide (351).

To a solution of methapyrilene (2.61 g, 10 mmol) in 20 ml THF at 0° was added a 10% aqueous solution of H_2O_2 (15 mmol). The solution was allowed to warm to room temperature. After 36 hours, the reaction mixture was rotary evaporated to a viscous oil. Water was added to the oil, the resulting mixture was made basic with K_2CO_3, extracted twice with diethyl ether (to remove starting methapyrilene), and then with CH_2Cl_2 (to recover product). The CH_2Cl_2 extracts were dried over K_2CO_3 and evaporated to an oil which solidified to a white solid upon trituration with benzene and and hexane: mp (sealed tube) 69-72°; NMR ($CDCl_3$) δ 3.22 (s, 6H, CH_3), 3.48 (t, 2H, CH_2), 4.20 (t, 2H, CH_2), 4.89 (s, 2H, thienylmethyl CH_2), 6.60-7.46 (m, 6H, aromatic), 8.15 (br d, 1H, aromatic); GC-EIMS: decomposition products (see Figure 35, Section V.B.2.).

Anal. Calc'd. for $C_{14}H_{19}N_3OS$: C, 60.65; H, 6.86; N, 15.16. Found: C, 57.91; H, 7.32; N, 14.49.

B. METABOLIC STUDIES

1. 1-BENZYLPYRROLIDINE

a. Liver Preparations

Most incubations were performed with rabbit liver 100,000 x g microsomal pellet preparations. Preparation of the microsomal pellet

was done at 0-4° in a cold room. Male dutch rabbits 6 months to 1 year old were stunned and decapitated. The livers were excised and rinsed in cold isotonic (1.15%) KCl. The liver tissue (12.5 g) was homogenized in 25 ml of cold 0.2M pH 7.4 tris HCl buffer using a Potter-Elvehjem Teflon pestle homogenizer. The homogenates were centrifuged at 10,000 x g for 20 min in a Sorvall RC-2 refrigerated centrifuge at 0-4° to yield the 10,000 x g supernatant fraction. The supernatant fractions were recentrifuged at 100,000 x g for 1 hour at 0-4° C in a Spinco Model L refrigerated centrifuge to obtain the 100,000 x g microsomal pellet. The sediment (microsomal pellet) was resuspended in the amount of cold 0.2 M pH 7.4 tris HCl buffer to make 1 ml of solution correspond to 0.5 g liver. The resuspended pellet was then recentrifuged at 100,000 x g for 1 hour at 0-4° C. The resultant pellet was then resuspended as described above for use in incubations. A Lowry assay was done to determine microsomal protein concentration.[231]

b. Incubations

All substrates were synthesized or purchased as previously described. NADPH was purchased from Sigma Chemical Co.

A typical incubate (5.0 ml total volume) contained 4 ml of microsomal fraction (0.5 g of liver/ml), $MgSO_4$ (15 mM), 1.0 mg of the desired substrate, and NADPH (4 mg added every 20 min unless indicated). Incubations in the presence of sodium cyanide unless otherwise indicated contained 1.0 mM or 10 mM NaCN (in H_2O). Incubations were done at 37° C in air for 1 hour in a metabolic shaker. In preincubation experiments, NADPH was added at time 0 and incubations were allowed to proceed for 1 hour before the addition of NaCN. At the end of the incubation period,

the incubates were chilled in ice and the internal standards were added (0.5 mg 1-benzylpiperidine and 0.2 mg 1-benzyl-2-piperidone). Analysis for the phenol 248-α,α-d_2 employed 10 μg 248 as internal standard. The incubates were made basic with solid $NaHCO_3$ and then extracted with fresh diethyl ether (2 x 12 ml). Analysis for 248 involved extraction with ethyl acetate at pH 9. The ether extracts were dried over Na_2SO_4 and were reduced in volume to 0.3 ml under a N_2 stream with gentle heating (28-32°C) and then analyzed by gc or gc-eims. The gc column oven temperature was held at 125° for 2 min 40 sec and then programmed at 6° per minute to 250°. Quantitation of gc peaks was achieved by measuring peak heights.

In the protein binding studies, the incubations were conducted as described above. After the incubation period, the reactions were terminated by chilling in ice and by adding either 1M ethanolic $HClO_4$ or EtOH. The resulting suspension was centrifuged and the supernatant removed. The protein pellet was homogenized with the aid of Potter-Elvehjem Teflon pestle homogenizer in 5 ml of either 0.4 N ethanolic $HClO_4$ or EtOH and recentrifuged. This washing process was repeated 7 times. The pellet was then dissolved in 2N NaOH, Aquasol scintillation fluid (New England Nuclear) was added and the mixture counted in a liquid scintillation counter.

2. METHAPYRILENE

a. Liver preparations

Incubations were performed with either rat or rabbit liver 100,000 x g microsomal pellet preparations. Male Sprague-Dawley rats (150-200 grams) were induced with phenobarbital (80 mg/kg, I.P., daily over 4 days before sacrifice). Male Dutch rabbits were used untreated. Animals

were stunned by a blow to the head and decapitated. Preparation of the 100,00 x g microsomal pellet was performed as described above (Section VI.B.1).

b. Incubations

Methapyrilene, NADPH, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. Sodium cyanide, [^{14}C]-(50-60 mCi/mmol), was purchased from New England Nuclear and diluted to desired specific activity (ca. 0.25 mCi/mmol).

A typical incubation (5.0 ml total volume) contained 4 ml of the microsomal fraction (corresponding to 0.5 g of liver/ml), $MgCl_2$ or $MgSO_4$ (15 mM), methapyrilene (1 mM), and NADPH (4 mg every 20 min) or an NADPH-generating system (0.4 mM NADP, 7.5 mM glucose-6-phosphate, 0.5 units/ml glucose-6-phosphate dehydrogenase, 5.0 mM $MgCl_2$). Incubations in the presence of sodium cyanide contained 1.0 mM NaCN or $Na^{14}CN$ (in H_2O); the specific activity of the $Na^{14}CN$ was adjusted such that the amount of radiolabel in a 5 ml incubation was ca. 5 x 10^4 dps. Incubations were done at 37° C, in a metabolic shaker in air for 1 hour unless otherwise indicated.

Analytical incubations for scintillation counting were adjusted to pH 10-11 by the addition of $K_2CO_{3(s)}$ and extracted with CH_2Cl_2 (2 x 6 ml). The CH_2Cl_2 extracts were washed once with 5 ml saturated K_2CO_3 solution and the resulting aqueous wash was back-extracted with CH_2Cl_2 (2 x 3 ml); where necessary, phase separation was achieved by centrifugation. The combined organic extracts were dried over $K_2CO_{3(s)}$ (10 min) and evaporated to dryness under N_2 stream at 25-30° (N_2 gas conducted through Teflon tubing). The residue was taken up in 10 ml Aquasol scintillation fluid

and counted in a liquid scintillation counter. Incubations for the quantitation of methapyrilene metabolism were made basic with $K_2CO_{3(s)}$ and chilled in ice. The internal standard pyrilamine (maleate salt, 200 µl of a 10.0 mg/ml aqueous stock solution) was added immediately and the postincubate was extracted with CH_2Cl_2 (3 x 6 ml). The combined CH_2Cl_2 extracts were dried over K_2CO_3, evaporated to near dryness under N_2 stream (25-30°), reconstituted to 0.3 ml with CH_2Cl_2, and analyzed by gc (3% OV-25; column oven 150° isothermal for 2 min 40 sec then programmed at 6°/min to 250°). Quantitation of gc peaks was achieved by measuring peak heights.

The pH of the preparative incubations was adjusted to 10-11 by the addition of solid K_2CO_3 and the alkaline postincubate was extracted with distilled CH_2Cl_2 (3 x 6 ml). The combined organic extracts were washed with 10% NaCl solution and dried over $K_2CO_{3(s)}$. The organic extract was treated with 1 N HCl, the aqueous layer was made basic (pH 11) with 4N NaOH, and extracted with distilled CH_2Cl_2. The CH_2Cl_2 extracts was dried over $K_2CO_{3(s)}$ and rotary evaporated to dryness. This residue was reconstituted with CH_2Cl_2 or MeOH to a desired volume for gc, gc-eims, or hplc analysis. The crude extract could be purified by preparative tlc (2000 µ silica gel plates; pre-eluted twice with 1:1 benzene/MeOH and reactivated by heating at 120° prior to use) with $CH_2Cl_2/CH_3CN/Et_3N$ (90/10/1) as the developing solvent. The desired radioactive zone (by radioscan and UV, R_f = 0.55) was scraped and extracted with 1:1 CH_2Cl_2/CH_3CN. The organic extract was dried over K_2CO_3 and evaporated to near dryness for gc, gc-eims, and nmr analysis. Development of the scraped tlc plate (in the same solvent) resulted in the resolution of several UV-absorbing components. These zones were scraped and extracted with CH_3CN for gc, gc-eims

or hplc analysis. Analysis by hplc was performed either on a reverse phase C-8 column using 40% CH_3CN/pH 3 phosphate buffer as the mobile phase or on a silica gel column using 1% MeOH/CH_2Cl_2 as the mobile phase. HPLC eluent fractions for cims analysis or liquid scintillation counting were collected directly from the detector outlet. The hplc eluent samples for cims analysis were evaporated to near dryness under a stream of N_2 with gentle heating; 10 ml of Aquasol scintillation fluid was added to the hplc eluent fractions (1.32 ml each) for liquid scintillation counting.

Several different methods were used in attempts to extract polar metabolic products. All were implemented only after the initial extraction of the preparative incubations at pH 11. The postincubate was treated with 4N HCl such that the pH of the solution was adjusted sequentially to 8, 7, 6, 5, and 4 and the aqueous mixture was extracted with CH_2Cl_2 (3x) at each pH. The organic extracts were dried over $K_2CO_{3(s)}$, evaporated and analyzed by gc or gc-eims. Another method involved adjusting the pH of the postincubate to 7.4 and extracting the aqueous solution with CH_2Cl_2 in a continuous extractor for 24 hours. The resulting organic extract was dried over $K_2CO_{3(s)}$, evaporated to near dryness, treated with diazomethane (in MeOH), and analyzed by gc-eims. This procedure was repeated after adjusting the pH of the aqueous postincubate to 6 and then to 4.5 with HCl. A last method involved adjusting the pH of the postincubates to 6 and then 4.5 with buffer. The buffered solutions were vigorously extracted with CH_2Cl_2 (8x). The CH_2Cl_2 extracts were dried over $K_2CO_{3(s)}$, evaporated to near dryness, and analyzed by gc or gc-eims. Amberlite XAD-2 resin was washed prior to use.[222] The postincubates (50-75 ml total volume) were column chromatographed on a 100 g of XAD-2 resin. After application of the microsomal mixture, the column

was washed with several volumes of H_2O (∿ 500 ml) and the desired organic material was eluted with MeOH (∿ 400 ml). The MeOH eluents were evaporated to near dryness *in vacuo* and analyzed by gc, gc-eims, and hplc.

CHAPTER VII

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