I. STRUCTURE AND BIOSYNTHESIS OF PYRIDINE ALKALOIDS FROM *TRIPTERYGIUM WILFORDII* HOOK

II. METABOLISM OF RICININE IN *RICINUS COMMUNIS* L.

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PART ONE

STRUCTURE AND BIOSYNTHESIS OF PYRIDINE ALKALOIDS FROM *TRIPTERYGIUM WILFORDII* HOOK

CHAPTER I

INTRODUCTION

During the past fifteen years several new pyridine alkaloids have been discovered; however, more emphasis has been placed on determining the biosynthetic route of the well known pyridine alkaloids such as ricinine and nicotine.

A naturally occuring insecticidal material, which was non-toxic to warm blooded animals, was discovered in the early 1950's. Studies conducted by the United States Department of Agriculture scientists showed this material to be a mixture of complex ester alkaloids containing a substituted pyridine moiety (1, 2). The insecticidal material is contained in Tripterygium wilfordii Hook, a plant known as the "Thunder God Vine" in its native Chinese habitat. Its toxic qualities come from the root alkaloids which have been used for centuries by Chinese gardeners to protect their crops against chewing insects. Upon alkaline saponification, the complex ester alkaloids yield one mole of a derivative(s) of nicotinic acid; either wilfordic acid or hydroxywilfordic acid, as one of the acidic subcomponents. The complete structure of these alkaloids is not yet known. Promising research work on elucidating the structure of these alkaloids was abandoned with the advent of organic insecticides in the 1940's and early 1950's. The purpose of this study was to determine the structure of the pyridine moiety as well as the original ester alkaloids by using

modern techniques.

Another object of this research project was to determine if nicotinic acid and nicotinamide adenine dinucleotide, obligatory members of the recently discovered pyridine nucleotide cycle (3, 4), could serve as precursors of the pyridine moiety of the Tripterygium wilfordii Hook alkaloids. Positive results from such a study would serve to confirm and extend the pyridine nucleotide cycle-pyridine alkaloid inter-relationships.

CHAPTER II

LITERATURE REVIEW

A. Isolation and Chemical Studies

The early efforts made in the 1930's to isolate insecticidal compounds from the roots of Tripterygium wilfordii Hook were generally unsuccessful. From extracts of the root bark Chou and Mei (5) isolated dulcitol and the insecticidally inert red pigment, tripterine, which Schechter and Haller (6) found to be identical with the red pigment celasterol isolated from Celastrus scandens, the common American bittersweet. Chou and Hwang (7) described the isolation of a toxic alkaloid fraction which they named "tripterygine" which was precipitated by the usual alkaloid reagents and was assigned the formula $C_{38}H_{38}O_{11}N$.

The isolation of wilfordine, an insecticidal ester alkaloid from the roots of T. wilfordii Hook was first reported in 1950 by Acree and Haller (8). They found wilfordine to be an ester alkaloid consisting of a polyhydroxy nucleus, esterified with 5 moles of acetic acid, 1 mole of benzoic acid, and 1 mole of a nitrogen-containing dicarboxylic acid; however, they reported that the formula for the sum of the component parts of wilfordine, $C_{43}H_{49}O_{18}N$ did not agree with the molecular formular, $C_{43}H_{47}O_{19}N$, calculated from the elemental analysis of the entire alkaloid. It was later shown that this discrepancy was due to the fact that wilfordine was a mixture of several alkaloids (9)

possessing similar structure.

The detailed studies on the isolation and structure of the complex ester alkaloids from the root of T. wilfordii Hook have been exclusively reported from 1951 to 1953 by Morton Beroza. It was reported (10) that the first two similar alkaloids, designated wilforine and wilfordine, were isolated from the mixture by partition chromatography and proven pure by countercurrent distribution. The reported formulae of wilforine and wilfordine, calculated from elemental analysis, were $C_{43}H_{47}O_{18}N$ and $C_{43}H_{49}O_{19}N$, respectively. The compounds were insecticidally active ester alkaloids which upon saponification yielded 1 mole of benzoic acid, 5 moles of acetic, and 2 moles of non-steam -volatile acid per mole of compound.

The isolation of two additional alkaloids, designated wilforgine and wilfortrine from the plant was reported by Beroza (11). The formulae of wilforgine and wilfortrine were calculated to be $C_{41}H_{47}O_{19}N$ and $C_{41}H_{47}O_{20}N$, respectively, based on elemental analysis. Wilforgine and wilfortrine were ester alkaloids and appeared to be very similar to wilforine and wilfordine. Upon saponification they yielded 5 equivalents of steam-volatile acids and 2 equivalents of non-steam -volatile acids per mole of alkaloid.

A small quantity of one other alkaloid, designated wilforzine, has been reported (12) from the root of the plant. The formula for the sum of the components of wilforzine was in agreement with the molecular formula $C_{41}H_{47}O_{17}N$ calculated from elemental analysis of the intact alkaloid. Wilforzine therefore appeared to be identical with wilforine, except that it contained one less acetyl group.

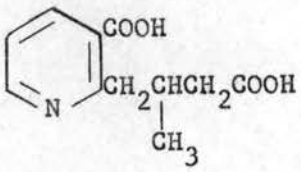
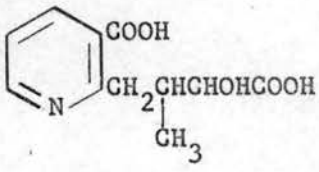
Beroza (13) studied in detail all the fragments resulting from the alkaline hydrolysis of the ester alkaloids wilforine, wilfordine,

wilforgine and wilfortrine and reported that the formula for the sum of the components of each alkaloid was in agreement with the molecular formula calculated from the elemental analysis of each alkaloid (Table I). It was shown by elemental analysis, paper chromatography, and x-ray diffraction patterns that the four alkaloids possessed the same polyhydroxy nucleus, $C_{15}H_{26}O_{10}$. This nucleus contained ten hydroxyl groups, but only eight of them were esterified in the intact alkaloid - five with acetic acid, one with either benzoic or with 3-furoic acid, and two with a nitrogen-containing dicarboxylic acid.

TABLE I

COMPONENTS OF TRIPTERYGIUM WILFORDII HOOK ALKALOIDS

(Results after Saponification) (13)

Sub-component \ Alkaloid	Wilforine	Wilforgine	Wilfordine	Wilfortrine
Poly-hydroxy moiety	$C_{15}H_{16}(OH)_{10}$ + 5 acetic acids + benzoic acid +	$C_{15}H_{16}(OH)_{10}$ + 5 acetic acids + 3-furoic acid +	$C_{15}H_{16}(OH)_{10}$ + 5 acetic acids + benzoic acid +	$C_{15}H_{16}(OH)_{10}$ + 5 acetic acids + 3-furoic acid +
Acidic moieties				
Sum of Sub-components (-8 H_2O)	$C_{43}H_{49}NO_{18}$	$C_{41}H_{47}NO_{19}$	$C_{43}H_{49}NO_{19}$	$C_{41}H_{47}NO_{20}$

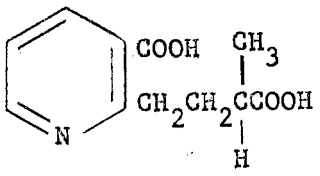
Beroza (10) reported that the alkaloids had almost identical ultraviolet absorption spectra in absolute ethanol and in dilute hydrochloric acid and that their infrared absorption spectra in carbon tetrachloride were also similar. Table II is a summary of reported alkaloids from the root of T. wilfordii Hook and their physical properties.

TABLE II

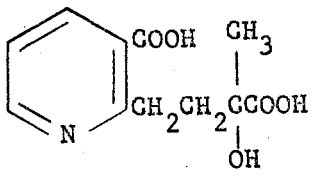
ALKALOIDS OF TRIPTERYGIUM WILFORDII HOOK

Alkaloid	Molecular weight	Melting point (°C)	$[\alpha]_D$	Reference
Wilforine	867	170	+30	10
Wilfordine	883	176	+12	10
Wilforgine	857	211	+25	11
Wilfortrine	873	238	+10	11
Wilforzine	827	178	+6	12

The most recent proposed structure of the two pyridine dicarboxylic acids, which were obtained after alkaline saponification of the alkaloids were those of Beroza (14).



[I]



[II]

Figure 1. Structure of Wilfordic acid [I] and Hydroxywilfordic acid [II]

Nonane was obtained from wilfordic acid and hydroxywilfordic acid by hydrogenolytic gas chromatography. This result, coupled with a study of the nuclear magnetic resonance spectra of wilfordic acid and hydroxywilfordic acid, has lead to a proposed structure of these acids as 3-carboxy-α-methyl-2-pyridinebutyric acid and 3-carboxy-α-methyl, hydroxy-2-pyridinebutyric acid.

The isolation of a similar alkaloids from the seed of Eunomus europaeus L. was reported by Pailer and Libiseller (15). By alkaline saponification of evonine, the main alkaloids of E. europaeus L., they isolated a dibasic acid, evoninic acid, which contained a 2, 3-dimethylpropionic acid in place of the 2-methyl-butyric acid side chain of wilfordic acid. They also found the polyhydroxy nucleus of evonine had the same empirical formula $C_{15}H_{26}O_{10}$ as that from T. wilfordii.

Molecular formulae of the intact alkaloids and the structure of the subcomponents, with the exception of the polyhydroxy moiety, have been proposed. But the exact structure of these alkaloids remain unknown.

B. Biosynthesis of Pyridine Alkaloids in Plants

The biosynthesis of pyridine compounds in higher plants is known to differ from that in animals (16). The metabolic conversion of the indole nucleus of tryptophan to nicotinic acid is well established in mammals, fowl, Neurospora crassa, Xanthomonos pruni, and Fusarium oxysporum (17, 18). The observation in 1945 by Krehl et al. (19) that tryptophan maintained the growth of niacin deficient rats provided the data that caused a number of scientists to conduct research in this area. It is now known that the pathway from

tryptophan goes through kynurenine, 3-hydroxykynurenine, 3-hydroxy-anthranilic acid, an unstable intermediate, quinolinic acid and then to nicotinic acid by way of the pyridine nucleotide cycle (20). Tryptophan is not a precursor of nicotinic acid or its derivatives in plants (21), Escherichia coli, Mycobacterium tuberculosis, Clostridium butylicum, and Seratia marcescens (22). The biosynthesis of the pyridine ring in living organisms has been reviewed by Leete (23), Spenser (24), Robinson (25), Mothes and Schuette (26) and Waller and Nowacki (18).

Mortimer (27) in 1954 suggested that ricinine, trigonelline and the pyridine ring of nicotine was derived from tryptophan. However, efforts to demonstrate a tryptophan-niacin relationship using isotopes in higher plants (21, 28, 29) and in certain bacteria (22) have not been successful. Henderson reported that conversion of tryptophan to compounds containing the pyridine ring did not occur in tobacco and corn (21). Aronoff (29) reported that 3-hydroxyanthranilic acid was not a precursor of trigonelline in soybeans. Yanofsky (22) found that tryptophan-G-^{14}C was not converted to nicotinic acid by E. coli or B. subtillus.

An important experiment describing the biosynthesis of pyridine compounds in E. coli was carried out by Ortega and Brown (30, 31). They found that radioactive nicotinic acid was synthesized from carbon labelled succinic acid, glycerol and pyruvate. They also demonstrated that the carbonyl carbon of nicotinic acid might be come directly from one of the carbonyl carbons of succinate by showing incorporation of succinate-1,4-^{14}C into nicotinic acid. Griffith et al. (32) studied the incorporation of propionate-2-^{14}C, glycerol-1,3-^{14}C and glycerol -2-^{14}C and found that most of the activities were in the pyridine ring

of nicotine. Waller and Henderson (33) studied the biosynthesis of ricinine with various 2-,3- and 4-carbon compounds and reported the following order of efficiency: succinate, propionate, β-alanine, acetate and glycerol. They also reported that all of the radioactivity in ricinine biosynthesized from succinate-2,3-^{14}C was in the pyridone ring. However, the radioactivity in the ricinine formed from succinate -1,4-^{14}C was located both in pyridone ring and in cyano group.

Yang and Waller (34) established by in vivo experiments with young Ricinus communis L. plants using aspartate-4-^{14}C, succinate-1,4-^{14}C and -2,3-^{14}C, glycerol-1,3-^{14}C and -2-^{14}C that carbons-2, 3 and 8 of ricinine arise from a four-carbon dicarboxylic acid such as aspartate and that carbons 4, 5 and 6 arise from intact glycerol. They suggested that the condensation of a three-carbon unit with a four carbon dicarboxylic acid might be from quinolinic acid which would then be decarboxylated to form nicotinic acid and ultimately the pyridine alkaloids. The incorporation of glycerol-1,3-^{14}C and glycerol-2-^{14}C into carbons-4, 5 and 6 of ricinine and succinate-1,4-^{14}C into carbons-2, 3 and 8 of ricinine in young castor bean plants was also demonstrated by Essery et al. (35) and Juby and Marion (36). It was also demonstrated that glycerol is incorporated without randomization into carbons-4, 5 and 6 of the pyridine ring of nicotine by using a stepwise degradation method for the pyridine ring of nicotine (37). Yang et al. (37) showed that quinolinic acid was an efficient precursor of ricinine in intact castor bean plants. The conversion of quinolinic acid into nicotinic acid mononucleotide was shown in plants (3) and other organisms (38, 39).

It is now well established (18) that the two main pathways for the formation of the pyridine nucleus in living systems involve either

tryptophan as a precursor or a four-carbon compound such as aspartate and a three-carbon compound such as glyceraldehyde-3-phosphate as precursors and both give arise to a common intermediate, quinolinic acid which is a key intermediate for the synthesis of the compounds in the pyridine nucleotide cycle and pyridine alkaloids derived from the cycle. The different pathways used to synthesize the pyridine nucleus are presented in Figure 2. Quinolinic acid was first isolated from mammalian sources by Henderson after feeding overloading doses of tryptophan. The enzyme quinolinate phosphoribosyl transferase, which catalizes the condensation between quinolinic acid with the loss of the α-carboxyl group as CO_2 and phosphoribosylpyrophosphate (PRPP), was partially purified from liver and bacteria (40, 41). Nicotinic acid mononucleotide reacts with ATP in the presence of nicotinate phosphoribosyl transferase to give desamido-NAD. NAD is then formed by the catalytic activity of NAD synthetase in the presence of either ammonia or glutamine and ATP. Degradation of NAD by NAD glycohydrolase yields nicotinamide which can be re-used by deamidation to nicotinic acid by nicotinamidase, an enzyme which has been found in plants (42) and mammals and certain bacteria (40, 43). The cycle is completed by the synthesis of nicotinic acid mononucleotide from nicotinic acid and PRPP by nicotinic acid mononucleotide pyrophosphorylase. Ryrie and Scott (44) observed in leaves of barley seedlings that nicotinic acid was rapidly metabolized with the formation of NAD. They also presented evidence suggesting that NAD was formed from nicotinic acid mononucleotide and desamido-NAD. Gholson (4) has summarized the evidence for the formation and degradation of the pyridine nucleotides in biological systems and has suggested the cycle exists in plants, animals and microorganisms. The control mechanism affecting the

metabolism of pyridine compounds in E. coli has been recently reviewed (18, 26).

Waller and his collaborators (3, 45) have established the relationship between the pyridine nucleotide cycle and the biosynthesis of nicotine and ricinine by showing that the pyridine moieties of nucleotides in the pyridine nucleotide cycle can be incorporated into nicotine and ricinine by the tobacco and castor bean plant respectively with an efficiency comparable to that of quinolinic acid, nicotinic acid and nicotinamide; compounds which had been established previously as being efficient precursors (10 - 30 %) of these alkaloids (45, 46).

The formation of N-methylnicotinamide from nicotinamide and N-methylnicotinic acid from nicotinic acid has been found in the caster bean plant and Nicotiana rustica L. (3, 45). Nicotinic acid can serve as a precursor of nudiflorine, N-methyl-5-cyano-2-pyridone (48), and ricinidine, N-methyl-3-cyano-2-pyridone (49). Sastry and Waller (50) recently identified a new pyridine alkaloid, N-methyl-5-carboxamide -2-pyridone which is a normal metabolite of nicotinic acid in mammals excreted in urine. They also established the biosynthesis of this alkaloid from nicotinic acid-6-^{14}C,-7-^{14}C and nicotinamide-7-^{14}C.

Mizusaki et al. (51) recently reported that nicotinic acid-6-^{14}C administered was incorporated into nine pyridine compounds during a three hour incubation in tobacco plants; two of these were identified as 6-hydroxy nicotinic acid and nicotinic acid-N-glucoside. The latter compound, which was the main product, was incorporated into nicotine with about the same efficiency as nicotinic acid; however, they suggested that the glucoside might not be involved in the direct route of nicotine biosynthesis since the rate of the incorporation was

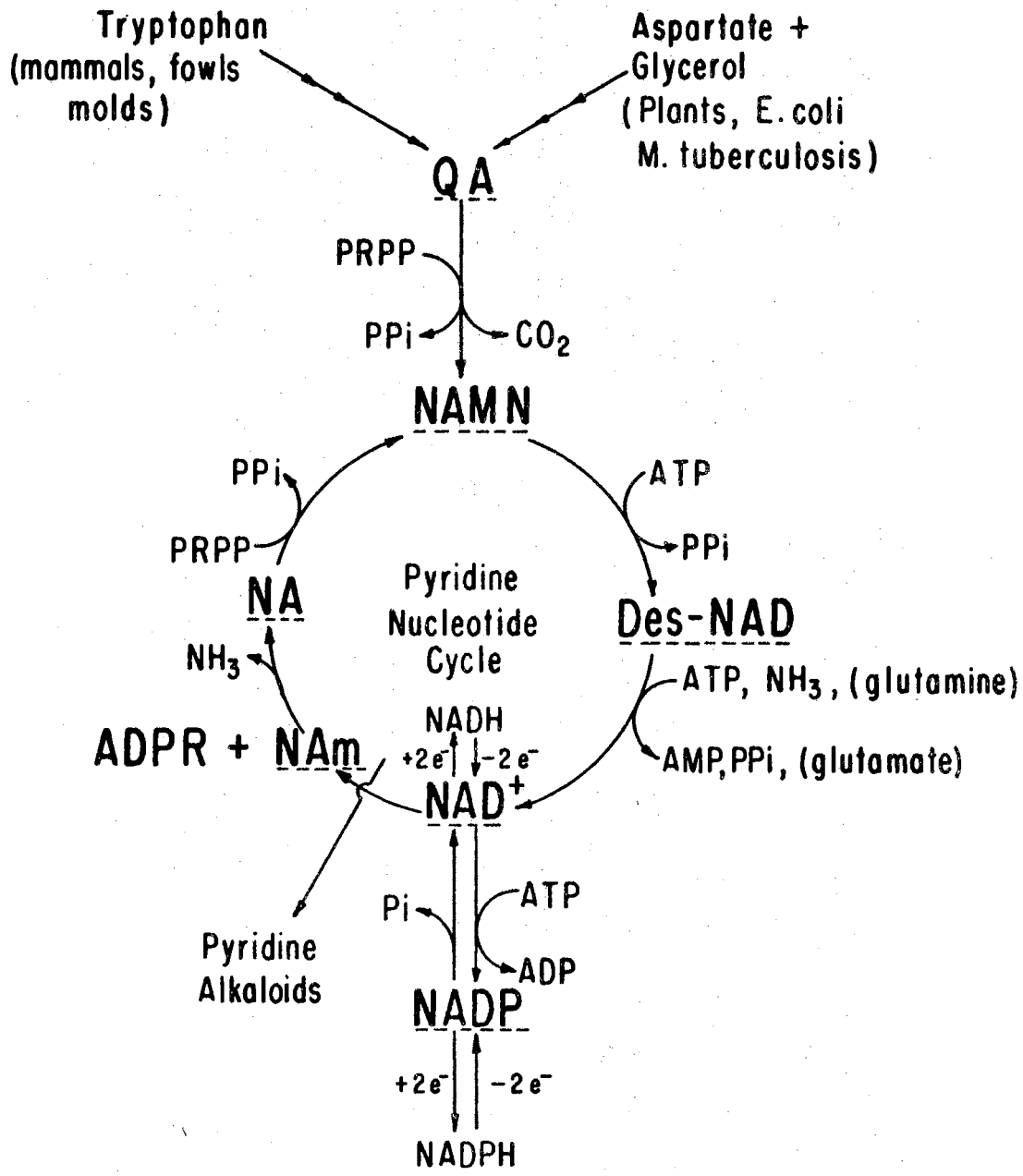


Figure 2. Metabolism of Pyridine Ring

markedly reduced when unlabelled nicotinic acid was fed.

It is now well established that the biosynthesis of pyridine compounds in higher plants is different from that in mammals and that some of the pyridine alkaloids are derived from the compounds in the pyridine nucleotide cycle. But the detailed biosynthetic steps for the formation of the pyridine alkaloids remain unknown.

C. Tripterygium wilfordii Hook Plant and its Insecticidal Effects

The first introduction into this country, and the results of preliminary entomological tests of Tripterygium wilfordii Hook, were reported in 1941 by Swingle (52). This plant, which belongs to the Celastraceae family, is a perrenial twining vine and its growth manner is much like that of the native North American bittersweet, Celastrus scandens L.

Tripterygium wilfordii Hook called lei kung teng, "Thunder God Vine", by the Chinese is cultivated rather widely in several Chinese provinces on the south side of the Yangtze River, especially in Checkiang Province, to kill chewing insects (8). Cuttings of the plant were imported into this country in 1936 and planted in the Plant Introduction Garden, Department of Agriculture, Glenn Dale, Maryland. Swingle (52) tested the insecticidal effects of T. wilfordii Hook root powder and reported in the early 1940's that the material was very toxic to first instar larvae of the diamondback moth, Plutella maculipennis Curt, and the imported cabbage worm, Pierie rapae L., and caused relatively low mortalities and in some instances practically no effect on instar larvae of the southern armyworm, Prodenia eridania Cram, and also to the second and larger instar larvae of the melonworm,

Diaphania hyalinata L. Tattersfield *et al.*(53) confirmed the insecticidal action of the root powder on the young larvae of the diamondback moth. Further tests (54) showed that *T. wilfordii* Hook root powder was effective against 8 species and was ineffective against 10 species of insects to their larvae.

The results against moth larvae were of sufficient interest to facilitate a detailed entomological and chemical study of the plant extract in the early 1950's. A study of the toxicities of four of the pure alkaloids to newly hatched larvae of the European corn borer was reported by Beroza and Botter (2). The insects were fed corn leaves treated with a spray containing 60 ppm. of the pure alkaloid. The percentage of larvae killed is shown in Table III.

TABLE III

THE INSECTICIDAL EFFECTS OF THE ALKALOIDS FROM THE ROOT OF *T. WILFORDII* HOOK ON THE EUROPEAN CORN BORER (2)

Alkaloid	Percentage killed After 2 days	After 3 days
Wilforine	88	100
Wilforgine	30	54
Wilfordine	54	100
Wilfortrine	48	73

Wilforzine, which was isolated two years later, exhibited definite, but much less insecticidal action than wilforine against larvae of the diamondback moth (12). In general, the smaller larvae were affected more than the larger ones. No symptoms of poisoning were observed when bait containing 20 % of the root powder or 0.5 % of the crystalline alkaloid was fed to rats (2).

CHAPTER III

EXPERIMENTAL METHODS

A. Materials and Chemicals Used

1. Plants

The cuttings of Tripterygium wilfordii Hook were imported from Taiwan in October, 1967 and September, 1968, and planted in pots with a mixture of clay loam and vermiculite at the green house of the Horticulture Department, Oklahoma State University, Stillwater, Oklahoma. Plants were propagated by cuttings. Cultivation was successful in the green house, however, attempts to cultivate T. wilfordii Hook outdoors were unsuccessful due to the high temperature and low humidity during summer months. Plants 2 years of age with similar appearance, fresh weight of roots ranging from 15 grams to 18 grams and that of leaves and stems ranging from 10 grams to 14 grams, were used for biosynthesis experiments.

A total 4 kilograms of dried root was imported in September, 1967 and stored at room temperature. This material was used for alkaloids isolation for the structure studies.

2. Radioactive Compounds

Nicotinic acid-6-^{14}C was purchased from Nuclear Chicago Corporation and purified before use by preparative thin layer chromato-

graphy using butanol : acetic acid : water (80 : 20 : 20, v/v/v) and n-butanol saturated with 3 % ammonia solution. The purified nicotinic acid-6-^{14}C had a constant specific activity of 20.6 mc/mM.

DL-glutamic acid-2-^{14}C was purchased from Tracerlab and purified by preparative thin layer chromatography using butanol : acetic acid : water (80 : 20 : 20, v/v/v). The purified DL-glutamic acid-2-^{14}C had a specific radioactivity of 3.7 μc/mg.

Nicotinamide-carbonyl-^{14}C-adenine-dinucleotide (specific activity of 26.2 mc/mM) was purchased from Nuclear Chicago Corporation and used without further purification.

3. Chemical Reagents

Solvents and chemical reagents were of analytical reagent grade unless otherwise noted. Solvents were redistilled using glassware. Non-radioactive nicotinic acid and other nucleotide used were purchased from Sigma Chemical, Biochemical Research Company or Nutritional Biochemicals Corporation.

Dowex 1-X8 Cl^- form, 200 - 400 mesh, was purchased from J. T. Baker Chemical Company and converted to the formate form by washing successively with several volumes of H_2O, 1 N NaOH, H_2O, 2 N HCl, H_2O, 8 N HCOOH, and then with deionized water until neutral pH.

Silicic acid (Bio-sil A, 100 - 200 mesh) was purchased from Bio-Rad Laboratories and purified by washing successively with methanol and ether. The washed material was activated by drying at 115°C overnight.

B. Isolation and Structure Identification

1. Isolation and Purification

In large-scale extractions the crude alkaloid fraction was isolated by a modification of Beroza's method as described below: Air-dried, finely grounded root powder was exhaustively extracted with ethylene dichloride using a soxhlet apparatus. The solvent was removed under reduced pressure at a temperature which did not exceed 40°C. The residue was dissolved in a minimum volume of ether and thoroughly mixed with an equal volume of 5 % hydrochloric acid. Upon removal of the ether under reduced pressure, the remaining yellowish water phase was filtered. This process of extraction of the tarry residue with ether and hydrochloric acid was repeated five times until a Dragendorff test of the extract spotted on a thin layer chromatography plate was negative. The acid extract was cooled to about 4°C and treated with concentrated ammonia to pH 9, the temperature of the solution being kept below 4° by keeping the flask in an ice-bath. Several hours later the precipitated crude alkaloid fraction was filtered, washed with cooled distilled water and dried. The yield of the crude alkaloid fraction was about 0.25 %. The dry crude alkaloid fraction was dissolved in hot methanol and was used for chromatography. A schemetic diagram of the isolation procedure is shown in Figure 3.

Small-scale extraction from the fresh plant and purification procedure for the alkaloids will be described in the Biosynthesis Section.

2. Saponification and Separation of Subcomponents

Fifty mg of the alkaloid fraction isolated from thin layer

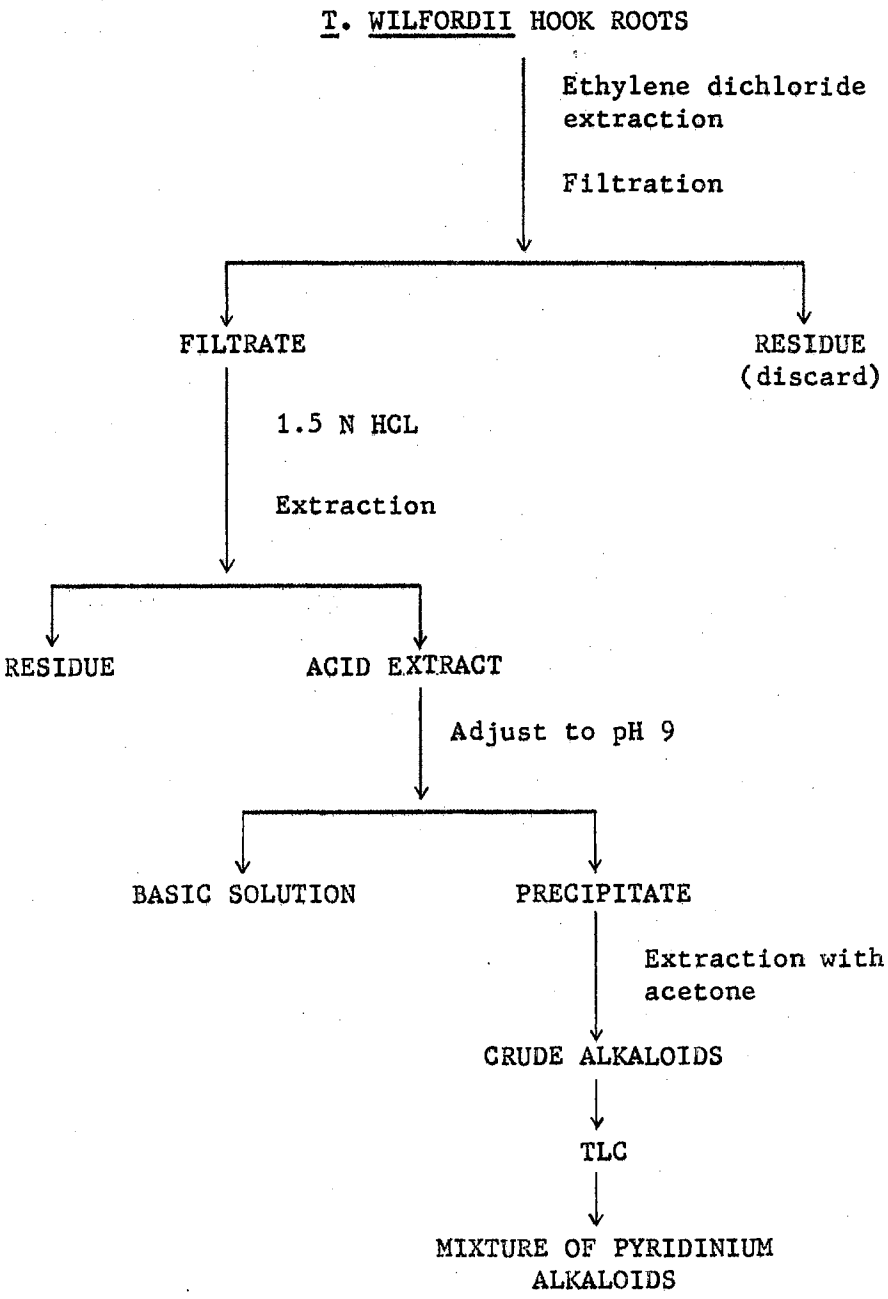


Figure 3. Isolation Procedure for T. wilfordii Hook Alkaloids

chromatography was hydrolyzed with 1 ml of 1 M potassium hydroxide in diethylene glycol for 30 minutes at 125° as described by Beroza (13). The saponification mixture was fractionated by liquid-liquid extraction at various pH values for the separation of the acidic moieties and the polyhydroxy moiety. The saponification mixture was made alkaline with a few drops of 1 N sodium hydroxide and then continuously extracted with ether in an all glass apparatus for 24 hours to remove the diethylene glycol. The ether extract was discarded. The extracted water solution was carefully acidified with 3 N sulfuric acid to pH 2.8 and continuously extracted with ether for at least 24 hours. If the pH of the water solution after the ether extraction differed from pH 2.8, then it was readjusted to this pH and again extracted for 24 hours. Extraction was completed when the ultraviolet absorption of the water solution could not be detected by spotting 0.01 ml on a thin layer chromatography plate and observing under UV light at 154 nm. The ether extract which contained all the acids resulting from saponification of the alkaloid was reduced in volume and stored for further studies.

The water solution containing the polyhydroxy nucleus of the alkaloid was adjusted carefully with 1 N sodium hydroxide to pH 7.0 and lyophilized to dryness. The dry residue was triturated with a glass rod and extracted repeatedly with hot absolute methanol. The filtered methanol extract was evaporated to dryness, weighed, redissolved in hot methanol, concentrated to a few ml and finally an equal volume of acetone was added. The compound crystallized slowly. After two more crystallizations a crystalline white compound was obtained.

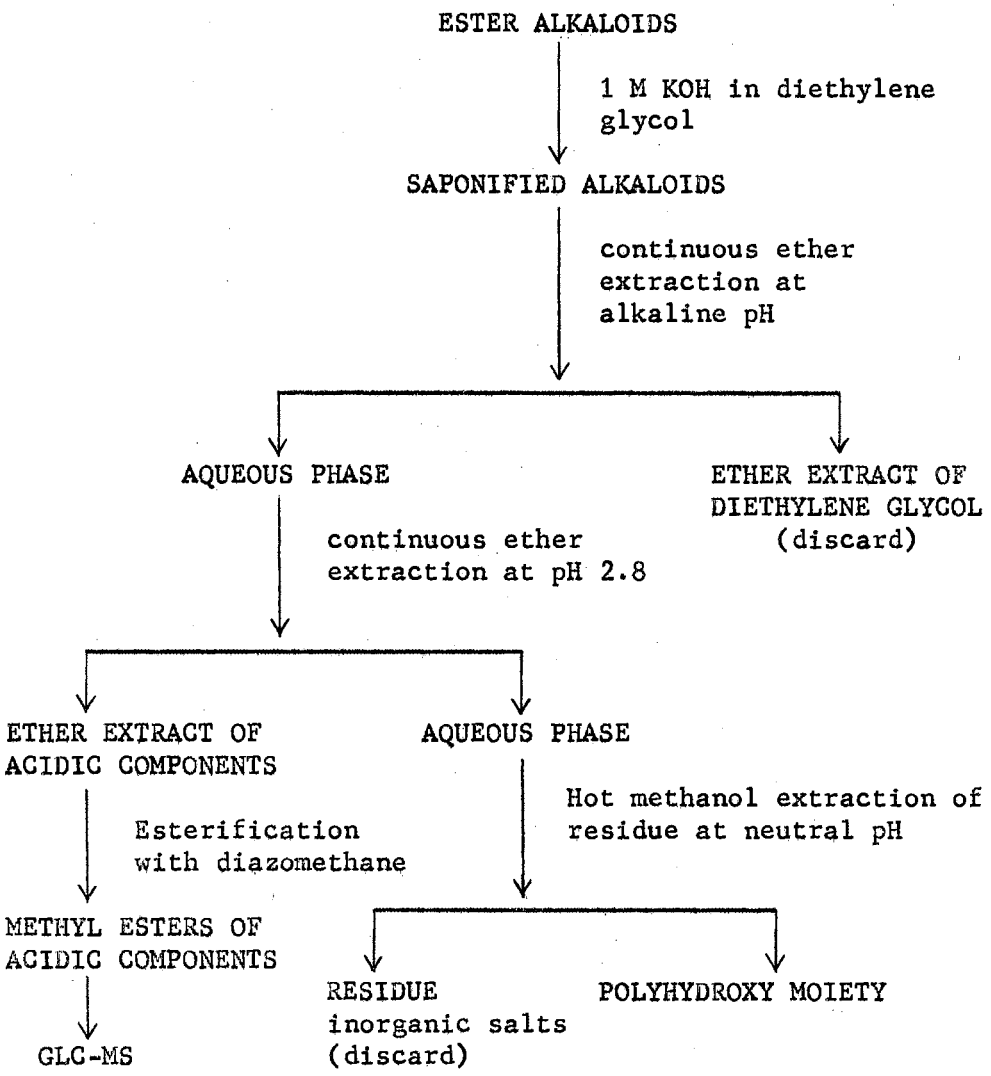


Figure 4. Isolation of Subcomponents from T. wilfordii Hook Alkaloids

3. Chemical Modification of Components

Esterification of Acidic Compounds (55): The acidic compounds were converted to their methyl esters with diazomethane which was prepared by the following method:

A solution of 12 grams of sodium hydroxide in 10 ml of water, 25 ml of carbitol (diethylene glycol mono ethyl ether), and 100 ml of ether in a flask were cooled to 0°C, and then 3.5 grams of N,N'-di-nitroso-N,N'-dimethyl tereaphthalamide(EXR-101) was added. Magnetic stirring was started and the reaction mixture was warmed slowly. The evolution of diazomethane became apparent at 15° to 20°C. In the 30° to 40°C range the diazomethane and ether were distilled and condensed to yield a bright yellow solution. By this method, and ether solution containing about one gram of diazomethane was obtained from 2.5 grams of EXR-101.

Formation of Trimethylsilyl-derivative of Polyhydroxy Compound (56): Polyhydroxy component from the ester alkaloids was converted to trimethylsilyl derivatives as described below: Bis-(trimethylsilyl) acetamide (BSA, 0.1 ml) was added to a vial containing the polyhydroxy compound (approximately one mg) which was then warmed to completely dissolve the compound and then trimethylchlorosilane (TMCS, 0.2 ml) was added. The sealed reaction mixture was kept at 55°C for three hours and then excess reagent was removed by passing a stream of nitrogen over it. The residue was dissolved in acetone and analyzed by gas liquid chromatography-mass spectrometry using the solid injection technique.

Selenium Dehydrogenation of Polyhydroxy Compound (57): The recrystallized polyhydroxy compound (30 mg) and Se metal (300 mg) were

placed in a dehydrogenation assembly consisting of a 10 ml round bottom flask fitted with an air condenser. The mixture was heated at 250 - 270°C in an electric furnace under a nitrogen atmosphere. The reaction mixture was cooled, extracted with ether, reduced in volume and then used for analysis.

4. Instrumental Analysis

Gas Liquid Chromatography (GLC): Gas liquid chromatographic analyses were performed on a modified Barber-Colman Model 5000 gas chromatography equipped with a hydrogen flame ionization detector (80). The column packing used was 10 % SE-30 on Gas-chrom Q, 100 - 200 mesh, unless otherwise noted. The column, 7 feet x 1/8 inches, was cured and silanized before use.

The following operational parameters were employed for the gas liquid chromatographic analysis of acidic components from ester alkaloids: the temperature of the column was either programmed 2°/min. from 115 to 200°C or fixed at two different temperatures, 115°C and 200°C, the injection port temperature was 215°C, and the helium flow rate was 40 ml/min.

Low and High Resolution Mass Spectrometry (MS): Low resolution mass spectra were obtained on a prototype of the LKB-9000 combination gas chromatograph-mass spectrometer which was constructed in the laboratory of Dr. Ragnar Ryhage, Karolinska Institutet, Stockholm, Sweden, and has been described by Waller (58). Mass spectra were obtained either using the direct inlet system or gas liquid column depending on compounds under following conditions unless otherwise specified: ionization voltage of 70 eV, 3.5 kV accelerating voltage, 40 amp. trap current, 1.7 to 2.1 kV electron multiplier voltage source

temperature of 310°C, separator temperature from 50 to 150°C, helium flow rate of 30 ml/min. A recording of the total ionization current obtained from the collector plate in the analyzer tube served as the gas chromatographic tracing. The vertical slash marks along the tracing indicate the points at which mass spectra were taken. Spectra were counted and the peaks height were measured manually. These data were introduced into the IBM 360/65 computer which was used to drive a Cal Comp Model 565 Plotter which plotted the mass spectra.

Mass spectra of the four intact alkaloid reference samples were obtained with approximately 50 μg of compound using the direct inlet system. The direct inlet temperature was programmed at 5°/min. from 50°C to 150°C and the source pressure was 5×10^{-6} to 1×10^{-7} mm/Hg. Mass spectra of the methyl esters were taken by following injection of the sample into the gas liquid chromatography column.

High resolution mass spectrometric (HRMS) analyses were conducted by K. Biemann and C. Hignite in the Mass Spectrometry Laboratory, Chemistry Department, Massachusetts Institute of Technology. The conditions for high resolution mass spectrometry were similar to those used for a low resolution mass spectrometry.

Low and High Resolution Nuclear Magnetic Resonance Spectrometry: Low resolution nuclear magnetic resonance spectra were taken on a Varian A-60 spectrometer equipped with a C-1024 time averaging computer. The operational condition was as follows: filter band width 1-4, sweep time 250 seconds, sweep width 500 H_z, sweep offset 017 H_z, and spectrum amp. 1.0, solvent used was dimethylsulfoxide-D_6 and the internal standard was trimethylsilane. The polyhydroxy compound was deuterated by introducing one drop of D_2O into the nuclear magnetic resonance tube. The time averaging computer was used under following operational

parameters: Offset value + 2417, nuclear magnetic resonance trigger sweep width 550, sweep time 250 seconds, counts from 18 to 140.

High resolution nuclear magnetic resonance spectra were obtained by P. F. Flanagan of the Continental Oil Company, Ponca City, Oklahoma. The operational parameters were as follows: frequency response and reference frequence attenuator 2 and 67 out respectively, sweep time 500 - 1000, sweep width 500 and spectrum amp. 1000. Abbreviation used for nuclear magnetic resonance spectral data are : s, singlet; t, triplet; q, quartet; and m, multiplet.

Ultraviolet and Infrared Spectrophotometry: Ultraviolet spectra were obtained either on a Bausch and Lomb Spectronic Model 505 recording spectrometer or on a Beckman DB recording spectrophotometer.

Infrared spectra were taken on a Perkin-Elmer 457 grating instrument with samples in dispersed potassium bromide pellets. Abbreviations used for infrared spectral data are: vs, very strong; s, strong; m, medium; w, week; vw, very week; sh, shoulder; v. br, very broad; and br, broad.

Melting Point Measurement: Melting point was measured on a Kofler Hot Stage Micro Melting Point apparatus (Arthur H. Thomas Company).

C. Biosynthesis

1. Administration of Labelled Compounds

Labelled compounds were administered using a micro syringe, in the upper part of the soft stems and the desired amount of labelled compound was injected slowly. Uptake of 50 µl of solution was usually completed within fifteen minutes.

2. Isolation of Metabolites

In the small-scale extraction from the fresh plant, the crude alkaloid fraction and its metabolites were isolated as follows. Plants were divided into an upper part which consisted of stems and leaves, and into a lower part, the roots. The divided plant part was weighed, frozen with liquid nitrogen, homogenized with Virtis "23" omnimixer in 80 % methanol and filtered using a sintered glass funnel (medium porosity). This extraction procedure with methanol was repeated four times until the remaining material was free of soluble pigments. The solvent was removed from the pooled extracts by evaporation at 40°C under reduced pressure. The residue was dissolved in a minimum volume of chloroform or ether and mixed with an equal volume of distilled water. Upon removal of the organic solvent at room temperature under reduced pressure, the remaining yellowish aqueous solution containing polar compounds of interest was filtered. This extraction procedure was repeated four times. The aqueous phase was reduced in volume and applied on an anion exchange column. The residue remaining on the flask filter paper after water extraction was extracted with acetone and used for thin layer chromatography. A flow diagram of the isolation procedure used for the plant metabolites is shown in Figure 5.

3. Chromatography

Anion Exchange Column Chromatography (44, 38, 60): A portion of the aqueous phase extract containing the polar metabolites of interest was placed on a Dowex 1-X8 formate column, 1.5 x 30 cm.

Elution was conducted by the application of a formic acid con-

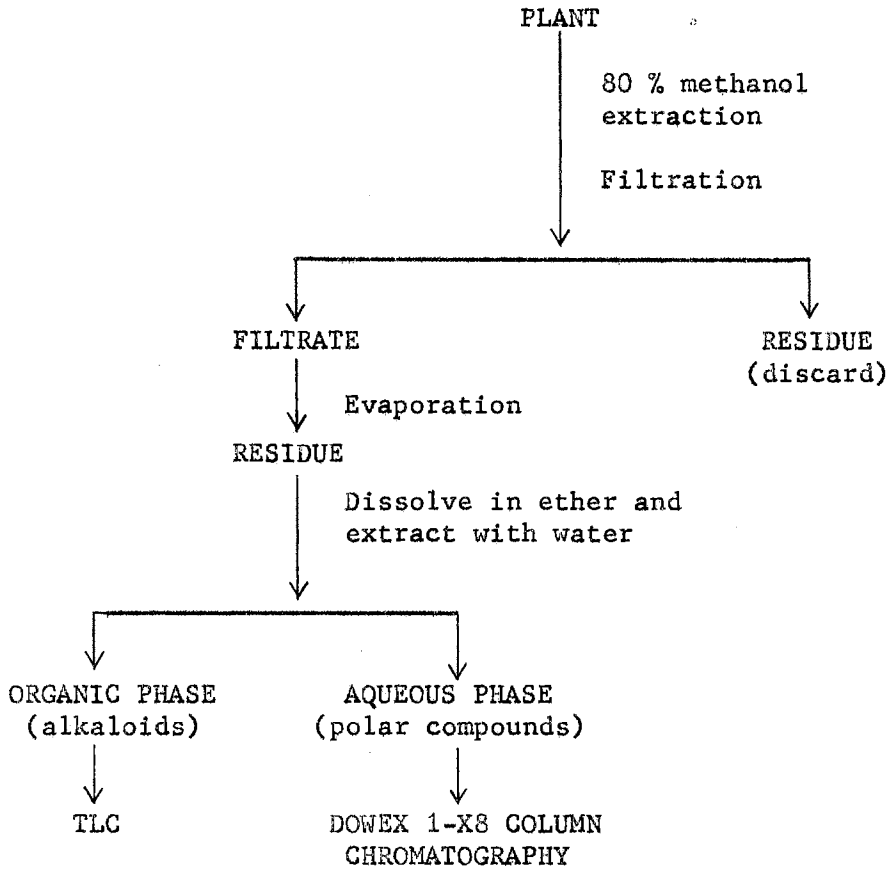


Figure 5. Isolation of Metabolites from Tripterygium wilfordii Hook after Administration of Labelled Precursors

centration gradient initially with 150 ml of water in the mixing chamber, into which 150 ml of deionized water, 250 ml of 0.25 N formic acid, 250 ml of 2 N formic acid and 250 ml of 4 N formic acid were successively introduced. A fraction collector equipped with an ISCO UA-2 flow ultraviolet analyzer, 254 nm, was adjusted at the rate of about 35 ml per hour. The pooled fractions of each tube, 10 ml, were used for the measurement of radioactivity and ultraviolet absorption at 260 nm.

Partition Column Chromatography: The acetone extract from the thin layer chromatography zone of interest was scraped from the plate, extracted and chromatographed on a silicic acid column. The immobile solvent, dilute hydrochloric acid, was equilibrated with the mobile solvent, ether, at the column temperature ($10^{o}C$) by means of a steady flow of cooled tap water. The column was prepared and developed as described below.

In a mortar 25 grams of silicic acid, and 14 ml of 0.6 % hydrochloric acid was thoroughly mixed. The fine slurry prepared by stirring in ether in the mixture was added to the column which was then tapped to settle the gel. The alkaloid fraction was dissolved in 30 ml of ether, added and washed into the gel with several small portions of ether. The flow rate of the column was adjusted to 60 ml/hr. and 10 ml fractions of the effluent were collected. The column was never permitted to run dry and the solvent added was at the same temperature as the column. The absorbancy of each fraction was measured at 270 and 255 nm as described by Beroze (10, 61).

Thin Layer Chromatography: The acetone extract containing the alkaloids of interest, lipids, pigments and other compounds were applied

on preparative thin layer chromatography plates. The plates, 0.75 mm thick, were prepared from Silica Gel PF 254 + 366 (Merck Co.). The solvent system consists of acetone and hexane (8 : 2, v/v). The alkaloids were detected on the chromatograms either by observing their fluorescence at 254 nm or by spraying Dragendorff reagent. The radioactivity on the chromatograms was located with a Nuclear Chicago 4 π Actigraph-III strip counter. For scanning thin layer chromatography plates the appropriate adaptor was used. The alkaloid zone on the thin layer chromatography plates was scraped off, put in small column and eluted with acetone and used for further studies.

Paper Chromatography: The fractions from the Dowex 1-X8 column containing radioactive products from the anion exchange column were combined and lyophilized to dryness. The residue was dissolved in distilled water and then spotted on Whatman No. 1 paper 1 inch strip with authentic compounds and descending chromatography was carried out with two different solvent systems: a) 1 M ammonium acetate : 95 % ethanol (3 : 7, v/v) adjusted to pH 5.0 with HCl, b) isobutyric acid : ammonia : water (66 : 1.7 : 33, v/v/v, pH 2.8). A Nuclear Chicago 4 π chromatogram scane and the ultraviolet lamp (254 nm) were employed to locate radioactive and quenching spots respectively.

4. Measurement of Radioactivity

The radioactivity of the pooled 10 ml fractions collected from the anion exchange column was measured using 1 ml of aqueous solution from each test tube in 10 ml Bray's scintillation solution which was prepared with 4 grams of 2,5-diphenyloxazole (PPO), 0.2 grams of p-bis-2-(5-phenyloxazolyl) benzene (POPOP), 60 grams of naphthalene, 20 ml of ethylene glycol, 100 ml of methanol and the proper volume of p-dioxane

for 1 liter of solution. Bray's solution could hold 2 ml of 4 N HCOOH without noticeable turbidity.

The counting efficiency of 10 ml of Bray's solution with 1 ml of formic acid from 0.25 N to 2 N was 75 %. The effects of formic acid concentration on the efficiency of scintillation counting are shown in Figure 6.

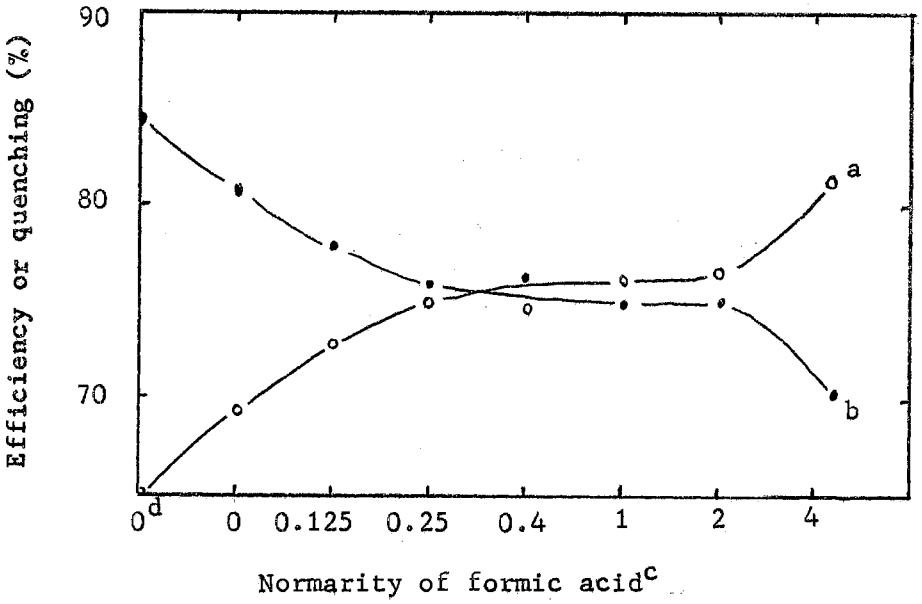


Figure 6. Efficiency of Radioactivity Counting at Various Concentration of Formic Acid using Liquid Scintillation Spectrometry

[a]Quenching effect by concentration of HCOOH
[b]Efficiency at various concentration of HCOOH
[c]1 ml of aqueous phase in 10 ml of Bray's solution
[d]Bray's solution only

Counting efficiency (%) = cpm/dpm x 100
Quenching effects (%) = x - y/x x 100
x - counting efficiency of Bray's solution without aqueous phase
y - counting efficiency of Bray's solution with 1 ml of aqueous phase

CHAPTER IV

RESULTS AND DISCUSSION

A. Isolation and Purification of Alkaloids

Two isolation procedures were used for the preparation of the alkaloid fractions as described under "Experimental Method". Large and small scale isolation procedures were employed for structure and biosynthesis studies respectively. Considerable difficulty was encountered in separating the alkaloid fraction from the plants since the plants do not contain a high amount of alkaloids. In the large scale preparation two modifications of Beroza's method (10) were made to prevent the possibility of alkaline hydrolysis of the ester alkaloids which might have resulted in a net loss of alkaloid: a) the dried plant powder was extracted thoroughly with ethylene dichloride without previously moistening the material with 10 % ammonium hydroxide, and b) instead of using a methanol fractionation procedure, the crude alkaloid fraction was purified by thin layer chromatography since the alkaloids are slightly soluble in methanol. It was found that the NH_4OH moistening process was not necessary because the yield of crude alkaloid fraction without moistening of the plant material was 0.2 - 0.25 % of dried plant weight which was comparable to Beroza's 0.2 % using the moistening treatment. The yield of the purified alkaloids from the crude alkaloid fraction was about 25 %; a value much higher than the previously reported one of 13 % (10).

In an early biosynthesis experiment, precipitation of the alkaloids from the aqueous extract at pH 9 was carried out since this procedure was followed in the large scale isolation procedure. It was observed that some alkaloids remained in the supernatant (about 10 %) and that some non-alkaloidal polar compounds coprecipitated with the alkaloid fraction after standing 24 hours about 38°F. A thin layer chromatography scan of the alkaline precipitated fraction is shown in Figure 7, and it is clearly evident that a substantial amount of the radioactivity did not move in the solvent system used for the alkaloids. This partial fractionation and contamination of the alkaloid fraction with polar compounds was undesirable so an improved procedure for isolating alkaloid that avoided the alkaline precipitation step following biosynthetic studies was developed.

Attempts to separate four structurally related alkaloids from the alkaloid fraction by use of thin layer chromatography was unsuccessful mainly due to their high and similar molecular weight and small differences in their functional groups. The thin layer chromatography R_f values of the four reference alkaloids[1] and that of a prepared alkaloid fraction developed with various solvent systems are shown in Table IV. Several relative polar solvent systems were examined; however, extensive tailing was observed, therefore, they are not listed in Table IV.

The alkaloid zone gave a positive brownish-yellow color upon spraying with Dragendorff's reagent and quenched UV light at 254 nm. The alkaloid zone was scraped off the plate, extracted and crystallized as previously described and the crystallized mixture of alkaloids was

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[1]Four alkaloids were provided by Morton Beroza

subjected to mass spectrometric analysis using the direct probe.

The results shown in the bottom of Figure 8 clearly show that the alkaloids zone on the thin layer chromatography plate is a mixture of four alkaloids. The figure shows the effect of a change in the probe temperature on the molecular ion peak height of the four ester alkaloids. Wilforgine and wilfortrine show maximum molecular ion peak height at 135°C and wilforine and wilfordine show maximum peak height above 135°C. Detection and identification of compound(s) from a mixture of compounds in one peak by taking a series of spectra during the peak emerging period time was reported (58).

B. Partition Chromatography of the Alkaloid Mixture

The results obtained when the alkaloid mixture was subjected to partition chromatography on silicic acid using hydrochloric acid as an immobile solvent and ether as a mobile solvent are shown in Figures 9 and 10. The concentration of hydrochloric acid in the immobile phase had a significant effect on the elution time of the constituents (compare Figures 9 and 10). The higher concentration of hydrochloric acid (Figure 9) caused a slower movement of the alkaloids with improved resolution. The general chromatographic pattern is similar to the result obtained by Beroza (61) who discussed in detail the conditions, advantages, limitations and sources of error of silicic acid column chromatography and the use of the absorbancy ratio for the detection of impurities in T. wilfordii Hook alkaloids.

Each of the two main peaks showed a shoulder and was widely separated. The two main peaks had an absorbancy ratios of about 1.5 and the two shoulders (tube no. 8-12 and 69-72) of each peak which had an absorbancy ratios of about 1.0 were pooled, rechromatographed on

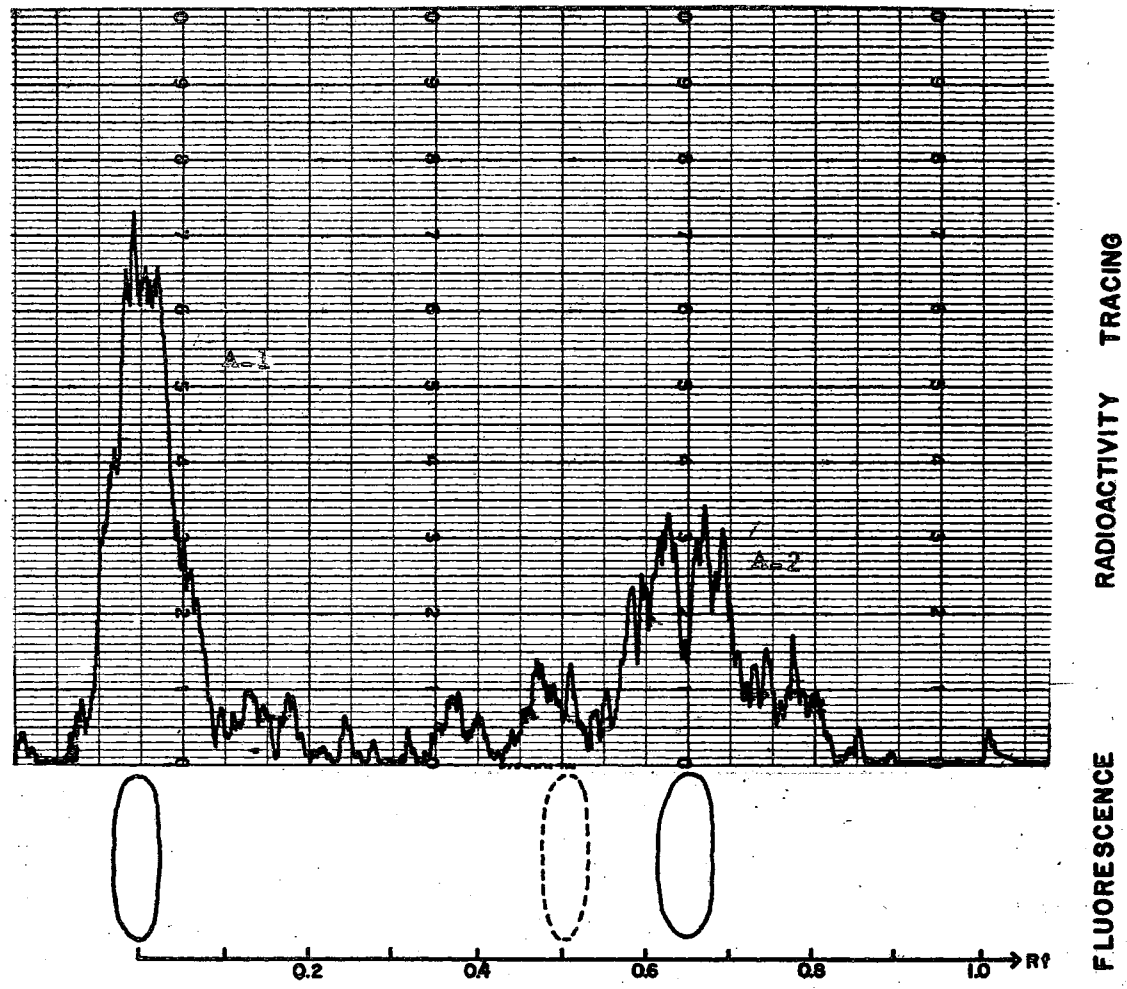


Figure 7. Thin Layer Radiochromatogram of Alkaline Precipitate from the Extract of a Plant fed with Nicotinic acid-6-^{14}C after 4 days (Solvent, acetone;hexane,8:2)

TABLE IV

THIN LAYER CHROMATOGRAPHY R_f VALUES OF THE ALKALOIDS FROM T. WILFORDII HOOK

Solvent system / Alkaloid	I	II	III
		R_f value	
Wilforine	0.12	0.26	0.74
Wilforgine	0.16	0.26	0.72
Wilfordine	0.17	0.28	0.78
Wilfortrine	0.16	0.25	0.77
Isolated alkaloid fraction	0.09-0.2	0.23-0.26	0.7-0.81

I - n-butanol : hexane (40 : 60, v/v)
II - n-butanol : hexane : chloroform (40 : 50 : 10, v/v/v)
III - acetone : hexane (80 : 20, v/v)

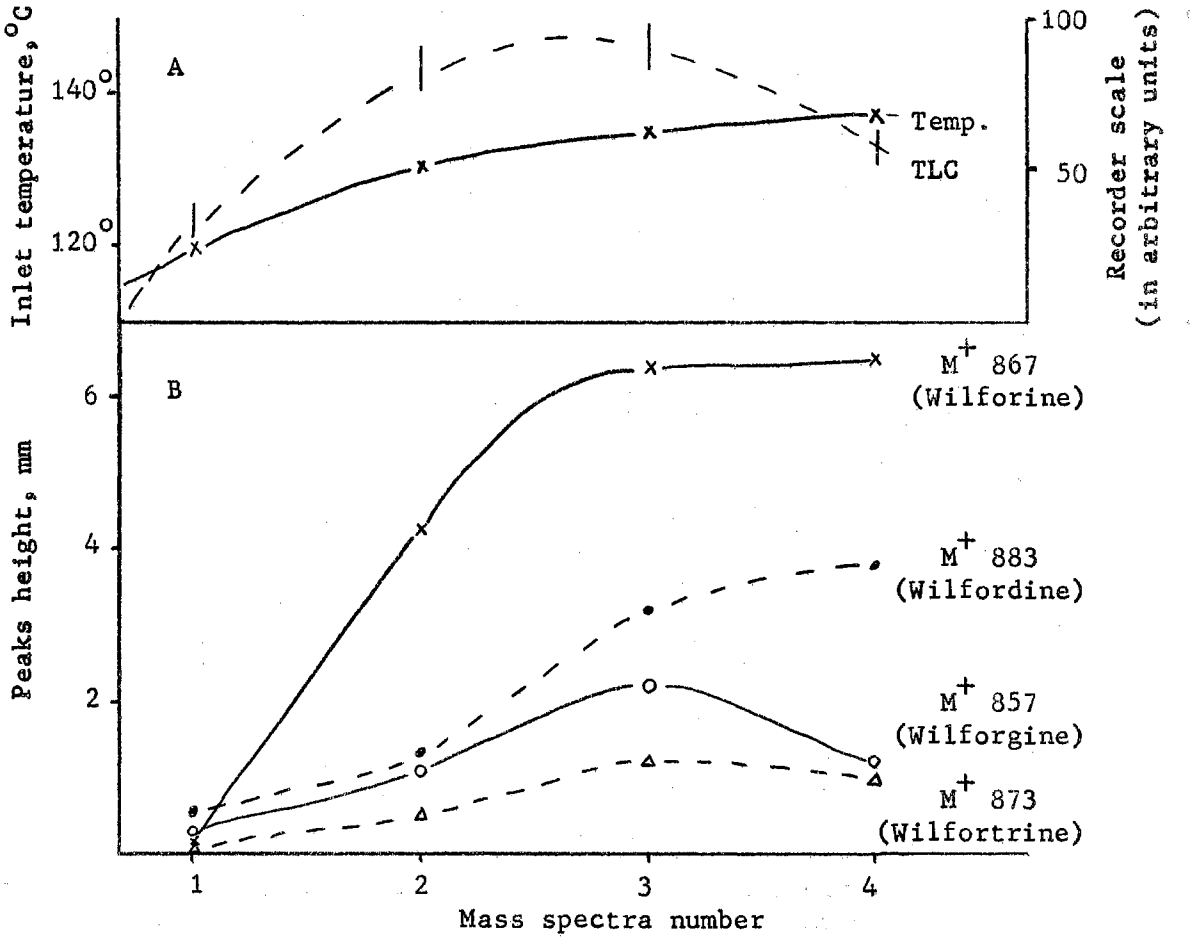


Figure 8. Molecular Ion Peak Height of T. wilfordii Hook Alkaloids (B) as a Function of the Direct Probe Temperature and Total Ion Current (A)

Slash marks denote where mass spectra were taken and molecular ion intensity was measured from each mass spectrum.

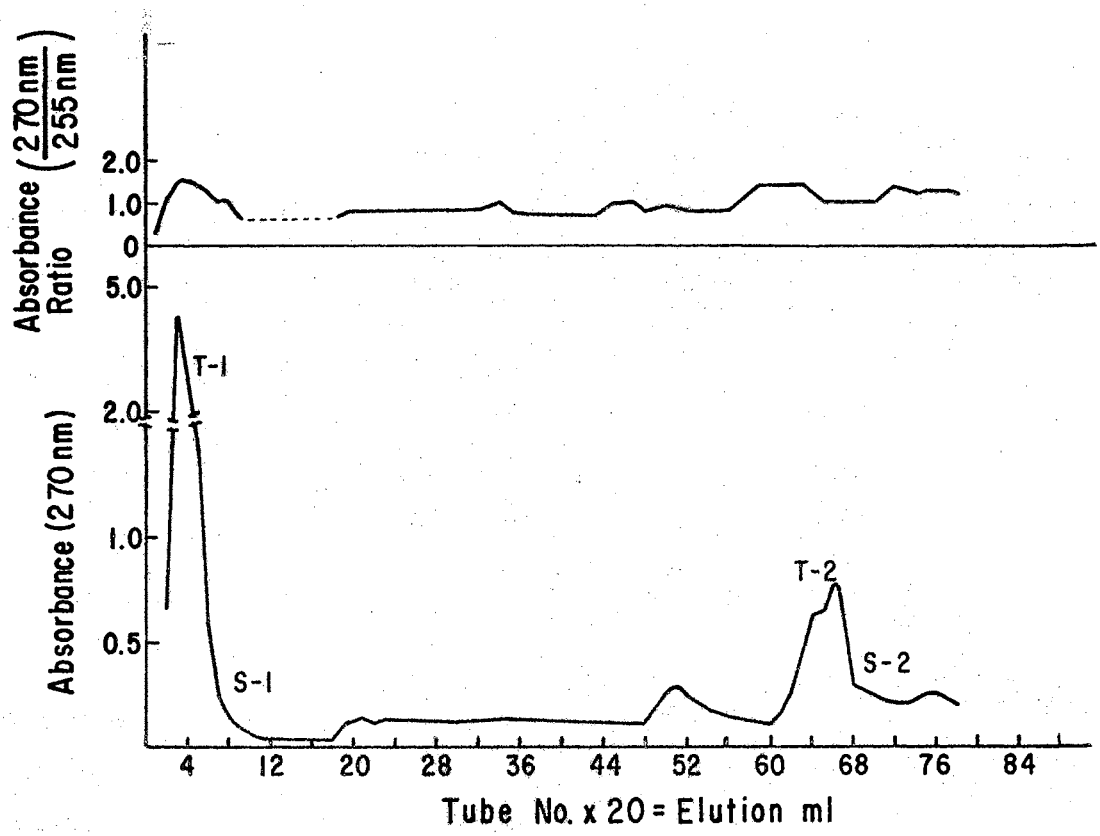


Figure 9. Partition Column Chromatography of T. wilfordii Hook Alkaloids using Absorbancy Ratios. Silicic acid column, 1.5 x 45 cm, was developed with 2 % HCl as an immobile solvent and ether as a mobile solvent. The flow rate of the column was adjusted 60 ml/hr.

T-1 - Wilforine
S-1 - Wilforgine
T-2 - Wilfordine
S-2 - Wilfortrine

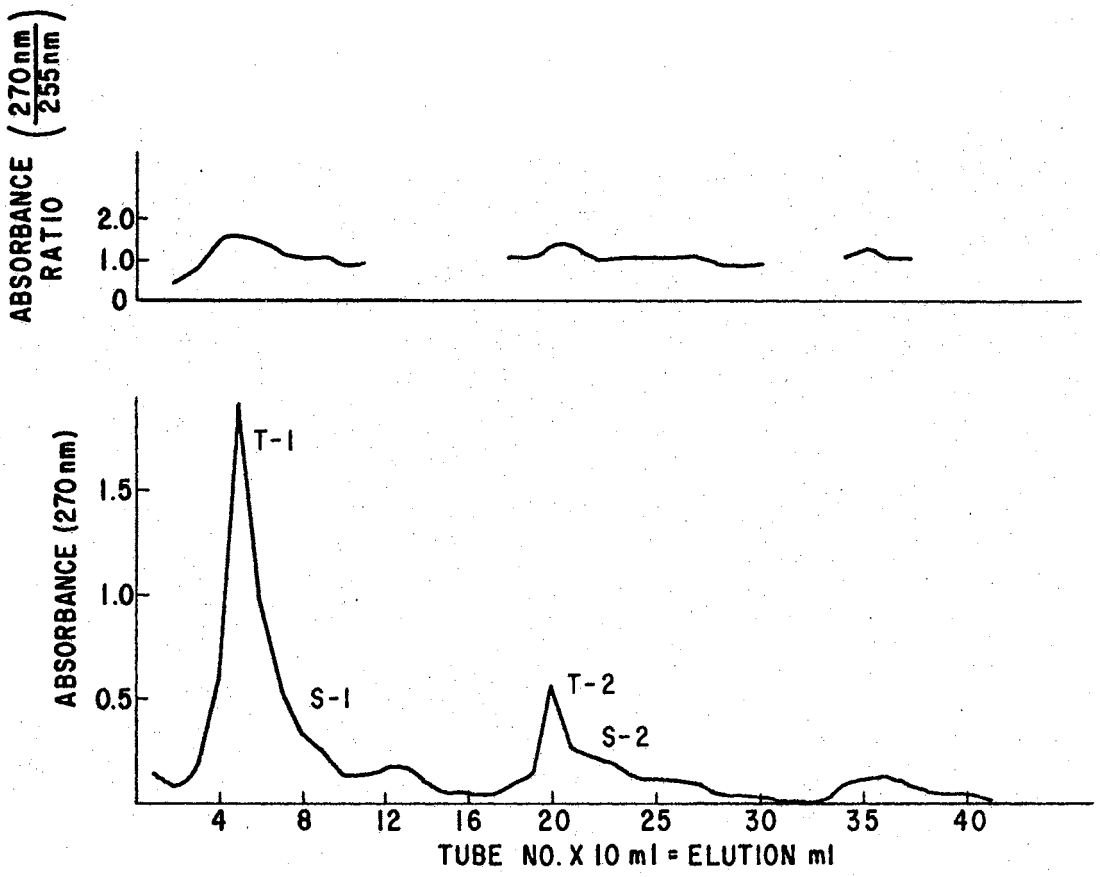


Figure 10. Partition Column Chromatography of T. wilfordii Hook Alkaloids using Absorbancy Ratios. Silicic acid column, 1.5 x 45 cm, was developed with 0.6 % HCl as an immobile solvent and ether as a mobile solvent. The flow rate of the column was adjusted 60 ml/hr.

T-1 - Wilforine
S-1 - Wilforgine
T-2 - Wilfordine
S-2 - Wilfortrine

thin layer chromatography, recrystallized from acetone and methanol and identified mass spectrometrically. The two main peaks, T-1 and T-2, were identified as wilforine and wilfordine and the two shoulders, S-1 and S-2 as wilforgine and wilfortrine respectively.

C. Mass Spectrometric Studies on the Ester Alkaloids

The mass spectra of wilforine, wilforgine, wilfordine and wilfortrine are shown in Figures 11 and 12 respectively. The molecular ions of the four ester alkaloids were found to be identical with the reported molecular weights (13). The molecular formula of each compound was determined by high resolution mass spectrometry and it was found that the molecular formulae were in agreement with the reported molecular formulae obtained by Beroza based on elemental analysis in 1953 as shown in Table V. The relative intensity of each ester alkaloid molecular ion in both the low and high resolution mass spectra was significantly higher than expected for compounds of such high molecular weight (e.g. glucose TMS derivatives) (56). There were discrepancies between the relative intensity values of the high and low resolution mass spectra because the conversion of optical densities recorded on the high resolution photographic plates into intensity values is not straightforward and may be subject to inherent error such as variation in the thickness and composition of the emulsion (62).

It is understandable that the fragmentation pattern of the four ester alkaloids should be somewhat similar, since these alkaloids have the same polyhydroxy moiety which is esterified with structurally related compounds. This is especially true in the high and low mass region. The relative intensity of the ten most intense peaks of each

alkaloid is shown in Table V.

The fragmentation pattern of wilforine and wilforgine which contain wilfordic acid and that of wilfordine and wilfortrine which contain hydroxywilfordic acid are similar. The molecular ion of wilforine and wilforgine constitute one of the ten most intense peaks whereas that of wilfordine and wilfortrine do not, probably because of the presence of the additional hydroxy group. The base peaks of wilforine and wilforgine are m/e 178, $C_9H_8NO_3$, and m/e 93, C_6H_7N, respectively. The ion, m/e 43, C_2H_3O, is the base peak in both wilfordine and wilfortrine.

The high resolution mass spectrometric data of the four ester alkaloids with their relative intensities, formulae and accuracy in measurement are attached in Appendix. The common mode of fragmentation of the four ester alkaloids in the high mass region over m/e 780, are the loss of the neutral fragments CO, CH_3CO, CO_2, $COOCH_3$ and CH_2COOCH_3 which are summarized in Table VI. The loss of a methyl group is another characteristic fragmentation of these alkaloids. Since each alkaloid has only one methyl group and the only subcomponents which contain a methyl group are the substituted pyridine moieties wilfordic acid and hydroxywilfordic acid, it may be concluded that the loss of the methyl group is from the side chain of these subcomponents. Therefore, it is possible to quickly identify each of these compounds by observing their respective molecular ion, the fragment ion produced by loss of the methyl group $(M-CH_3)^+$ and the ions $[(M-CO)^+$, $(M-COOCH)^+$, etc.] produced by the cleavage of their ester bonds. The loss of fragments resulting from cleavage of the ester bonds are also supported by observed metastable peaks which are listed in Table VII along with the denoted transitions; however, the only metastables observed were in the low mass range so that they indicate a different set of cleavage

reaction than those representing the loss of the functional groups in the high mass region.

There are no interpretable intense peaks from m/e 250 to m/e 700 in both the low and high resolution mass spectra of the four ester alkaloids. The structure and fragmentation of the ions containing nitrogen and/or oxygen under m/e 250 will be discussed in the mass spectra of individual acidic subcomponents since most of the peaks of that region are fragments derived from acidic subcomponents upon cleavage of the ester linkages.

The fragment ions above m/e 100 containing only carbon and hydrogen are listed in Table VIII together with their proposed structures. The origin of these ions is noteworthy since the only subcomponent which may contribute to these ions, especially those which contain more than 10 carbon atoms, is the polyhydroxy moiety, unless the pyridine moieties undergo extensive rearrangement, a phenomenon which is highly unlikely. If we assume that these ions containing more than 10 carbon atoms, such as $C_{10}H_9$ through $C_{15}H_{10}$ are derived from the polyhydroxy moiety, then it is possible that the carbon skeleton of the polyhydroxy compound is a substituted tricyclohexane.

The fragmentation of the intact alkaloids (see Figures 11 and 12 for mass spectra) will not be discussed, because the proposed fragmentation of a compound without knowing its structure has little significance.

D. Infrared Spectra of Ester Alkaloids

The infrared spectra of wilforine, wilforgine, wilfordine and wilfortrine are shown in Figures 13 and 14 respectively. Infrared spectra of wilforine and wilfordine in CCl_4 and in nujol were reported

by Beroza (11). The infrared spectra of the four ester alkaloids using the KBr pellet are very similar except for a few peaks. A comparison of the infrared spectra of the four ester alkaloids with correlation of the most significant peaks to their infrared functional group frequencies is given in Table IX. A very broad peak from 2.7 to 3 microns which indicated the presence of a hydroxyl group is present in the spectra of each of the four alkaloids. The strong absorption at 5.75 and 7.3 microns indicated the presence of an ester bond(s) in the four compounds. A weak absorption peak at 6.07 microns which might indicate the presence of an aromatic group in the alkaloids was present in the infrared spectra of wilforine and wilfordine which contain benzoic acid as one of the acidic subcomponents. A sharp absorption peak with medium intensity at 13.25 microns, which might represent a carbon-carbon double bond in the molecule, is present in the infrared spectra of wilforine and wilfortrine. An interesting peak in the infrared spectra of all four alkaloids is the moderately strong CH_2 "scissoring" absorption at 7.2 microns indicating the presence of a cycloalkane structure.

The infrared spectra of four ester alkaloids indicated that the four alkaloids are closely related structurally and that each had one or more hydroxyl groups and multiple ester linkages.

E. Mass Spectra of the Acidic Subcomponents

All the mass spectra of the acidic subcomponents were obtained using the gas chromatographic inlet system following esterification with diazomethane. Analyses were performed either by temperature programming or by isothermal using two different temperatures; the latter method required two times injection into the column; however,

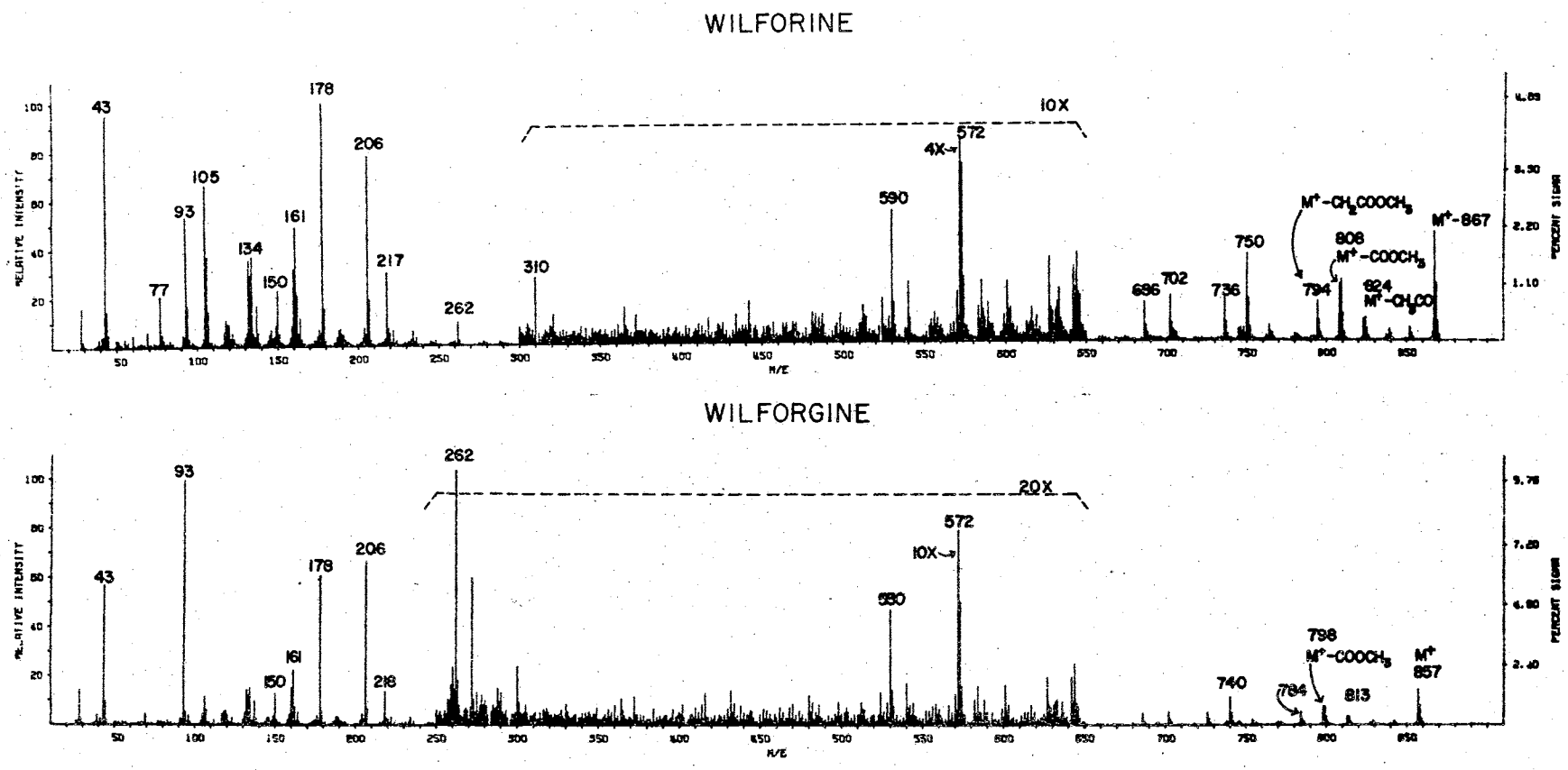


Figure 11. Mass Spectra of Wilforine and Wilforgine

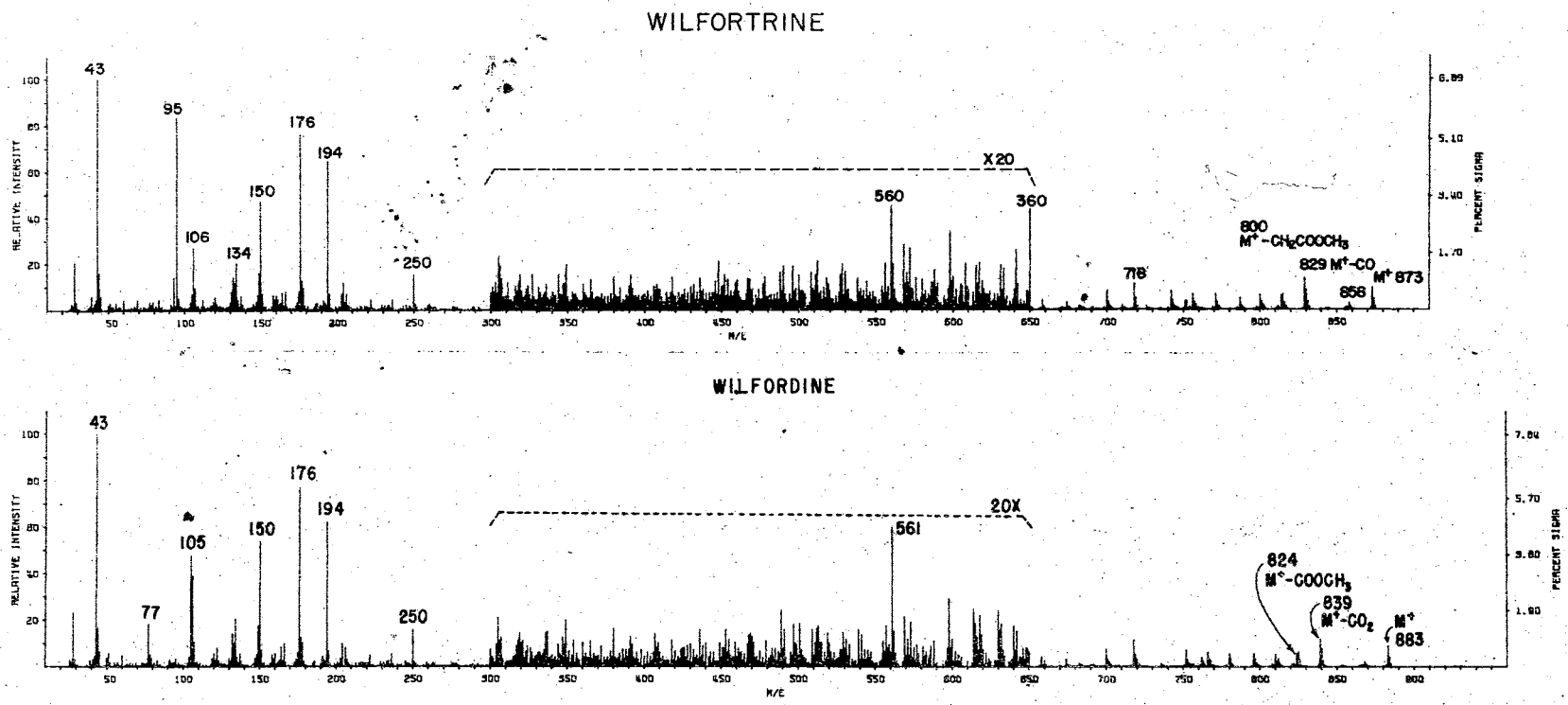


Figure 12. Mass Spectra of Wilfordine and Wilfortrine

TABLE V

SUMMARY OF THE MASS SPECTRA OF THE FOUR ESTER ALKALOIDS FROM *T. WILFORDII* HOOK

T. wilfordii alkaloid	Mol. formula (10, 11)	Mol. ion $(M)^{+1}$ (Lee, 1971)	Ten most intense peaks (relative intensity in percent)									
Wilforine	$C_{43}H_{49}O_{18}N$	$C_{43}H_{49}O_{18}N^{+\cdot}$	43 (95)	93 (53)	105 (67)	106 (57)	132 (55)	134 (67)	161 (49)	178 (100)	206 (78)	867 (44)
Wilforgine	$C_{41}H_{47}O_{19}N$	$C_{41}H_{47}O_{19}N^{+\cdot}$	43 (57)	93 (100)	132 (15)	134 (13)	150 (13)	160 (15)	161 (22)	178 (61)	106 (86)	857 (13)
Wilfordine	$C_{43}H_{49}O_{19}N$	$C_{43}H_{49}O_{19}N^{+\cdot}$	28 (23)	43 (100)	77 (18)	95 (85)	105 (47)	106 (38)	134 (20)	150 (74)	176 (78)	194 (63)
Wilfortrine	$C_{41}H_{47}O_{20}N$	$C_{41}H_{47}O_{20}N^{+\cdot}$	28 (22)	43 (100)	44 (15)	95 (82)	106 (27)	134 (20)	149 (15)	150 (46)	176 (77)	206 (65)

[1]Molecular ion was determined by high resolution mass spectrometry

TABLE VI

COMMON FRAGMENTATION MODES OF THE FOUR ESTER ALKALOIDS FROM T. WILFORDII HOOK (OVER MASS 780 REGION)

	M^+	$(M - CH_3)^+$	$(M - CO)^+$	$(M - CH_3CO)^+$	$(M - CO_2)^+$	$(M - COOCH_3)^+$	$(M - CH_2COOCH_3)^+$
Wilforine	867	852	839	824	823	808	794
Wilforgine	857	842	829	814	813	798	784
Wilfordine	883	868	855	840	839	824	810
Wilfortrine	873	858	845	830	829	814	800

TABLE VII

METASTABLE PEAKS IN THE MASS SPECTRA OF WILFORINE, WILFORGINE, WILFORDINE AND WILFORTRINE

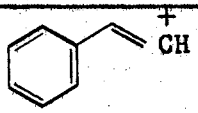
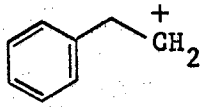
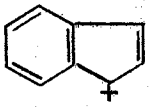
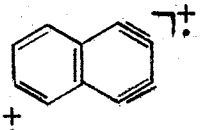
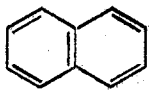
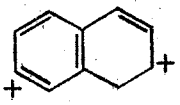
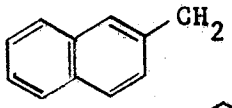
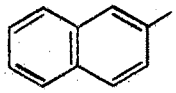
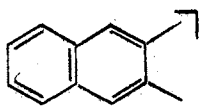
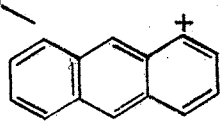
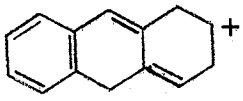
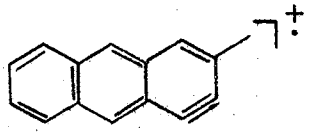
Apparent mass of metastable ion	Transition denoted	Possible natural product	I	II	III	IV
92.83	$(150)^+ \rightarrow (118)^+ + 32$	O_2 or CH_3OH		+		
102.02	$(176)^+ \rightarrow (134)^+ + 42$	CH_2CO			+	+
104.90	$(206)^+ \rightarrow (147)^+ + 59$	$COOCH_3$	+			
109.22	$(206)^+ \rightarrow (150)^+ + 56$	$2CO_2$	+	+		
132.01	$(134)^+ \rightarrow (133)^+ + 1$	H		+		
143.82	$(178)^+ \rightarrow (160)^+ + 18$	H_2O	+	+		
153.81	$(206)^+ \rightarrow (178)^+ + 28$	CO	+	+		
159.67	$(194)^+ \rightarrow (176)^+ + 18$	H_2O			+	+
178.09	$(207) \rightarrow (192) + 30$	CH_2OH	+	+		

+ - Indicate presence of metastable ions

I - Wilforine
II - Wilforgine
III - Wilfordine
IV - Wilfortrine

TABLE VIII

FRAGMENT IONS CONTAINING CARBON AND HYDROGEN IN THE MASS SPECTRA OF THE FOUR ALKALOIDS FROM T. WILFORDII HOOK

m/e	C H	Proposed structure	I	II	III	IV
103	C_8H_7		+	+	+	+
105	C_8H_9		+			+
115	C_9H_7		+	+		
124	$C_{10}H_4$		+		+	
128	$C_{10}H_8$		+	+	+	+
129	$C_{10}H_9$		+	+	+	+
141	$C_{11}H_9$		+		+	+
142	$C_{11}H_{10}$		+	+	+	+
156	$C_{12}H_{12}$			+		
177	$C_{14}H_9$				+	+
182	$C_{14}H_{14}$					+
187	$C_{15}H_7$			+		
189	$C_{15}H_9$			+		
190	$C_{15}H_{10}$			+		

I - Wilforine II - Wilforgine III - Wilfordine IV - Wilfortrine
Position of side chain is arbitrary. Detailed high resolution mass spectrometric data are shown in Appendix

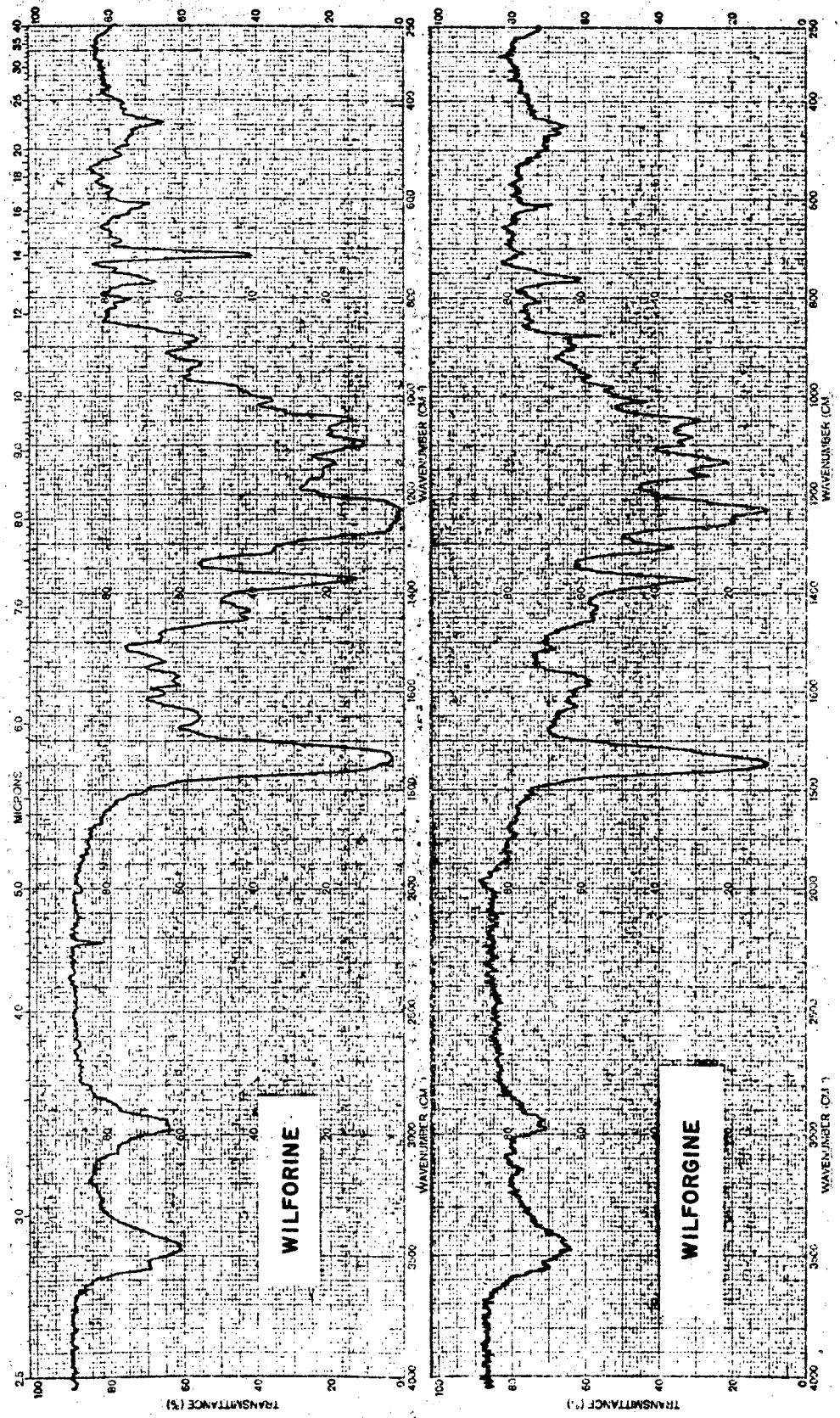


Figure 13. Infrared Spectra of Wilforine and Wilforgine

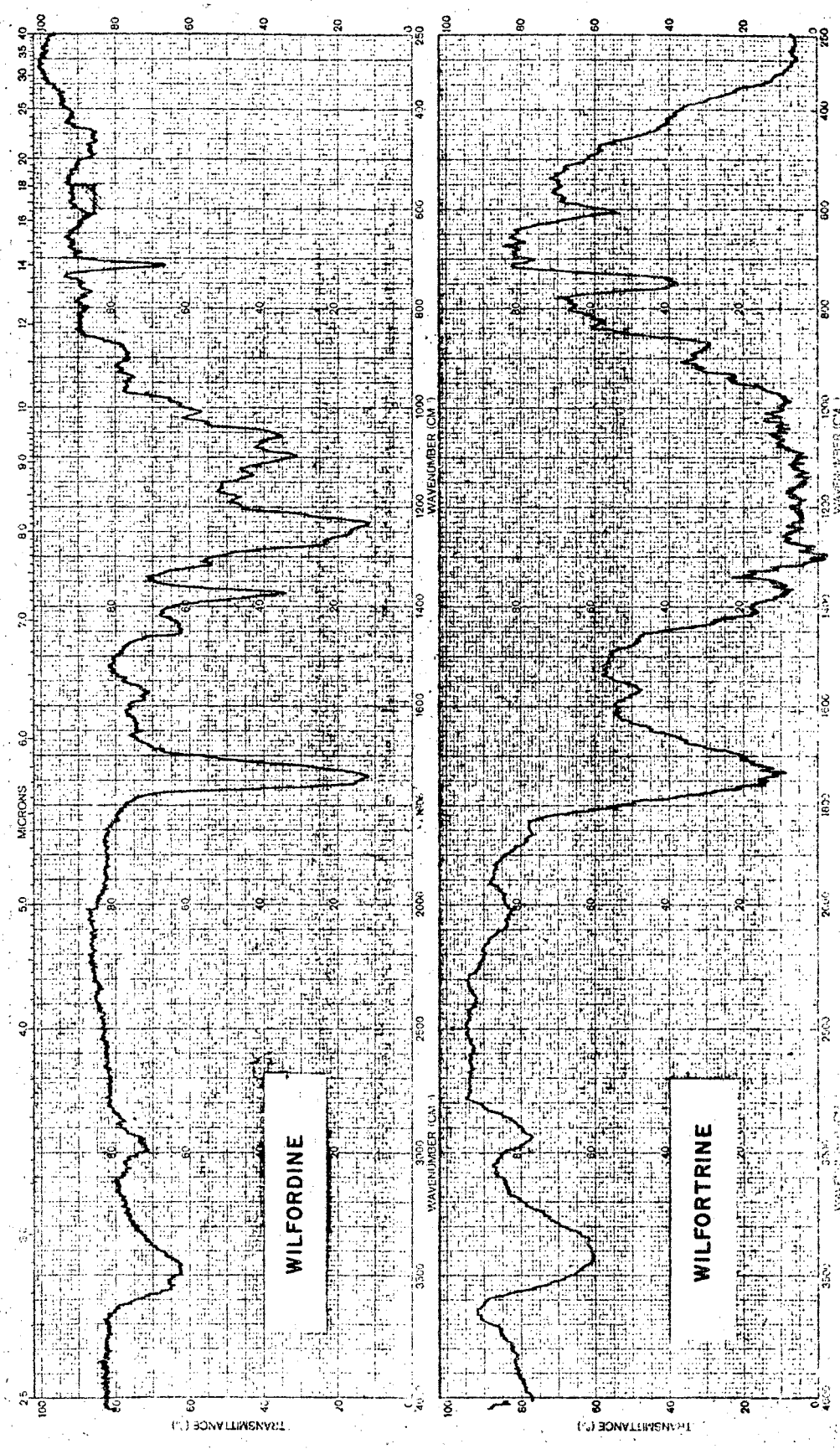


Figure 14. Infrared Spectra of Wilfordine and Wilfortrine

TABLE IX

COMPARISON OF INFRARED SPECTRA
OF FOUR ESTER ALKALOIDS

nm	Wilforine	Wilforgine	Wilfordine	Wilfortrine	Remarks
2.75-3.0	m (v.br.)	m (v.br.)	m (v.br.)	m (v.br.)	OH
3.2-3.5	w (br.)	w (br.)	w (br.)	w (br.)	
5.75	vs	vs	vs	vs	$R-\overset{O}{\overset{\|\|}{C}}$
6.07	w	-	vw	-	Aromatic
7.0	m	m	m	m	$-\overset{\|}{C}-\overset{\|}{C}-$ $\overset{\|}{O}$
7.3	s	s	s	s	$CH_3\overset{O}{\overset{\|\|}{C}}-O$
7.65	sh	s	sh	sh	
8.2	vs (br.)	vs (br.)	vs (br.)	vs (v.br.)	
8.7	s	s	s	s (br.)	$-\overset{\|}{C}-O$
9.2	s	s	s	s (br.)	$-C=C-$ Aromatic
13.25	-	m	-	m	
14.0	s	-	m	-	

Abbreviation given in "Experimental Methods".

the method made it possible to save time. The gas liquid chromatograms obtained from using the above two methods are shown in Appendix.

A typical total ion current tracing of the methylated acidic components is presented in Figure 15. The total ion current tracing peaks corresponding to the molecular ion of dimethylwilfordate, dehydrated dimethylhydroxywilfordate, methylbenzoate, methylfuroate and methylacetate, the methylated derivatives of the reported acidic components of the ester alkaloids, will be discussed in order.

The mass spectrum of component A-13 (refer to Figure 15, total ion current tracing, for designation of the components) is shown in Figure 16. It has a molecular ion at M^+ 251, which corresponds to the molecular weight of the dimethyl ester of wilfordic acid. The spectrum exhibited a characteristic fragmentation pattern of a pyridine compound with α-side chain (63). The proposed fragmentation of dimethyl wilfordic acid is shown in Figure 17. The molecular ion M^+ 251, $C_{13}H_{17}O_4N$, may lose a methoxy group, a common fragment lost from methyl esters (64, 65), to form the fragment ion a, m/e 220, $C_{12}H_{14}O_3N$. The base peak b, m/e 204, $C_{11}H_{10}O_3N$, may be formed either from a, by loss of one hydrogen atom and a methyl group or directly from the molecular ion by the successive loss of the neutral fragments CH_3, H and OCH_3. The base peak, b, might arise from both of these pathways; however, the latter fragmentation path is more plausible, since the fragment ion at m/e 204, b, is more intense than the ion at m/e 220, a.

The base peak, b, m/e 204, loses OCH_3 and CHCO to form the ion c, m/e 132, C_8H_6ON; further stepwise loss of CO and C_2H_2 can occur to form ions d, C_7H_6N, and e, C_5H_4N, respectively. The ion e, which is a common fragment formed from substituted pyridine compounds may be

formed either from the ion c, by loss of CO and C_2H_2 or from the ion f, by the loss of C_3H_3.

The fragment ion a, m/e 220, $C_{11}H_{10}O_3N$, may lose 28 mass units (CO) to yield ion g, $C_{11}H_{14}O_2N$, which may again lose C_2H_4 and OCH_3 to form ions h, m/e 164, $C_9H_{10}O_2N$, and c, m/e 132, C_8H_6ON, respectively. Another ion i, $C_9H_{10}N$, may be formed from molecular ion by the loss of $COOCH_3$ from pyridine ring at C-2 and mass unit 14, H and CH_3, from C-2 side chain.

Further successive loss of CH_3 and C_3H_3 from ion i, $C_9H_{10}N$, yields the ions f, C_8H_7N, and e, C_5H_4N, respectively. The ions m/e 132, 117 and 104, which have a positive charge at C-3 of the pyridine ring might be plausible in terms of the view that in pyridine the electron density is relatively high at para but low at the ortho and meta-position.

The mass spectrum of compound A-15 is shown in Figure 16. This spectrum does not show a peak corresponding to the molecular ion of dimethylhydroxywilfordate, M^+ 267, however the ion h, m/e 249, $C_{13}H_{15}O_4N$, which may be formed by the loss of water $[M^+ - 18(H_2O)]$ from the molecular ion is present. The absence of the molecular ion due to elimination of water from higher alcohols is a common phenomenon and was extensively studied by McFadden et al. and McLafferty with deuterium labelled compounds (66, 67). In higher alcohols, dehydration may also occur as a result of thermal decomposition prior to electron impact; a finding confirmed by the observation that the intensity of the $M^+ - 18$ is greatly reduced when the sample is inserted near the ion source (68).

The mass spectra of A-15 and that of A-13 are similar in the region from m/e 160 to m/e 100 but different in the high and low m/e regions. The difference in the fragmentation of dimethylhydroxy-

wilfordate in the high m/e region is most likely due to the tertiary hydroxyl group on the side chain at C-2. Once dimethylhydroxywilfordate lost the oxygen containing groups from the molecule, the fragmentation pattern of the remaining ion should be similar to that of dimethylwilfordate in the region of m/e 100 to 160. In the low m/e region, the contribution of oxygen containing fragments might make the mass spectrum of dimethylhydroxywilfordate different from that of dimethylwilfordate since it is a well known phenomenon that the positive charge on the oxygen has a substantial influence on the degradation of long chain alcohols (69).

The proposed fragmentation of dimethylhydroxywilfordate is shown in Figure 18. The base peak a, m/e 176, $C_{10}H_{10}O_2N$, may be formed by the loss of a methoxyl group from the side chain at C-3 and $HCOOCH_3$ from the side chain at C-2. The ion a, m/e 176, successively loses the neutral fragments, CH_3 and H, and then CH_2CO to from ions b, $C_9H_6O_2N$ and c, C_8H_8ON, respectively. The loss of carbon monoxide from ion b and two hydrogen atoms from ion c to form ion d, m/e 132, which is structurally stable due to the conjugation of double bond with the aromatic pyridine ring. The molecular ion, M^+ 267, may also lose CH_3COO and a hydroxyl group from the C-2 side chain to form the fragment ion e, m/e 191, $C_{11}H_{13}O_2N$; further successive loss of $COOCH_3$, CH_3 and C_3H_3 can occur to form ion f, m/e 117 and ion g, m/e 78, $C_6H_{14}N$. The ion j, m/e 104, C_7H_6N, may also be formed by the successive loss of two $COOCH_3$ fragments and acetylene from ion h, m/e 249, $C_{13}H_{15}O_4N$.

The mass spectrum of component A-13 along with the mass spectrum of methylbenzoate are shown in Figure 19. The spectrum of component A-3 was identical to that of methylbenzoate. The mass spectral behavior of benzoic acid, methylbenzoate and many related compounds have been

extensively studied (70, 71). Methylbenzoate undergoes facile α-cleavage and it was observed that the two abundant fragment ions in the mass spectra of methylbenzoate were due to the loss of the methoxyl and of the methylcarboxyl group.

The mass spectra of component A-2 and 2-methylfuroate are shown in Figure 20. It was reported that one of the acidic subcomponents of wilforgine and wilfortrine was 3-furoic acid, however, 3-furoic acid was not available, so 2-furoic acid was methylated with diazomethane, and its methylated derivative was used for comparison with component A-2. Component A-2 had an identical gas liquid chromatographic retention time with 2-methylfuroate, 7 minutes at a column temperature of 115°C on the SE-30 column, and also the same molecular ion, M^+ 126. However, the fragmentation pattern of component A-2 was so different from 2-methylfuroate and other related furan derivatives (63) that additional information will be necessary before the identity of A-2 can be postulated. Fragment ions such as m/e 67 $(M - COOCH_3)^+$ and m/e 95 $(M - OCH_3)^+$ which are present in the mass spectrum of 2-methylfuroate would be expected; however, the intensity of both major ions in mass spectrum of A-2 are unusually low compared to the other peaks, m/e 45 and m/e 76. The possibility of an unusual rearrangement of 3-methylfuroate by electron impact which could cause the fragmentation of 3-methylfuroate to be quite different from that of the 2-methylfuroate cannot be excluded, but it is extremely unlikely.

Acetate was confirmed as one of the acidic components of the ester alkaloids by observing the molecular ion of methyl acetate M^+ 74, and the major fragmentation peaks, m/e 43, CH_3O and m/e 31, OCH_3. The mass spectra of methylacetate always contained ions from the solvent since

the retention time were similar; however, the presence of methylacetate was unequivocally established.

F. Structural Identification on the Polyhydroxy Compound

1. Nuclear Magnetic Resonance Spectrometer

The recrystallized polyhydroxy compound from the pyridine ester alkaloid had a constant melting point of 197-198°C. The nuclear magnetic resonance (NMR) spectra of polyhydroxy compound and that of the deuterium exchanged compound are shown in Figure 21. The NMR spectrum of the polyhydroxy compound was somewhat simpler than expected based on the view that it was supposed to contain 26 hydrogen atoms. The strong absorption peak at 3.48 ppm which was broad and sharp at the lower and upper parts of the peak respectively and which showed as a hump on the integration curve, indicated that the absorption was contributed by two or more different kinds of hydrogen atoms. To simplify the peak by elimination of exchangeable protons, the polyhydroxy compound was deuterated simply by adding deuterium oxide in the sample holder. The nuclear magnetic resonance spectrum of the deuterium exchanged polyhydroxy compound showed that the size of the peak at 3.48 ppm. was reduced and that a new peak at 3.68 ppm. contributed by a proton on HOD appeared as expected (a comparison of the two nuclear magnetic resonance spectra are summarized in Table X). These results agree with our assumption that the peak at 3.48 ppm. is due to the absorption by two kinds of hydrogen atoms, the first type being a proton on the oxygen of a hydroxyl group and the second type being a proton on a α-hydroxy substituted methine carbon atom. The exchangeability explains why the hydroxyl peak of the polyhydroxy

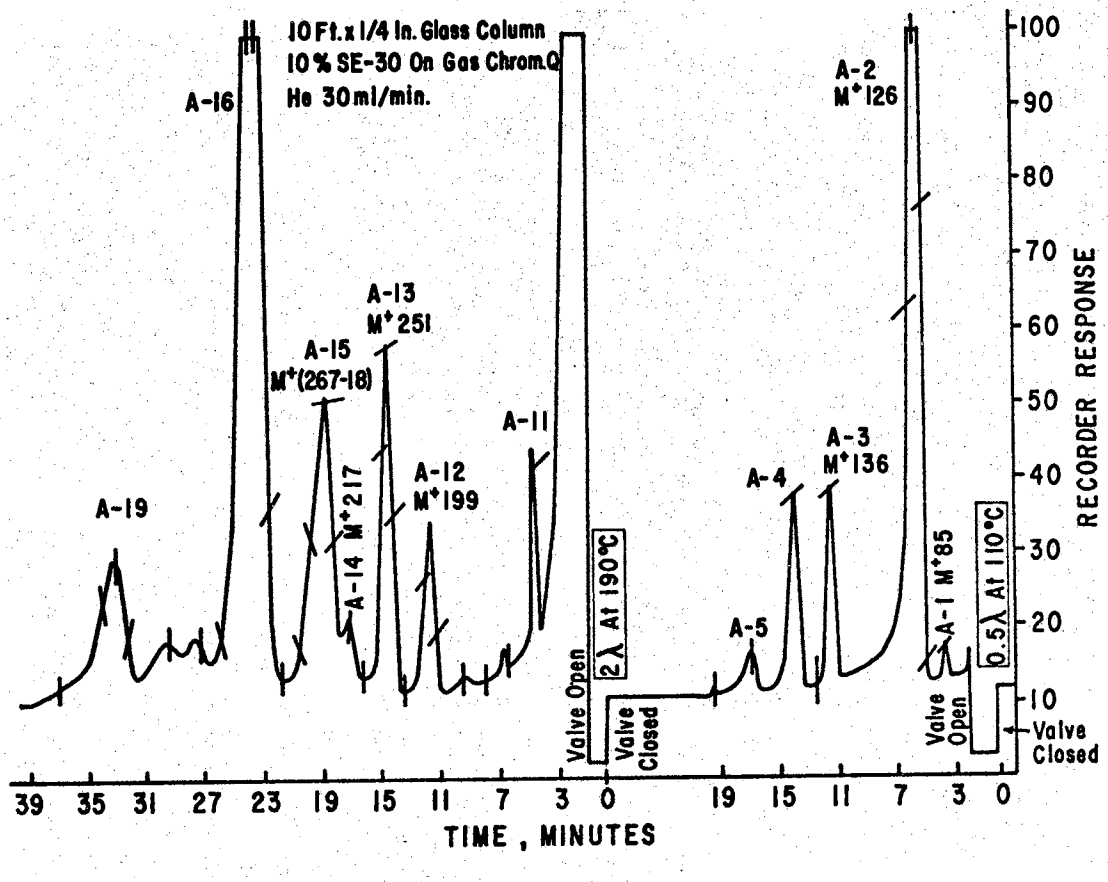


Figure 15. Total Ion Current Tracing of the Methyl Esters of Acidic Components of T. wilfordii Hook Alkaloids. The slash marks along the tracing indicate the point at which mass spectrum was taken. The column, 7 feet x 1/8 inches, was packed with 10 % SE-30 on Gas Chrom Q, 100 - 200 mesh. Operational parameters were as follows: column temperatures of 110°C and 190°C, injection port temperature of 215°C, helium flow rate of 40 ml/min.

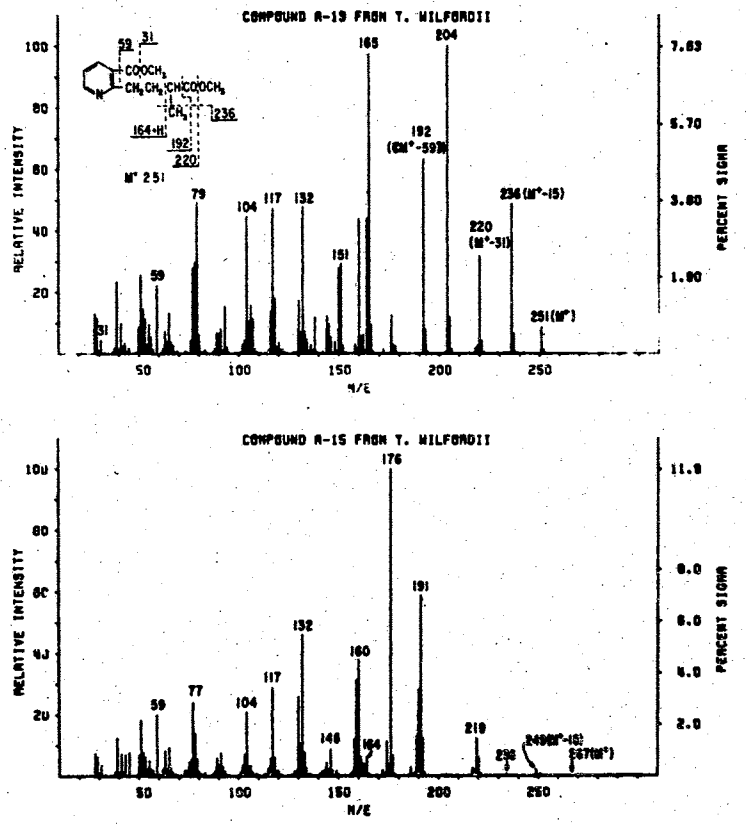


Figure 16. Mass Spectra of the Components A-13 and A-15 from Triptery-gium wilfordii Hook

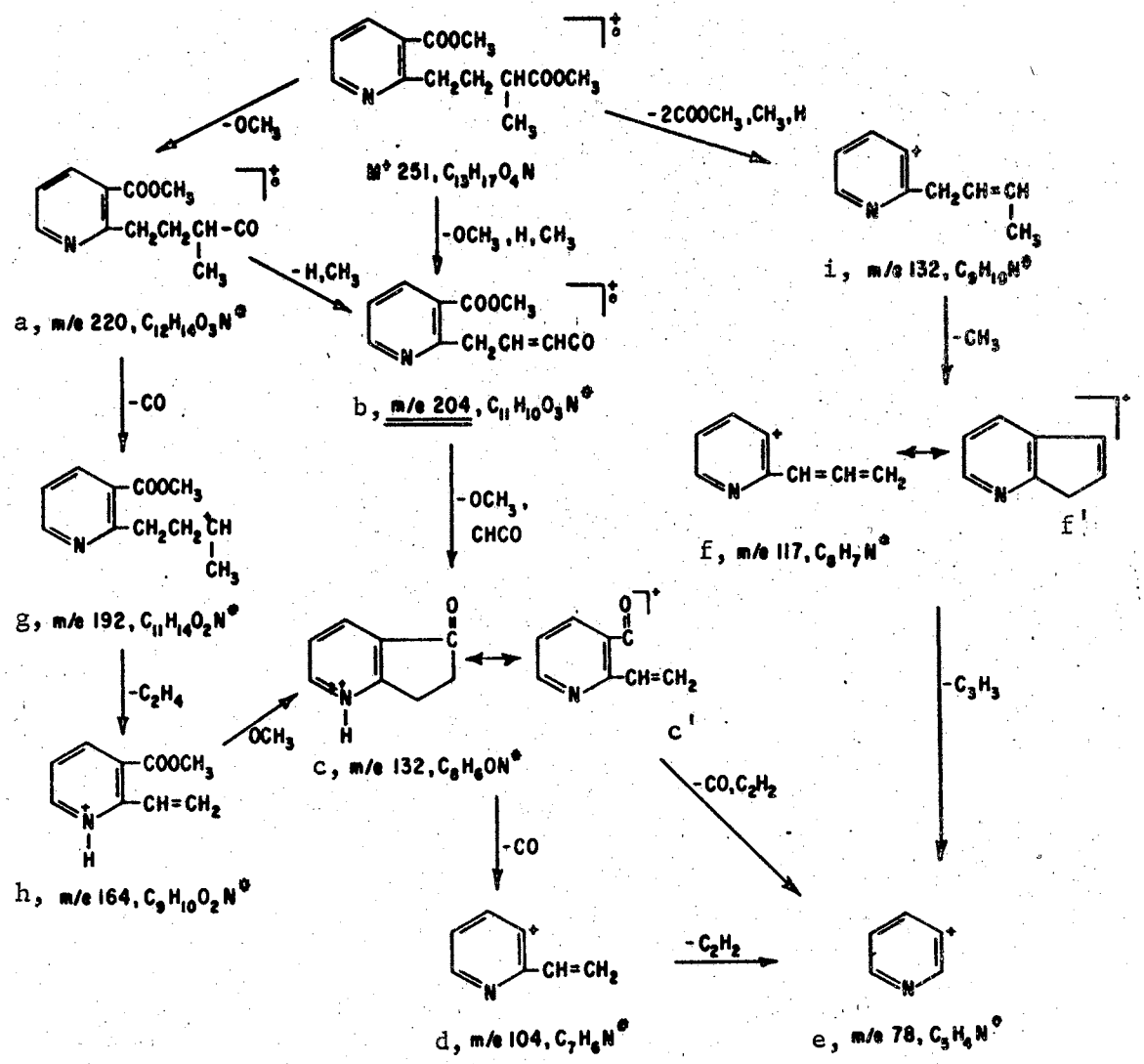


Figure 17. Proposed Partial Fragmentation of Dimethylwilfordate

*Molecular formulae were determined on high resolution mass spectrum of ester alkaloid.

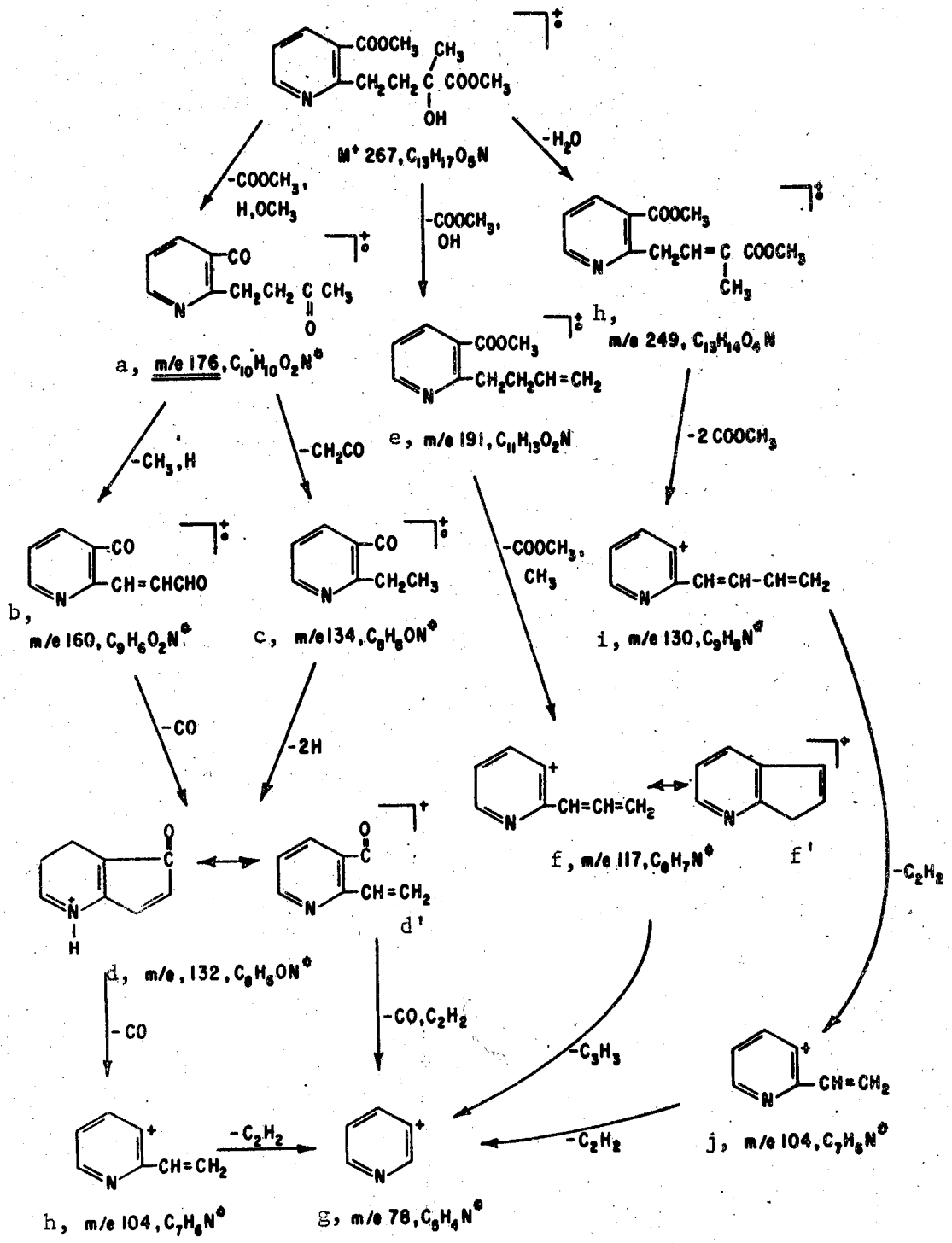


Figure 18. Proposed Partial Fragmentation of Dimethylhydroxy-wilfordate (legend is same as Figure 17)

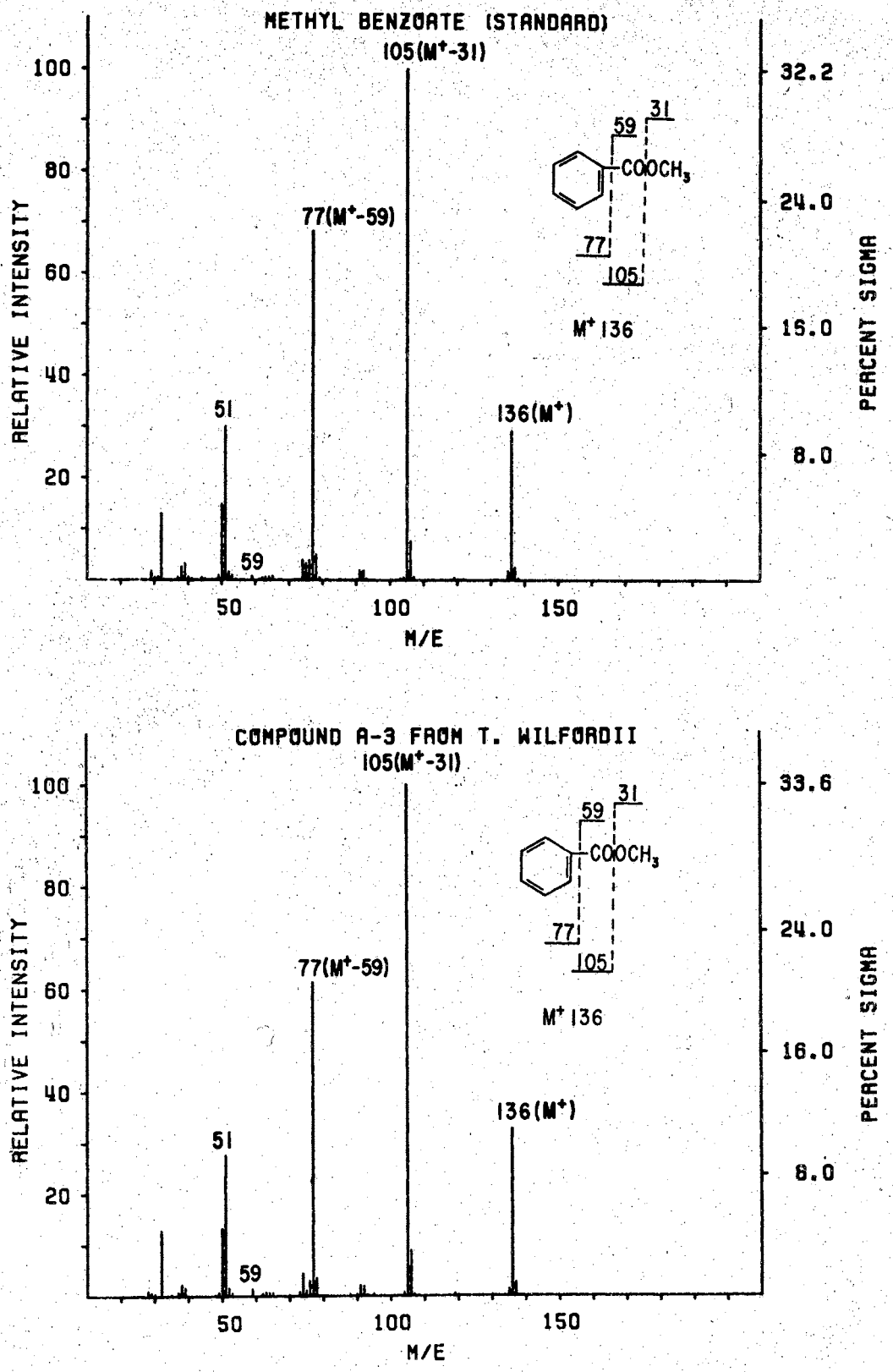


Figure 19. Mass Spectrum of Methylbenzoate (standard) and the Component A-3 from T. wilfordii Hook

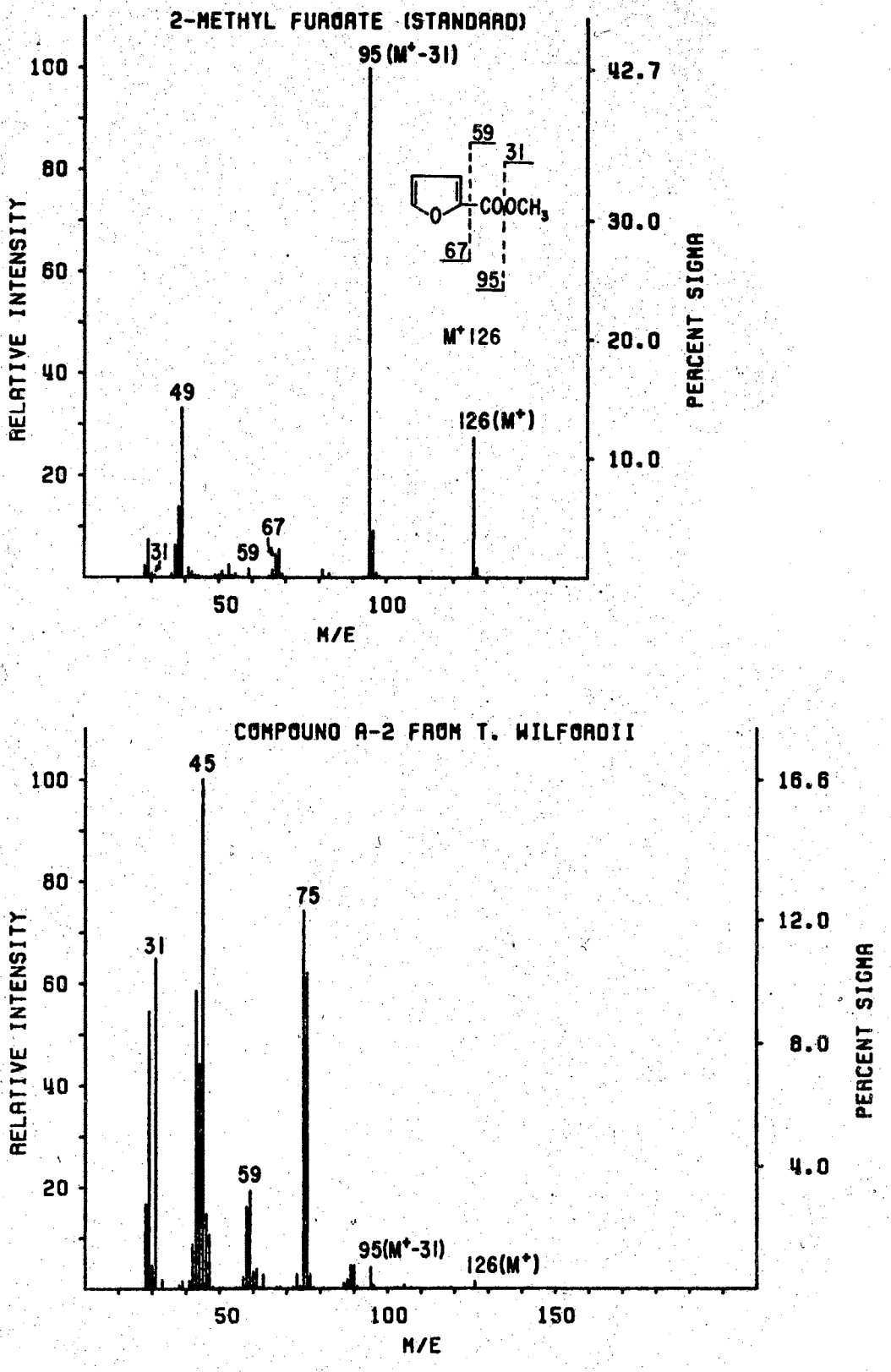


Figure 20. Mass Spectrum of 2-methylfuroate (standard) and the Component A-2 from T. wilfordii Hook

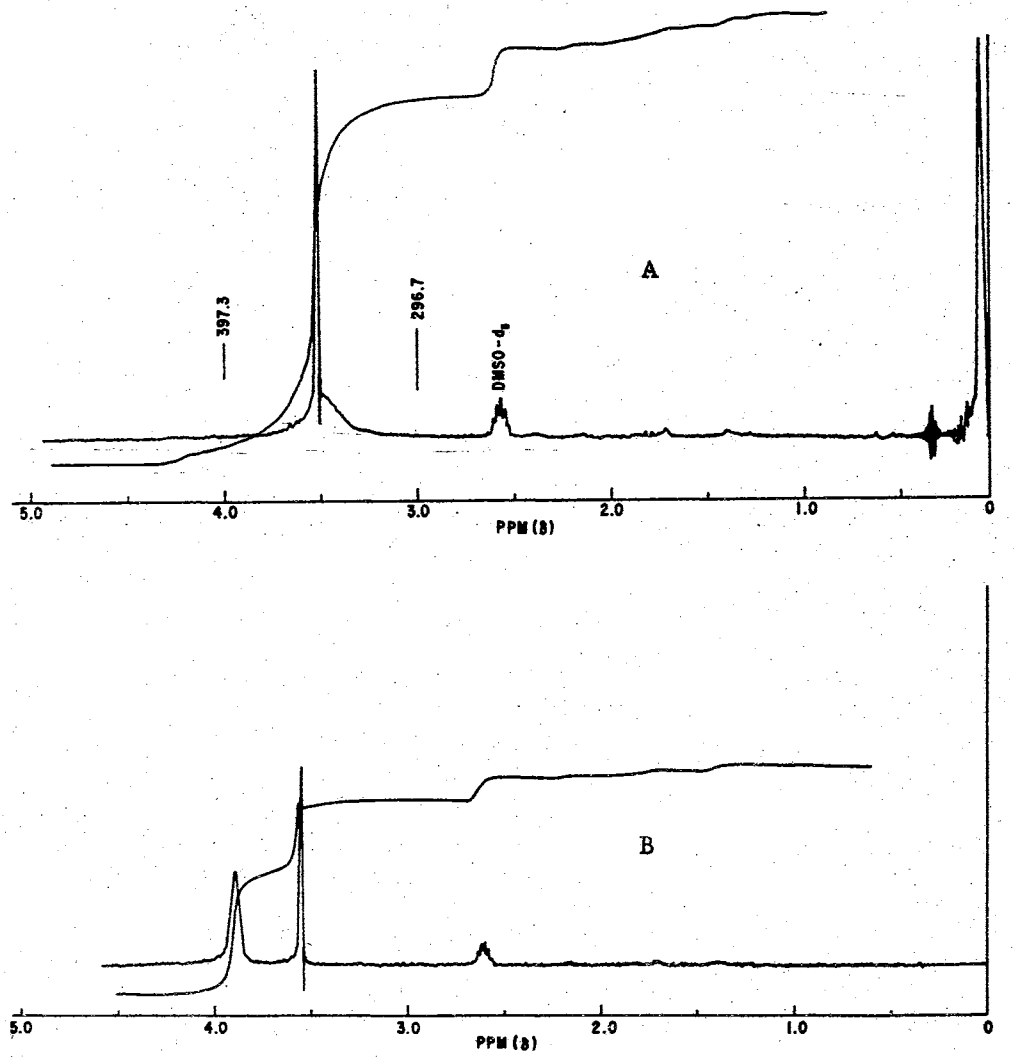


Figure 21. High Resolution NMR Spectra of Polyhydroxy Compound (A) and Its Deuterated Form (B)

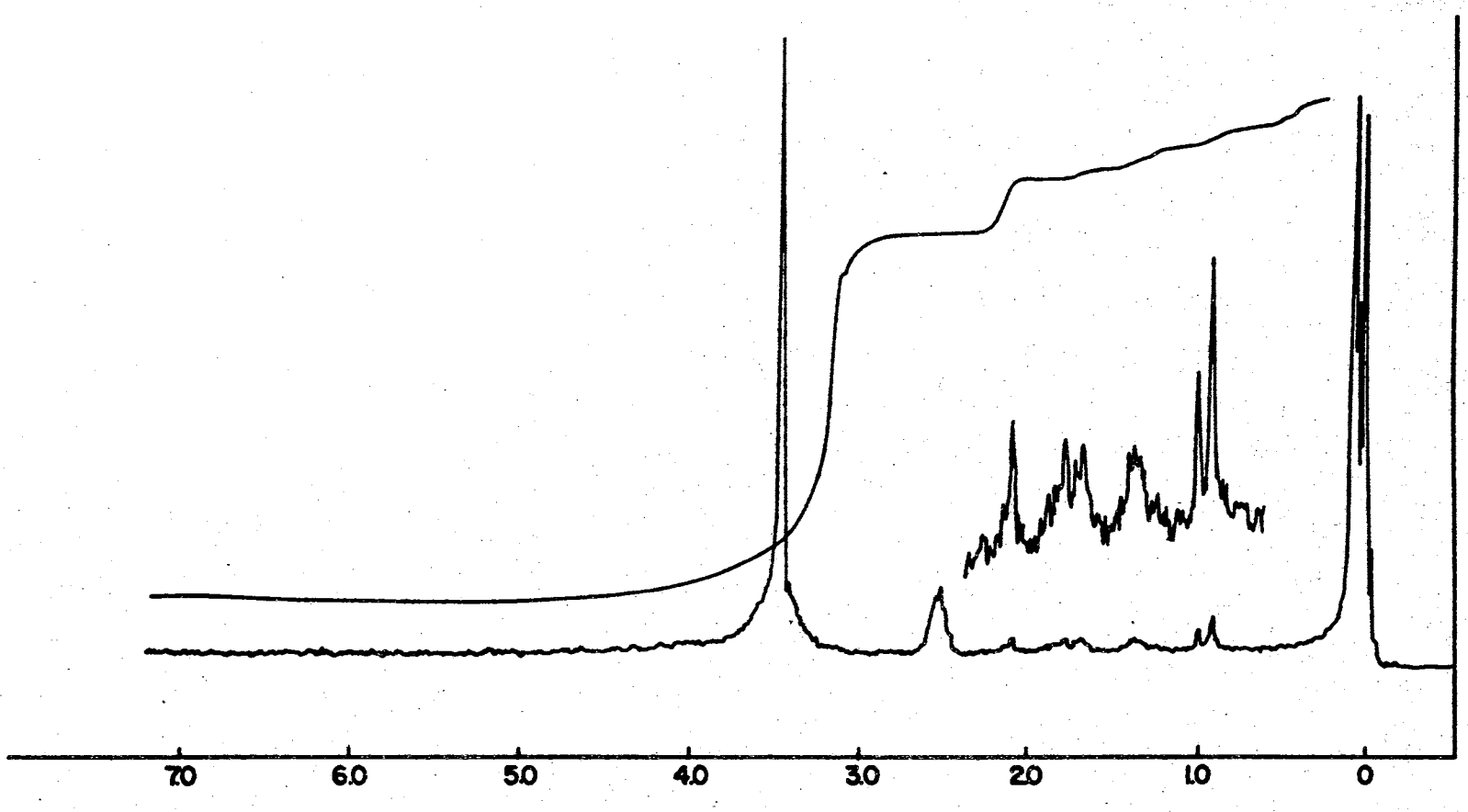


Figure 22. Low Resolution Nuclear Magnetic Resonance Spectrum of the Polyhydroxy Compound

TABLE X

COMPARISON OF THE NUCLEAR MAGNETIC RESONANCE SPCETRA OF THE POLYHYDROXY COMPOUND

ppm.	Spin-spin coupling[1]	Integration ratio I	II
3.86	S (B)	0	13
3.48	S	21	8
2.10	S	1	1
1.76	D	1	1
1.38	T	1	1
0.95	D	2	2

I - Polyhydroxy compound
II - Deuterium exchanged polyhydroxy compound

[1]Abbreviation given in the "Experimental Methods".

compound is a broad singlet; the proton is not on the oxygen atom long enough for hydrogen to see the proton on a α-hydroxy substituted carbon and consequently there is not coupling (72). A polyhydroxy alcohol would hardly show separate absorption peaks for each hydroxylic proton; in this case the rate of exchange in cycles per second is higher than the difference between the separate absorptions and as a result, the absorption peaks of the hydroxyl hydrogens broaden and then merge to form a singlet broad peak (73). The integration ratio shows that the peak at 3.86 ppm. represents 13 protons where the polyhydroxy compound has been reported to contain 10 hydroxyl groups. This discrepancy might be due to the impurity of water which may be present either in the polyhydroxy compound or in the D_2O. Both nuclear magnetic resonance spectra indicate the presence of 5 protons (2.10, 1.76, 1.38 and 0.95 ppm.) on carbon atoms which are at least not substituted at the α-position by a hydroxyl group.

2. Mass Spectrometric Study

The recrystallized polyhydroxy compound was treated with bis -(trimethylsilyl)-acetamide (BSA) and trimethylchlorosilane for a silylating reagent as described in "Experimental Methods". The excess reagent in the reaction mixture was removed by passing a stream of nitrogen over it and the residue was dissolved in acetone and immediately analyzed by gas liquid chromatography-mass spectrometry using the solid injection technique (56).

Figure 23 is the total ion current tracing obtained from a gas chromatographic-mass spectrometric analysis of the reaction mixture on a 10 % SE-30 column. More than 10 peaks were observed and none of

these peaks contained a series of molecular ions[1] which agreed with values that could be calculated by stepwise adding 72 mass units to the molecular weight of the polyhydroxy compound, 366. Moreover, it was noted that all the mass spectra obtained from the reaction mixture were those of trimethylsilyl (TMS) derivatives. An indication of the presence of silicon in the ions was studied by measurement of the relative height of the recorded isotope peak (M+1) and by observing fragmentation pattern.

The ion at m/e 73 corresponding to the molecular weight of trimethylsilyl group was present in the 11 mass spectra obtained from the reaction mixture. The common silicon containing fragment ions of the trimethylsilyl derivatives such as m/e 43 ($SiCH_3^+$), m/e 44 ($SiCH_4^{+\cdot}$), m/e 45 ($SiOH^+$ or $SiCH_5^+$), m/e 55 ($SiC_2H_5^+$), m/e 73 [$Si(CH_3)_3^+$] and m/e 75 [$Si(CH_3)_2OH^+$] were usually observed in the mass spectra of the trimethylsilyl polyhydroxy compound (74).

The use of the trimethylsilyl derivatives for gas chromatographic -mass spectrometric analysis overcomes the main difficulty in the analysis of alcohols and sugars, namely the determination of the molecular weight (75), by giving them the required volatility. However, the fact that absence of the molecular ion and/or explainable calculated fragment or molecular ions, usually M-15 and M-73, in the mass spectra of the trimethylsilyl derivatives of the polyhydroxy compound in this study is not easily explainable. It might be related to the fact that the attempted addition of 10 trimethylsilyl group to the polyhydroxy compound might be stereochemically unfavored so that the remaining

---

[1] $M^+ = 366 + n(73-1)$ where n is a number of trimethylsilyl groups introduced in the molecule.

hydroxyl group(s) might have a significant influence on the course of the reaction of the compound when subjected to electron impact.

3. Field Desorption Mass Spectrometer

Recently a number of authors have reported studies on field desorption mass spectra of substances which are biologically significant (76); particularly polyhydroxy compounds such as monosaccharides (77). In the field desorption mass spectrometer heating of the field anode wire by a current of several mA may increase the molecular ion intensity (M or M+1) much more than the fragment ion intensity.

Several attempts to measure the field desorption mass spectra of the polyhydroxy compound were carried out by Dr. H. D. Beckey[1]. However, it was not possible for him to obtain either the parent or fragment peaks. Although there is presently no reasonable explanation for that results, it might be that the solubility of the sample is not high enough to yield absorption on the field desorption emitter (78).

4. Selenium Dehydrogenation

Attempts to dehydrogenate the crystallized polyhydroxy compound with selenium were made as described. The ether extracts of the reaction mixture, which did not show UV absorption at 254 nm, were injected in the SE-30 column and no peaks were observed. These results might indicate that no aromatic compound was formed by selenium dehydrogenation.

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[1]Insitut fur Physikalische Chemie der Universitat Bonn, Wegelerstrasse 12, 53 Bonn, West Germany.

Although no conclusive evidence was obtained to determine the structure of polyhydroxy compound, it is postulated that the skeleton of the compound might be tetradecahydroanthracene or tetradecahydrophenanthracene (Figure 24). This postulation is based on the following preliminary evidences; a) fragment ions corresponding to the structural formulae of anthracene derivatives such as $C_{14}H_9$, $C_{14}H_{14}$ and $C_{15}H_{10}$ and those of naphthalene such as $C_{10}H_8$, $C_{10}H_9$, $C_{11}H_9$, $C_{11}H_{10}$ and $C_{12}H_{12}$ were found in the high resolution mass spectrometric data of ester alkaloids (refer to Table VIII), b) a moderately strong CH_2 "scissoring" frequency was observed at 7.2 microns in the infrared spectra of all four ester alkaloids indicating the presence of a cycloalkane structure in the molecule, c) relative upfield proton absorptions (2.10 - 1.02 ppm.) of the nuclear magnetic resonance spectra of polyhydroxy compound may account for the protons on the cyclic alkane skeleton, d) the presence of multiple hydroxyl groups in the molecule was proved by infrared spectra of the ester alkaloids and nuclear magnetic resonance spectra of the polyhydroxy compound, e) the possibility of some kind of tripentose structure is excluded since among the 10 hydroxyl groups in the compound 8 are esterified in the intact alkaloid and 2 are free.

G. Summary and Suggestions for Further Study

The purpose of the study described in this section was to isolate and identify the ester alkaloids and their subcomponents by the use of modern micro analytical instruments such as mass spectrometry, combination gas chromatography-mass spectrometry, field desorption mass spectrometry, nuclear magnetic resonance spectrometry and infrared spectrometry.

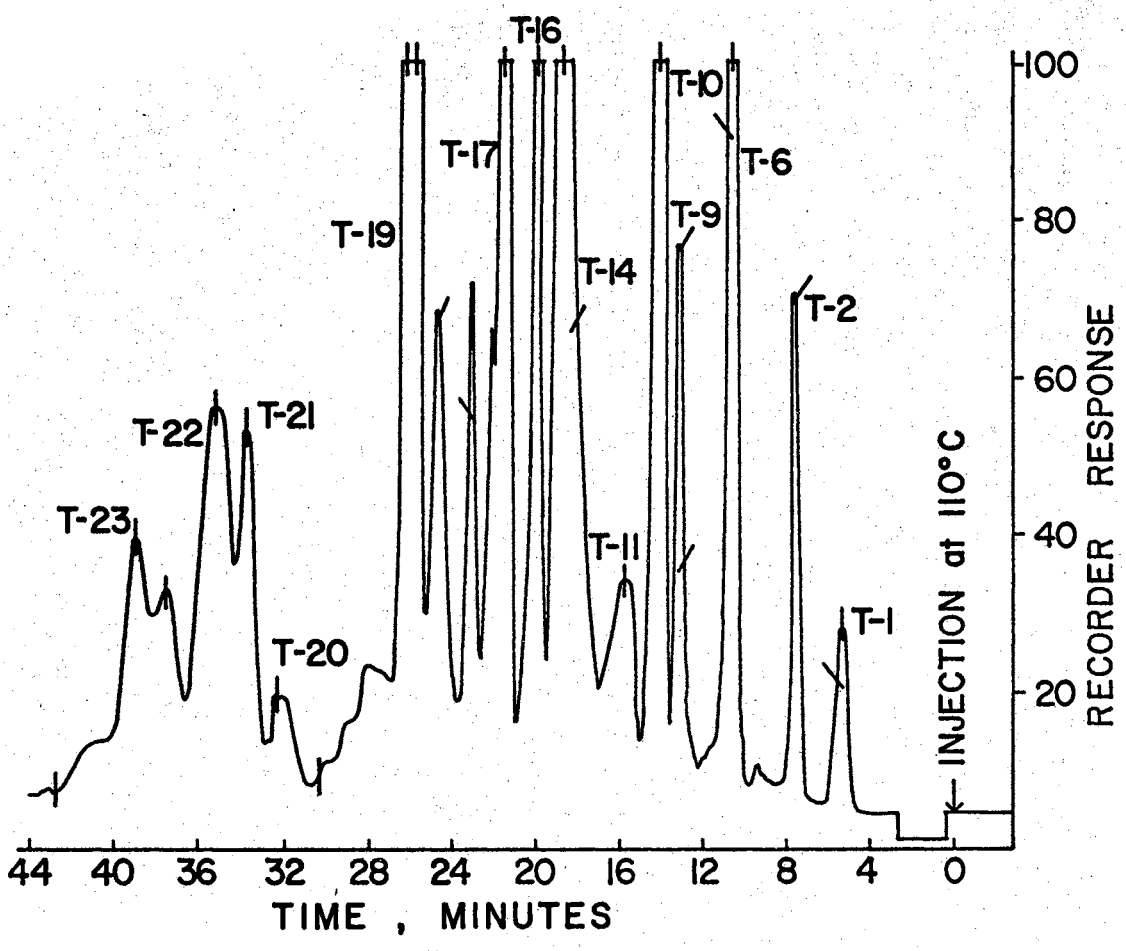


Figure 23. Total Ion Current Tracing of the Trimethylsilyl Derivatives of the Polyhydroxy Compound. The slash marks along the tracing indicate the point at which mass spectrum was taken. The column, 7 feet x 1/8 inches, was packed with 10 % SE-30 on Gas-Chrom Q, 100 - 200 mesh. Operational parameters were as follows: column temperature was programmed 2°C/min. from 110°C to 250°C, and helium flow rate was 40 ml/min.

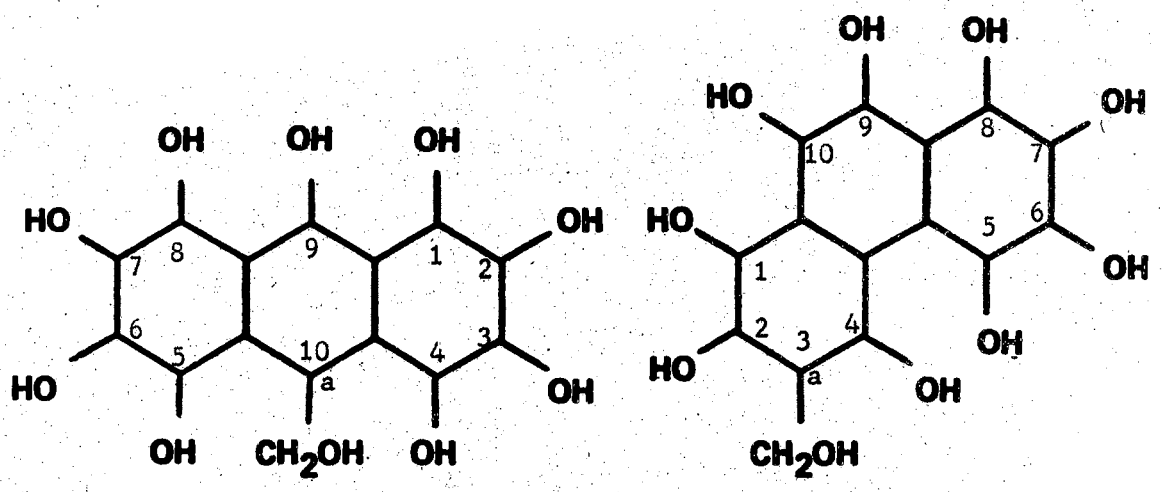


Figure 24. Proposed Structure of the Polyhydroxy Moiety $[C_{15}H_{16}(OH)_{10}]$ from T. wilfordii Hook Alkaloids

[I] 10-hydroxymethyl-tetradecahydroanthracene-1,2,3,4,5,6,7,8, 9-nonaol

[II] 3-hydroxymethyl-tetradecahydrophenanthracene-1,2,4,5,6,7, 8,9,10-nonaol

[a]Position of hydroxymethyl group is arbitrary.

The high and low resolution mass spectral data of the four ester alkaloids were presented. These data confirmed molecular weight and elemental formulae of the four ester alkaloids agreed with the values and elemental formulae reported by Beroza based on his elemental analysis in 1953.

Among the five acidic components of the ester alkaloids reported, the structures of four components have been identified mass spectrometrically; wilfordic acid, hydroxywilfordic acid, benzoic acid and acetic acid. The other reported acidic component, 3-furoic acid, was questionable since the fragmentation of the components, A-2, which had identical retention time and molecular weight with 2-methylfuroic acid was quite different.

For the identification of the reported 3-furoic acid as one of the acidic components of the ester alkaloids, it is desirable to isolate the compound by preparative gas chromatography and to study with carbon-skeleton chromatography (79). The interesting application of carbon-skeleton chromatography in the study of wilfordic acid and hydroxywilfordic acid has been reported (14). Chemical degradation and formation of derivatives of the compound may also give useful information.

The structure of the polyhydroxy compound is proposed from the preliminary various instrumental analysis data. Much more evidence is needed to prove or reject the proposed structure. Determination of the complete structure of a polyhydroxy compound requires complementary analysis, the most important of which are graded chemical hydrolysis of etherified derivatives (80, 81), followed by isolation and identification of the product formed.

H. Biosynthesis of T. wilfordii Hook Alkaloids

1. Isolation and Identification of Nicotinic Acid and NAD Metastables

When a portion of the 80 % methanol extract of the plant fed with nicotinic acid-6-^{14}C was subjected to thin layer chromatography and the plates were developed with a relatively non-polar solvent system (acetone : hexane, 8 : 2), two main radioactive zones; alkaloids zone (A-1 in Figure 25) and polar compounds (A-2 in Figure 25), were observed. More than 90 % of the isolated radioactivity was located at the polar compound zone.

Radiochromatograms of the aqueous and organic phases from the different part of plants fed with either nicotinic acid-6-^{14}C or NAD-carbonyl-^{14}C are shown in Figures 26 and 27. In most cases the aqueous phase and the organic phase contained more than 99 % of the radioactivity as polar compounds and alkaloids respectively. In case extraction with aqueous phase was not completed and polar compounds remained on the thin layer chromatography plate, the zone was scraped off, extracted with 80 % methanol and added to the appropriate fraction.

The polar compounds were subjected to Dowex 1-X8 formate anion exchange column chromatography as described. Figure 28 shows the results of the metabolism of nicotinic acid-6-^{14}C in the upper part (including leaves and stems) and the roots of T. wilfordii Hook respectively. In Figure 28, more than 10 radioactive peaks were found. The height and number of the peak (6 main peaks) were smaller in the radiochromatogram of root extracts. The chromatographic pattern of the NAD-carbonyl-^{14}C metabolites from the upper part and the roots are given in Figure 29. In both chromatograms of these NAD metabolites, the number of peaks were reduced when compared to those of the nicotinic

acid metabolites. Tubes representing each peak were pooled, lyophilized to dryness and identified by paper chromatography with authentic samples in two different solvent systems.

Two well known solvent systems (60) for paper chromatographic identification of the pyridine nucleotide were employed. The R_f values from descending paper chromatography of the nicotinic acid and NAD metabolites are given in Table XI. Based on R_f values from paper chromatography, elution volume of anion exchange chromatography and ultraviolet absorption spectra, the following radioactive peaks, N-1, N-2, N-7, N-8, N-11 and N-13 were tentatively identified as N-methyl-nicotinamide, nicotinamide, nicotinic acid, NAD, NaMN and desamido-NAD respectively. The possibility of contribution of compounds other than the one assigned to the radioactive peaks is not excluded. There are indeed some chromatographic evidences that peak N-1 contains more than one compound. The minor peaks remain unknown in this study since the low concentrations and weak radioactivity indicated that they were not major metabolites.

2. Nicotinic Acid-6-^{14}C and NAD-carbonyl-^{14}C as Precursors

Biosynthesis of the pyridine alkaloids, expecially ricinine (3), nicotine (82) and anabasine (83, 84), in higher plants has been studied extensively by measuring the incorporation of various isotopically labelled precursors into the alkaloids and chemically degrading them to locate the position of labelled atoms. However, no attempt has been made to elucidate the origin of pyridine moieties of the T. wilfordii Hook ester alkaloids, wilfordic acid and hydroxywilfordic acid. The results of four day T. wilfordii Hook alkaloids biosynthesis from the plants administered nicotinic acid-6-^{14}C and NAD-carbonyl-^{14}C

are shown in Tables XII and XIII. The radioactivity of alkaloids in the roots was much higher than that in the leaves and stems when nicotinic acid-6-^{14}C was the precursor. Both precursors were injected into the stems. The total percentage of the incorporation of nicotinic acid into the root alkaloids was about twice as high as that into the alkaloids from the top part of the plant. It is interesting to note that the difference in extent of total incorporation between nicotinic acid and NAD is due to their different efficiencies of incorporation into the root alkaloids but not in the alkaloids of the upper part of the plant. The radioactivity in the alkaloids of the leaves and stems was actually slightly higher when NAD was fed than when nicotinic acid was fed (1.8 % compared to 1.2 %); however, the radioactivity of the alkaloids in the roots was remarkably lower when NAD was administered than when nicotinic acid was administered (3.2 % compared to 0.5 %). The incorporation efficiencies of nicotinic acid and NAD into T. wilfordii Hook alkaloids are comparable if we assume that the differences in the radioactivity of the alkaloids in the roots of the plants fed nicotinic acid and NAD are due to other factors such as site of synthesis of the alkaloids (85), and translocation and/or uptake of the precursors. It has been proven that each compound which is a member of the pyridine nucleotide cycle can serve as a ricinine (3) or nicotine (32) precursors, each with an efficiency of the same order of magnitude. In the present study, it was shown that the pyridine moiety of NAD could be readily incorporated into the pyridine component of T. wilfordii Hook alkaloids with comparable efficiency as nicotinic acid by the leaves and stems of the plants but was less efficient as a precursor in the root.

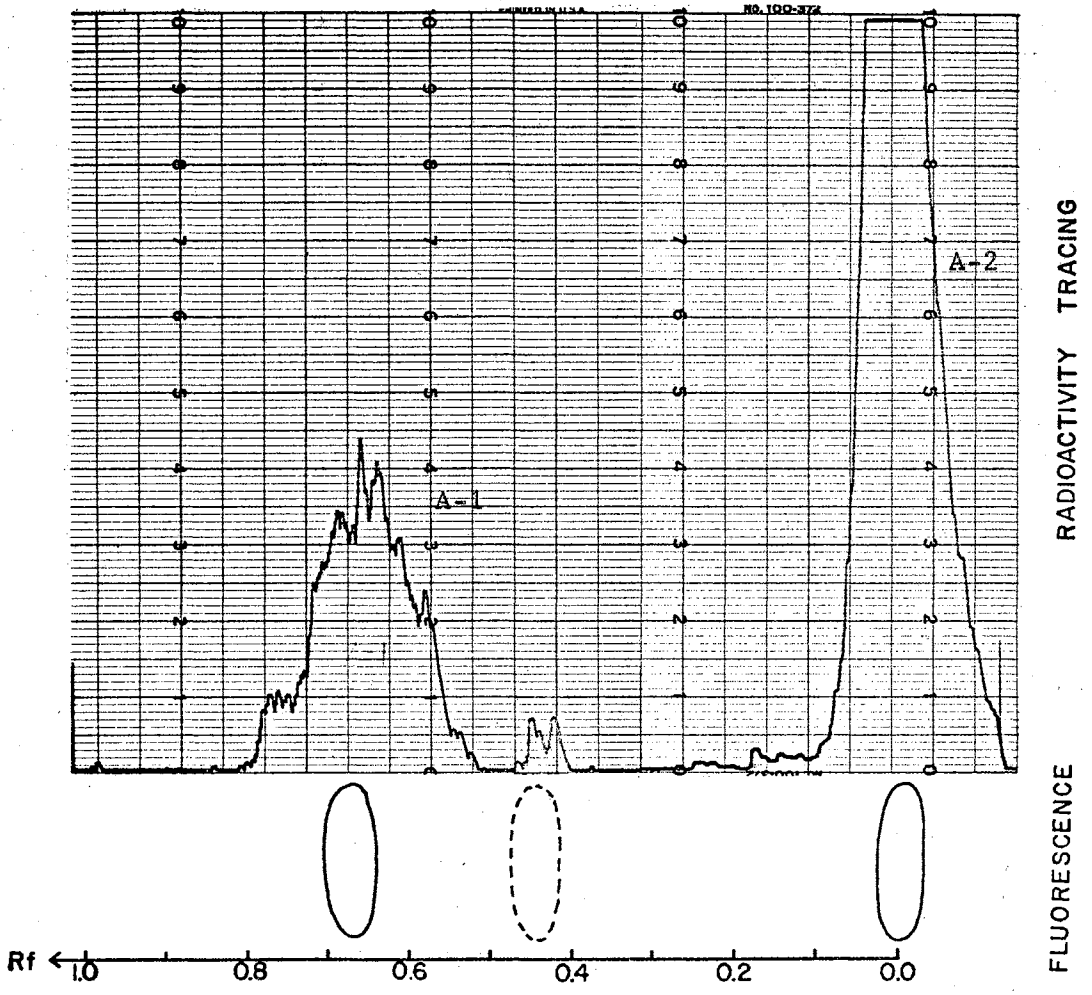


Figure 25. Thin Layer Radiochromatogram of 80 Percent Methanol Extract from the Plant which was Fed with Nicotinic acid-6-^{14}C after 4 days (Solvent, Acetone : hexane, 8 : 2, v/v)
A-1 - Mixture of the alkaloids
A-2 - Polar compounds

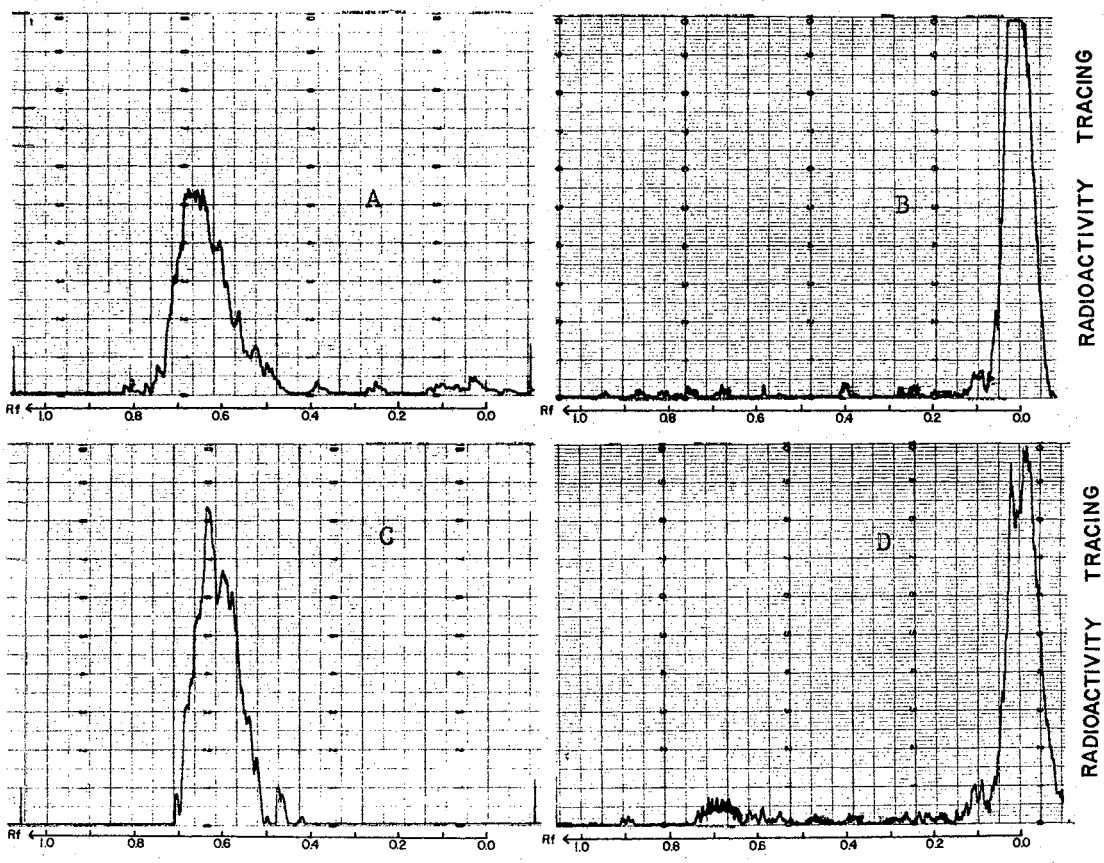


Figure 26. Thin Layer Radiochromatograms of an Extract of the Plant Fed with Nicotinic acid-6-^{14}C. Duration of experiment was 4 days. Solvent system used was acetone : hexane (8 : 2, v/v).

A - Organic phase of an extract from the top
B - Aqueous phase of an extract from the top
C - Organic phase of an extract from the roots
D - Aqueous phase of an extract from the roots

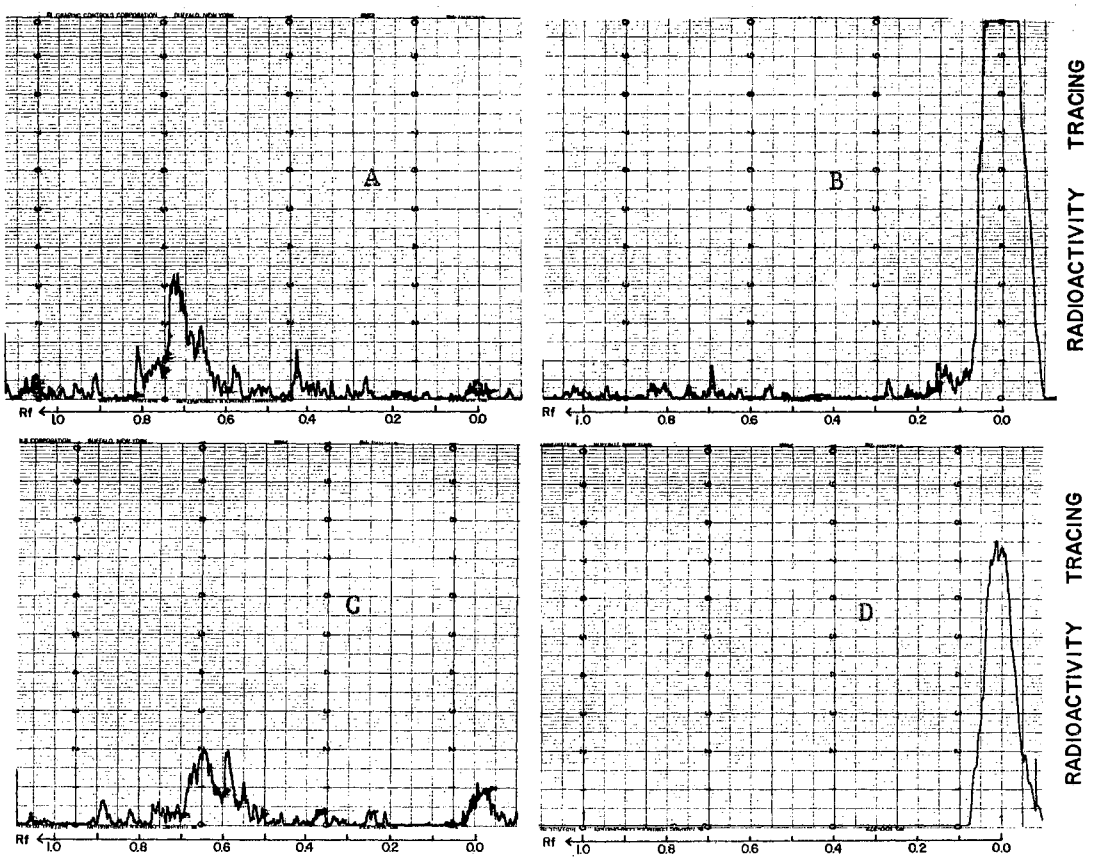


Figure 27. Thin Layer Radiochromatograms of an Extract of the Plant Fed with NAD-carbonyl-^{14}C. Duration of experiment was 4 days. Solvent system used was acetone : hexane (8 : 2, v/v).

A - Organic phase of an extract from the top
B - Aqueous phase of an extract from the top
C - Organic phase of an extract from the roots
D - Aqueous phase of an extract from the roots

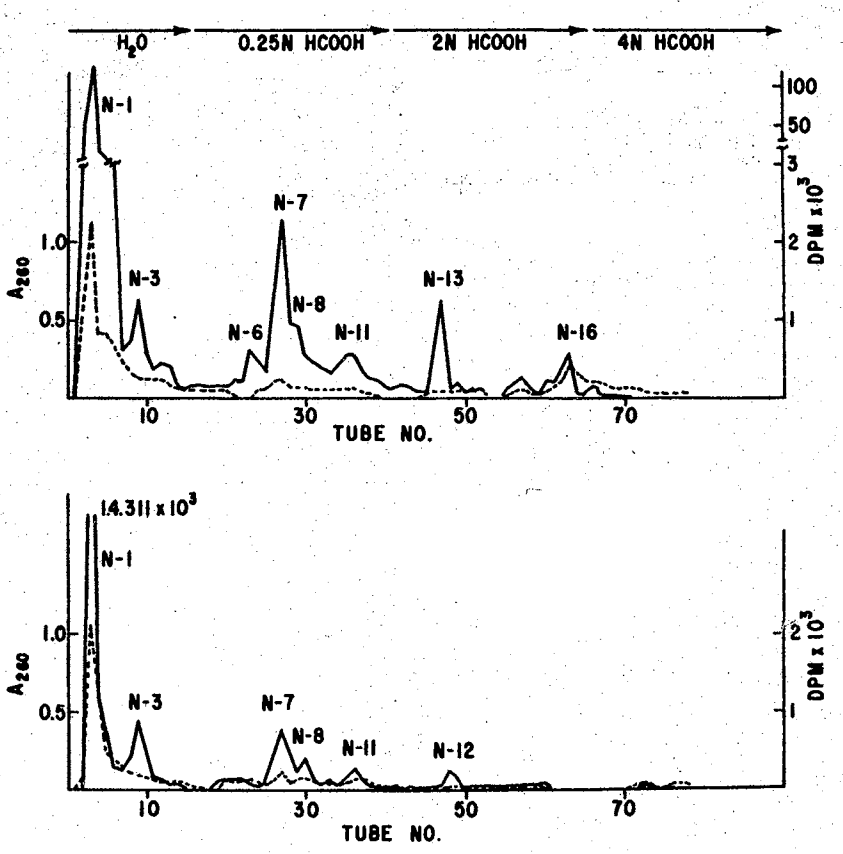


Figure 28. Dowex 1-X8 Formate Column Chromatography of the NA-6-^{14}C Metabolites from T. wilfordii Hook. Upper and lower figures are the chromatograms of the metabolites from the top and the roots of the plant respectively. The solid and broken lines represent radioactivity and absorbancy at 260 nm respectively. The radioactivity peaks, N-1, N-2, N-7, N-8, N-11 and N-13 were tentatively identified as N^1-methylnicotinamide, nicotinamide, nicotinic acid, NAD, NaMN and desamido-NAD respectively.

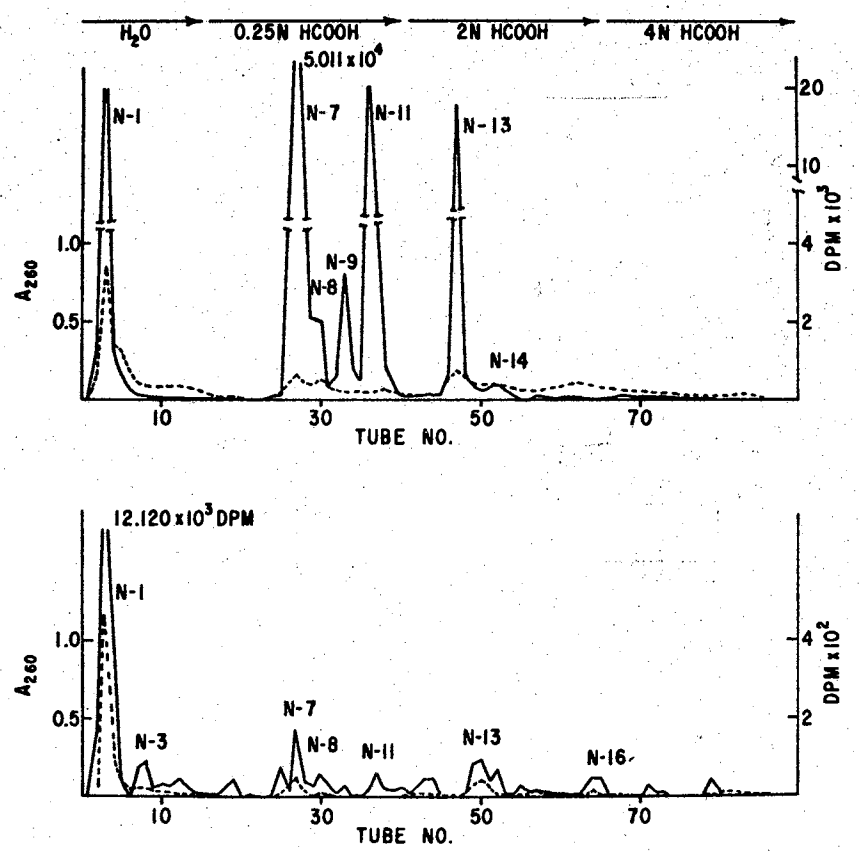


Figure 29. Dowex 1-X8 Formate Column Chromatography of the NAD-carbonyl-^{14}C Metabolites from T. wilfordii Hook. Legend is the same as described under Figure 28.

TABLE XI

R_f VALUES FROM DESCENDING PAPER CHROMATOGRAPHY OF THE METABOLITES OF NICOTINIC ACID AND NAD IN T. WILFORDII HOOK

Compound	R_f value Sol. I	Sol. II
N-1 (D-methylnicotinamide)	0.81	0.88
N-3 (nicotinamide)	0.89	0.89
N-7 (nicotinic acid)	0.75	0.76
N-8 (NAD)	0.29	0.32
N-11 (nicotinic acid mononucleotide)	0.38	0.35
N-13 (desamido-NAD)	0.17	0.19
N-2	0.89	0.86
N-6	0.46	0.38
N-9	0.20	0.10
N-14	0.28	0.08

Solvent system I - 95 % ethanol : 1 M ammonium acetate (7 : 3, v/v, pH 5)
Solvent system II - isobutyric acid : ammonia : water (66 : 1.7 : 33, v/v/v, pH 3.8)

TABLE XII

DISTRIBUTION OF RADIOACTIVITY IN T. WILFORDII HOOK AFTER ADMINISTRATION OF NICOTINIC ACID-6-^{14}C

Exp. No.	Compound	Roots		Leaves & Stems		Total	
		dpm x 10^4	%	dpm x 10^4	%	dpm x 10^4	%
I	Alkaloids	2.232	3.5	.704	1.1	2.936	4.6
	Polar compounds	2.817	4.4	21.387	33.2	24.203	37.5
	Total	5.049	7.8	22.091	34.3	27.139	42.1
II	Alkaloids	1.912	2.97	.825	1.28	2.738	4.25
	Polar compounds	1.717	2.66	18.825	29.19	20.542	31.85
	Total	3.629	5.63	19.650	30.47	23.280	36.10
Average	Alkaloids	2.072	3.21	.764	1.19	2.837	4.40
	Polar compounds	2.267	3.52	20.106	31.18	22.373	34.69
	Total	4.3390	6.73	20.870	32.36	25.210	39.09

Duration of experiment was 4 days. The plants used were grown in the green house. Nicotinic acid-6-^{14}C (1.1×10^{-2} μM) with a total radioactivity of 64.487×10^4 dpm was administered. Percentage of incorporation was determined by dividing total radioactivity administered by the total amount recovered.

TABLE XIII

DISTRIBUTION OF RADIOACTIVITY IN T. WILFORDII HOOK AFTER ADMINISTRATION OF NAD-CARBONYL-^{14}C

Administered compound	Isolated compound	Roots		Leaves & Stems		Total	
		dpm x 10^4	%	dpm x 10^4	%	dpm x 10^4	%
NAD-carbonyl -^{14}C	Alkaloids	0.242	0.5	0.777	1.4	1.019	2.3
	Polar compounds	1.486	3.4	16.686	37.6	18.172	41.0
	Total	1.728	3.9	17.463	39.4	19.191	43.3

The plants used were grown in the green house. Duration of the experiment was 4 days. NAD-carbonyl-^{14}C (10^{-2} µM) with a total radioactivity of 44.353 x 10^4 dpm was administered. Percentage of incorporation was determined by dividing total radioactivity administered by the total amount recovered.

3. DL-glutamic acid-2-^{14}C as Precursor

DL-glutamic acid was administered to young T. wilfordii Hook plants for evaluation as a possible precursor for the side chain of the pyridine ring. The plant extract was divided into aqueous phase and organic phase as described. Upon subjecting the organic phase to thin layer chromatography, the alkaloid zone (G-6 in Figure 30, A) together with 5 more peaks were observed which was different from the results obtained with nicotinic acid and NAD which have only one peak, which was alkaloids. After two preparative chromatographic purifications by thin layer chromatography, the alkaloid zone was assayed for radioactivity. No other zone on the thin layer chromatograms of organic phase and aqueous phase was studied.

The incorporation of radioactivity from glutamic acid-2-^{14}C into the alkaloids (Table XIV) was too low to justify an attempt to locate the label by degradation analysis; however, the extent of incorporation was about the same order of magnitude as that observed for ricinine (86), and nicotine (31). The incorporation percentage, 0.84, should be doubled if it is considered that the plants used only the L-form. It is most likely that glutamic acid can be incorporated into the pyridine ring of the alkaloids via succinate which can be formed after glutamic acid enters into the citric acid cycle.

4. Metabolism of Nicotinic acid-6-^{14}C and NAD-carbonyl-^{14}C in the Plant

During the course of the study on the biosynthesis of the alkaloids produced by T. wilfordii Hook, 80 - 90 % of the isolated radioactivity remained in the aqueous phase after either extracting the alkaloids with organic solvent or as a result of the thin layer

chromatography using the relatively non-polar solvent system. To elucidate the relationship between the pyridine nucleotide cycle and biosynthesis of T. wilfordii Hook alkaloids, a study of the polar compounds in aqueous phase was undertaken.

As shown in Table XV, the total radioactivity recovered from the plant fed with NAD was higher than that of the plant fed with nicotinic acid and the distribution of radioactivity among the metabolites of above two precursors was remarkably different from each other. The ratio of radioactivity present in the roots compared to that found in the upper part of the plant was higher when nicotinic acid was administered than when NAD (0.2 % compared to 0.1 %) was administered. This difference might reflect the difference between nicotinic acid and NAD with respect to uptake and/or translocation. Lan and Henderson (87) reported that the uptake of nicotinic acid and nicotinamide by rat erythrocytes consisted of two processes, diffusion and conversion to nucleotides which did not readily diffuse from the cell. They studied the processes separately with the use of ^{14}C-labelled substrates and fluoride to prevent the formation of nucleotides and concluded that the two processes provided for a very rapid removal of these compounds, especially nicotinic acid, from the external medium. Two different views on the permeability of nucleotides in animal cells have been recently reported: Negishi et al. reported (88) that in 20 seconds 90 % of the radioactivity in the blood after the injection of NAD-carbonyl-^{14}C was found as nicotinamide-carbonyl-^{14}C which was reabsorbed and reutilized as a precursor of NAD in various mice tissues. In contrast to these results, Everse et al. (89) suggested the possibility that the NADH might enter the mice liver without cleavage. They reported a great increase in liver NAD^+, when NADH was

administered in comparison with oxidized NAD^+. Our finding, that total radioactivity present in the root was lower when NAD was injected than when nicotinic acid was injected, might be indicative of a lower uptake into the cells and/or a lower translocation rate of intact NAD compared to nicotinic acid. However, no definite conclusion could be drawn from those results, since involvement of the more complicated factors such as metabolic rate, the accumulation capability of different tissues and the polarization of compounds, not with respect to their structure, but with respect to their function, should be considered first.

The distribution of radioactivity among metabolites of nicotinic acid varied greatly. The incorporation percentage of nicotinic acid into the non-nucleotide compounds, especially N-methylnicotinamide, was very much higher than in the nucleotides (16). The radioactivity distribution among nucleotides such as NAD, nicotinic acid mononucleotide and desamido-NAD was rather uniform ranging from 0.3 to 0.48 % of the total administered radioactivity. More than 98 % of the administered nicotinic acid was metabolized in the plant after 4 days, if we consider that at least a portion of the radioactivity was contributed by resynthesized nicotinic acid.

The radioactivity distribution among the nucleotides such as NAD^+, nicotinic acid mononucleotide and desamido-NAD^+ was lower in the roots and much higher in the leaves and stems when NAD was fed than when nicotinic acid was fed. The fact that labelled NAD^+ gave arise to higher radioactivity in nicotinic acid mononucleotide and desamido-NAD in the leaves and stems than did nicotinic acid permits the speculation that phosphoribosylpyrophosphate may be the rate limiting compound in the cycle. It is known that degradation of the pyridine nucleotides are the only biological source of nicotinamide and subsequently

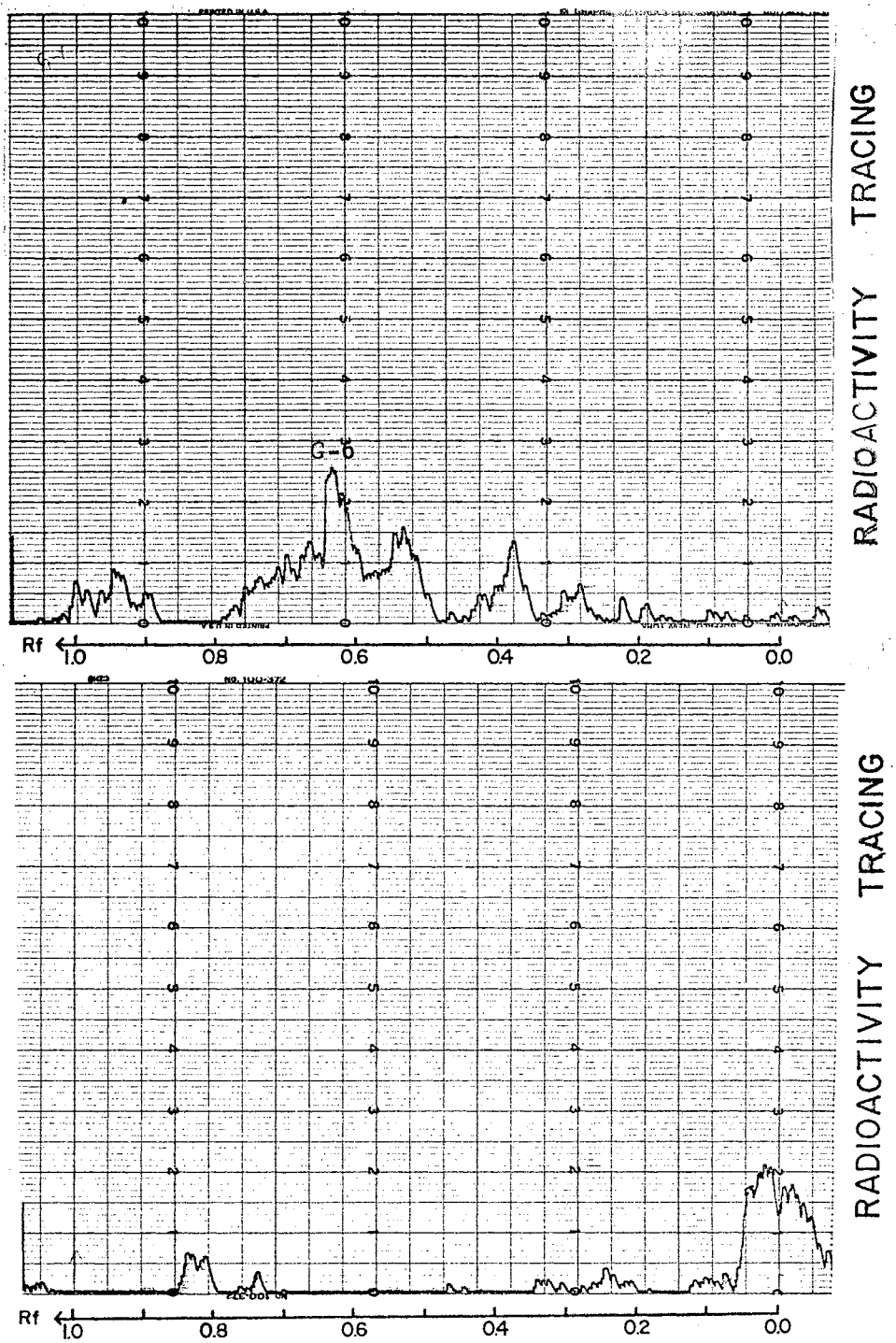


Figure 30. Thin Layer Radiochromatograms of Organic (A) and Aqueous (B) Phase of the Plant Extract Fed with Glutamic acid-2-^{14}C. Duration of experiment was 4 days and the solvent system used was acetone : hexane (8:2, v/v)

TABLE XIV

DISTRIBUTION OF RADIOACTIVITY IN T. WILFORDII HOOK AFTER ADMINISTRATION OF DL-GLUTAMIC ACID-2-^{14}C

Administered compound	Isolated compound	Roots		Leaves & Stems		Total	
		dpm x 10^4	%	dpm x 10^4	%	dpm x 10^4	%
DL-glutamic acid-2-^{14}C	Alkaloids	0.265	0.3	0.48	0.54	0.745	0.84
	Polar Compounds	0.3575	0.4	18.8325	21.2	19.19	21.6
	Total	0.6225	0.7	19.313	21.7	19.934	22.4

Duration of experiment was 4 days. The plants used were grown in the green house. DL-glutamic acid-2-^{14}C (8.1×10^{-3} μM) with a total radioactivity 88.94×10^4 dpm was administered. Percentage of incorporation was determined by dividing total radioactivity administered by the total amount recovered.

TABLE XV

RADIOACTIVITY DISTRIBUTION AFTER ADMINISTRATION OF NICOTINIC ACID-6-^{14}C AND NAD CARBONYL-^{14}C INTO T. WILFORDII HOOK PLANTS

Metabolite \ Administered compound / Plant part	Nicotinic acid-6-^{14}C[b]				NAD-carbonyl-^{14}C			
	Root	Top	Total		Root	Top	Total	
	dpm x 10^3			%	dpm x 10^3			%
N-demethyl nicotinamide	16.286	156.679	172.965	26.82	12.889	36.993	49.882	11.25
Nicotinamide	1.682	4.396	6.078	0.94	0.189	0.282	0.468	0.11
Nicotinic acid	1.753	8.739	10.492	1.63	1.219			
NAD	0.839	2.270	3.109	0.48	0.119	57.425	57.763	13.02
Nicotinic acid mononucleotide	0.593	1.330	1.923	0.30	0.081	26.775	26.861	6.06
Desamido-NAD	0.526	1.896	2.422	0.38	0.186	19.245	19.431	4.38
Alkaloids	20.732	7.648	28.371	4.40	2.426	7.765	10.200	2.29
Others[a]	0.931	27.249	28.237	4.38	1.269	26.160	27.323	6.16
Total	43.390	208.707	252.097	39.09	17.291	174.639	191.928	43.27

[a] - Unclassified polar compounds. [b] - Average of two experiments.

Duration of the experiment was 4 days. The plants used were grown in the green house. Nicotinic acid-6-^{14}C (1.1×10^{-2} µM) with a total radioactivity of 64.484×10^4 dpm, and NAD-carbonyl-^{14}C (10^{-2} µM) with a total radioactivity of 44.353×10^4 dpm was administered. Percentage of incorporation was determined by dividing the total radioactivity administered by the total amount recovered.

nicotinic acid which is the immediate precursor for nicotinic acid mononucleotide. Desamido-NAD is not formed from NAD. Therefore, the concentration of nucleotides in the pyridine nucleotide cycle might be governed by the concentration of phosphoribosylpyrophosphate (PRPP) since it is required for de novo and salvage synthesis of nicotinic acid mononucleotide. Furthermore the formation of N-methylnicotinamide might also be controlled by the concentration of PRPP.

An alternate circuit loop of the main cycle (NAD → NMN → nicotinamide) was found in yeast (90), and it was suggested that it occured in barley based on experiments by Ryrie et al. (44). This alternate pathway may not operate in T. wilfordii Hook, since no detectable nicotinamide mononucleotide was present. A pyrophosphatase, which catalysed the formation of nicotinamide mononucleotide from NAD, has been demonstrated in plant tissue (42).

It is well known that the biosynthesis of NAD^+ from nicotinic acid via nicotinic acid mononucleotide and desamido-NAD^+ occurs in the higher plants family such as Euphorbiaceae and Selanaceae, and certain bacteria (44, 91). The in vivo experiments reported here provide evidence for the function of this pathway in still another family of the plants, Tripterygium wilfordii Hook, Celastraceae.

In summary, labelled nicotinic acid and nicotinamide adenine dinucleotide were rapidly metabolized in T. wilfordii Hook with the formation of all the compounds in the pyridine nucleotide cycle, and the nicotinamide moiety of NAD can be readily incorporated into the alkaloids from T. wilfordii Hook with efficiency of the same order of magnitude as nicotinic acids. The possible role of phosphoribosylpyrophosphate on the control of the cycle was suggested.

PART TWO

METABOLISM OF RICININE IN

RICINUS COMMUNIS L.

CHAPTER V

INTRODUCTION

Advances in our knowledge of the biosynthesis of the alkaloids have little counterpart with respect to their further metabolism. The alkaloids have generally been considered as a final product of nitrogen metabolism in the plants. The occurrence of the possible alkaloid catabolic processes in the plants which produce them has become of interest in the last decade when isotopically labelled alkaloids became available.

The alkaloid selected for studying this metabolic process was ricinine, the major alkaloid produced by the castor bean plant, Ricinus communis L. Considerable information on its biosynthesis was available. Degradation of the -pyridone ring of ricinine to carbon dioxide by the castor bean plant had been demonstrated (92). Recently a rapid interconversion of exogenous ricinine and N-demethyl ricinine in senescent and green castor bean plant leaves was reported (93). The two compounds, N-demethyl ricinine and CO_2, are the only compounds which have been identified as ricinine metabolic products.

The experiments described herein were designed to identify the possible new ricinine metabolites and to provide further information on the translocation of ricinine within the plant and to study the possible role of demethylation and methylation reactions of these alkaloids in connection with translocation.

CHAPTER VI

LITERATURE REVIEW

A. Structure and Properties

Ricinine, an α-pyridone alkaloid produced by Ricinus communis L. was first discovered in the castor bean seed in 1864 by Tuson (94).

Boettcher (95) reported that the bulk of ricinine was located in mature castor seed with about 0.15 % in the seed coat and about 0.03 % in the kernel. Robinson (96) showed that measurable quantities of ricinine were in the roots. He also reported that the hypocotyls were higher in ricinine content than in the roots, with larger amounts being found in the top than in the bottom portion of the hypocotyls. Ricinine was shown to be present in all parts of the young castor bean plant, approximately 1 milligram per gram fresh weight; however, the content varies from one part of the plant to another depending on probably its physiological function (97).

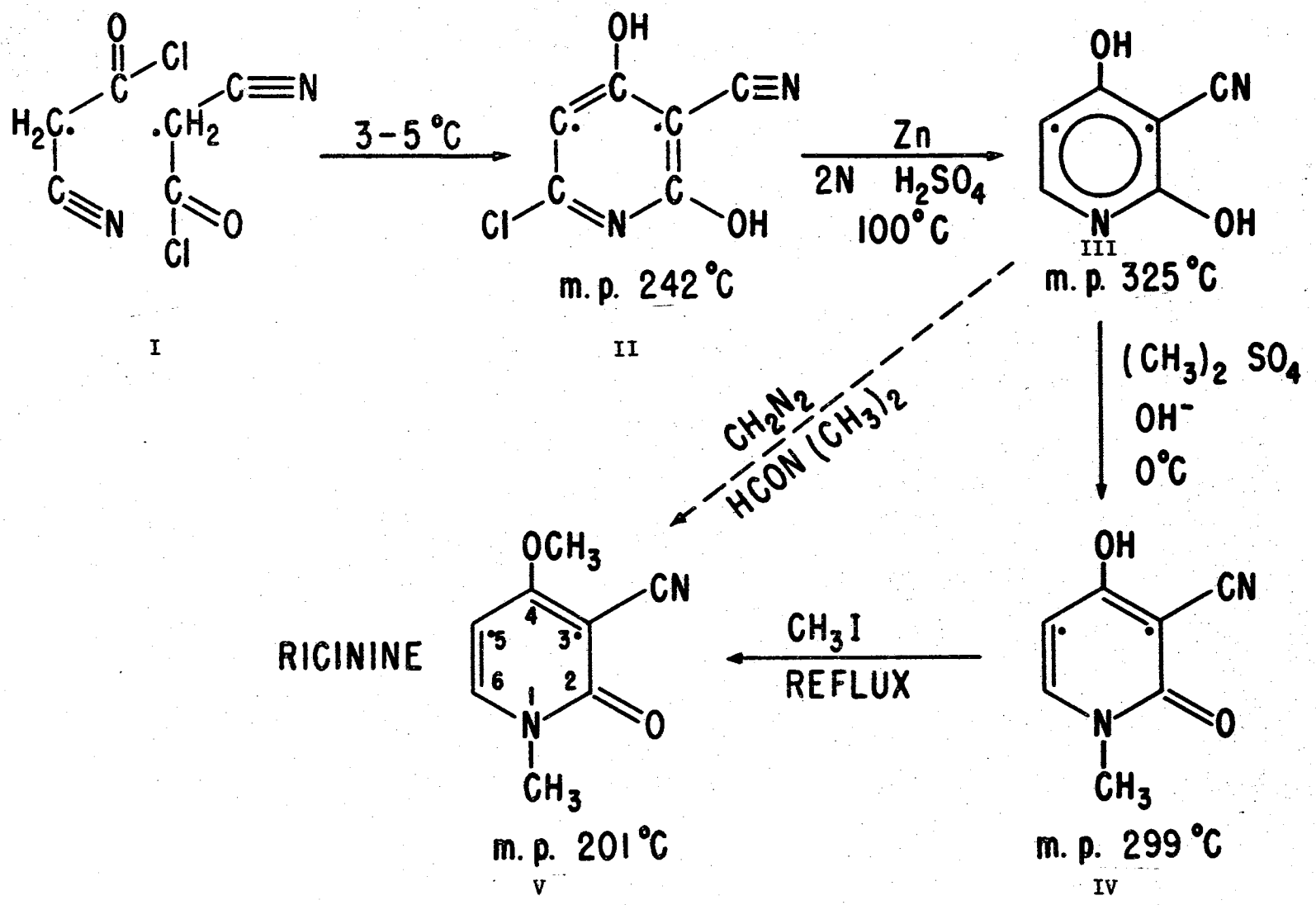
The first study on the structure of ricinine was published by Spaeth and Koeller (98). They were able to show the presence of a pyridine ring in the alkaloids. The structure of ricinine was proved to be N-methyl-4-methoxy-3-cyano-2-pyridone (99) and was confirmed by chemical synthesis from 4-chloroquinoline via the intermediate 4-chloro-2-aminoquinoline-3-carboxylic acid and 2,4-dichloronicotinanitrile by Spaeth and Koeller (100). Several researchers (101, 102) synthesized ricinine from different starting compounds and thus

confirmed the structure. Schroeter et al. (103) synthesized ricinine from the aliphatic compound cyanoacetyl chloride. This transformation was attributed to the intermediate formation of malanamoyl chloride and cyanoketene to give 2,4-dihydroxy-6-chloronicotinonitrile, II, which was then converted to 4-dihydroxynicotinonitrile, III, by removal of the halogen. III was converted to ricinine acid, IV, by methylating the nitrogen atom and IV was converted to ricinine V by methylating the hydroxyl group. The direct N- and O-methylation of III was developed by Robinson and Hook (104). Yang et al. (105) prepared ricinine-3,5-^{14}C with a specific activity of 567.0 μc/m mole from cyanoacetyl chloride-2-^{14}C by following the scheme presented in Figure 31.

Ricinine is a neutral and optically inactive compound (106). It melts at 201.5°C (corrected) and sublimes at 170 - 180°/760 mm. It is slightly soluble in water, chloroform, alcohol and pyridine and insoluble in ether. Ricinine does not react with the usual alkaloidal reagents such as Dragendorff's, but it gives positive color tests to the Weidol reaction, Fehling's solution, ferric complex and formation of an isonitrile which can be detected when treated with 2 N NaOH and 30 % H_2O_2 (107).

Ricinine forms chloride and mercuric chloride salts, and give three bromide derivatives which can be characterized by their different melting points (106). Ricinine undergoes hydrogenation in the presence of platinum as catalyst and yields a pyridine in the presence of zinc dust. The oscillopolarography, ultraviolet and infrared spectroscopy of ricinine have been reported (108, 109).

Skursky et al. (93) have recently reported ultraviolet



I II III IV V

Figure 31. Chemical Synthesis of Ricinine (. refers to the position of carbon-14 label)

absorption maximum in water at 307 and 255 mm on 10 times recrystallized ricinine with molar extinction coefficients 8.77×10^3 and 4.29×10^3 liters M^{-1} cm^{-1}, respectively which are moderately different from the previously reported data (110). They also reported that absorption at 219 nm was not suitable for analytical utilizations because of its sensitivity to impurity.

Waller et al. (111) have reported in detail the mass spectrometric analysis used for the assignment of labelled atoms of isotopically enriched ricinine together with the mass spectral fragmentation pattern of ricinine. Nuclear magnetic resonance spectra of ricinine, N-demethyl ricinine and O-demethyl ricinine were reported by Skursky et al. (93).

Yang (86) reported that ricininic acid (O-demethyl ricinine) was obtained from ricinine by alkaline hydrolysis. Ricininic acid crystallized from water melted at 298 - 299°/760 mm. was slightly soluble in water, ethanol, chloroform and ether.

B. Biosynthesis of Ricinine

The first experimental evidence indicating biosynthesis of ricinine in the castor bean plant seed was reported in 1932 by Weevers (112). He studied the ricinine content in the seed during germination in the dark and found that the ricinine nitrogen in 100 seeds increased in three weeks from 4 to 72 milligrams. James (113) developed a different view and showed that the accumulation of alkaloid in the cotyledons and hypocotyl during germination appeared to be due to the translocation, with or without decomposition, from the endosperm. He found an extractable amount of ricinine only in the cotyledons and hypocotyl and trace amounts in the endosperm and young emerging root,

when ricinine was extracted from younger seedlings in which the cotyledons were still retained within the endosperm. Bogdashevskaya (114) reported that the absolute amount of ricinine in the whole plant increased with the development of the plant, but percentage wise it was highest in the 20 days old plants, after which it fell sharply, increasing again slightly after flowering. Waller et al. (115) reported that the ricinine in the seed accounted for about 75 % of the total alkaloid in the plant at 20 weeks of age and the amount of ricinine per gram of leaves and stems decreased to 60 % but the ricinine content of the flowers and seed remained relatively constant in post flowering plants.

The biosynthesis of ricinine with the use of radioisotopes in the castor bean plant was first reported in 1952 by Dubeck and Kirkwood (116). They investigated the origin of the O- and N-methyl groups of ricinine by feeding germinating castor seeds L-methionine-methyl-^{14}C, choline-methyl-^{14}C and sodium formate-^{14}C and found that only the carbon-14 labelled methyl group of methionine was appreciably incorporated into the methyl groups of ricinine.

The inability of lysine to serve as a precursor of the α-pyridone ring of ricinine in higher plants was reported in 1958 by Grimshaw and Marion. However, a conflicting result was reported by Tamir and Ginsburg who found that lysine-2-^{14}C was incorporated into ricinine to the extent of 0.01 % with all of the radioactivity located in carbon-6 of ricinine (117). They also reported that α-amino-adipic acid-2-^{14}C gave arise to ricinine labelled in carbon-2 and -6. Juby and Marion (118) studied lysine as a precursor of ricinine and found that the extent of incorporation was very low. They considered that lysine was not an important precursor of the pyridine ring in higher plants; a

concept supported by studies made by Waller and Henderson (46).

Juby and Marion (118) found 93 % of activity in the cyano group of ricinine obtained from feeding sodium acetate-1-^{14}C to castor bean plants. The distribution of radioactivity in ricinine synthesized from acetate and glutamate could be accounted for by the operation of the citric acids cycle with or without the glyoxylate bypass. Anwar et al. (119) also reported that 90 % of the radioactivity from acetate-1-^{14}C, glutamic acid-2-^{14}C and propionic acid-3-^{14}C was located in the nitrile carbon of ricinine. These results agreed with and supported the earlier findings of Waller and Henderson (33) that succinic acid or a related four-carbon dicarboxylic acid found in the citric acids cycle was a direct precursor of ricinine. The four-carbon dicarboxylic acid would be incorporated in such a way that one of the carboxyl groups provided the carbon for the cyano group of ricinine and the methylene groups provided the carbon for the 2 and 3 positions of the pyridine ring respectively. The other carboxyl group must eventually be lost by decarboxylation. In these experiments the radioactivity of succinic acid-1,4-^{14}C was distributed 85 % in the cyano group and 15 % in the α-pyridone ring of ricinine. Juby and Marion (118) reported that succinic acid-2,3-^{14}C was incorporated into ricinine to an extent of 38.9 %, 38.3 % and 20.8 % in carbon atoms 2, 3 and 8 (cyano group) respectively. The incorporation of glycerol-1,3-^{14}C and glycerol-2-^{14}C into ricinine to the same extent as many other precursors was reported (106).

1. The Relationship Between the Pyridine Nucleotide Cycle and Biosynthesis of Ricinine

Leete and Leitz (120) first suggested that intermediates of what

is now called the pyridine nucleotide cycle might be involved in the biosynthesis of ricinine in the castor bean plant; however, the experimental proof was not attempted until the ^{14}C-labelled compounds became available.

Leete and Leitz (120) and Waller and Henderson (46) showed that the pyridine ring of nicotinic acid and nicotinamide could become the ∝-pyridone ring of ricinine. Leete and Leitz fed nicotinic acid-7-^{14}C to 3 week old castor bean seedlings and then after 14 days isolated, purified and treated ricinine with 57 % sulfuric acid to yield N-methyl -4-methoxy-2-pyridone which contained none of the radioactivity. Thus all of the radioactivity was found to be located in the nitrile group. Waller and Henderson (46) confirmed and extended their finding using ^{14}C and ^{3}H doubly labelled nicotinic acid to show that all the carbons of nicotinic acid were incorporated into the pyridine ring of ricinine. Yang and Waller (34) have established by in vivo experiments with young castor bean plants that quinolinic acid-2,3,7,8-^{14}C can serve as a more efficient precursor of ricinine than nicotinic acid-7-^{14}C. They also performed chemical degradation of ricinine formed from quinolinic acid -2,3,7,8-^{14}C, aspartate-4-^{14}C, succinate-1,4-^{14}C and -2,3-^{14}C, glycerol-1,3-^{14}C and -2-^{14}C and confirmed that carbons 2, 3 and 8 of ricinine arise from a four carbon dicarboxylic acid such as aspartate and that carbons 4, 5 and 6 arise from intact glycerol. The relative efficiency of various four carbon dicarboxylic acid and inorganic nitrogen sources for the ricinine was reported (45).

The relationship between the pyridine nucleotide cycle and the biosynthesis of ricinine was established by Waller and collaborators (3). They showed that each compound in the cycle could serve as an efficient ricinine precursor for the castor bean plant. The possible

role of the cycle in ricinine biosynthesis is illustrated in Figure 2.

A different view which suggests that NAD and ricinine are made from quinolinic acid by separate pathways (Figure 32) was reported recently by Hiles and Byerrum (122). Their view is based on competitive feeding experimental data exogenous NAD did not cause a decrease in the total radioactivity incorporated into ricinine from quinolinic acid, but instead caused an increase. However, their argument can only be justified when one assumes that NAD is an obligatory intermediate in the biosynthesis of ricinine from quinolinic acid and that exogenous NAD can cross the cellular membrane barrier intact as they mentioned. It is hard to understand the 2.9 fold increase in the incorporation of quinolinic acid into ricinine by NAD if pathways I and II (Figure 32) are independent. Their results might be more reasonably explained by assuming that ricinine might be derived from an intermediate, such as nicotinic acid mononucleotide, which is located before NAD in the cycle (see Figure 2).

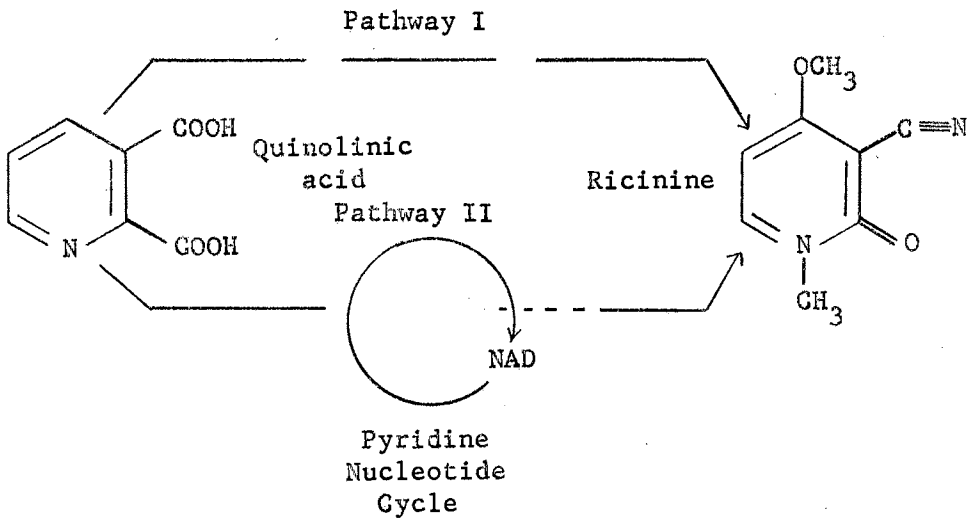


Figure 32. The Two Possible Pathways for the Biosynthesis of Ricinine from Quinolinic Acid

2. Factors that Affect the Biosynthesis of Ricinine

The physiological conditions that affect the biosynthesis of ricinine were studied at the beginning of this century by several authors (112, 123). Weevers reported that the quantity of ricinine in etiolated castor bean seedling was higher than in normal seedling. However, it has been found that ricinine production may be stimulated by sunlight. Bogdashevskaya (123) found an 18 % reduction in the content of ricinine in the leaves which were shaded from the light; however, the upper unshaded leaves of such plants produced ricinine at a 14 % higher level than normal plants.

Waller and Nakazawa (124) reported that the ricinine content of young castor bean seedlings growing in the sand increased from 30 to 50 fold in the 2 to 5 day period following planting of the seed. Waller et al. (115) also demonstrated that the ricinine production by individual castor bean plants was increased from 0.1 m mole in an individual castor bean seed to levels of about 1.2 m moles in a 17 week old plant.

Weevers (112) studied the effect of the availability of nitrogen in the soil on ricinine production and found that 100 seedlings which were germinated in light in the soil "as nitrogen-free as possible" yielded 273 milligrams of ricinine after 3 weeks and 175 milligrams after 5 weeks. He sought to implicate ricinine as a nitrogen source in the nitrogen depleted condition that could be used for other purposes.

Skursky and Waller (125) showed that nicotinic acid-7-^{14}C was significantly incorporated into both ricinine and N-demethyl ricinine during the first day of germination indicating that alkaloid

biosynthesis is one of the earliest metabolic processes initiated with growth and differentition in Ricinus communis L. In fact seeds which contained less than 0.8 % of ricinine and 0.04 % of N-demethyl ricinine (normal seed contain approximately 0.1 % and 0.07 % of these compounds respectively) failed to germinate; causing them to speculate that ricinine might have some metabolic or physiological role in the germination process.

Nowacki and Waller (126) recently reported that addition of ammonium nitrate to the plant decreased the incorporation of nicotinic acid into ricinine, however, addition of ammonium nitrate to the soil increased the content of ricinine and also the content of asparagine and glutamine, arginine and ammonia in the plant after 6 weeks. This was explained as being due to intensive new growth, i.e., the plants with additional nitrogen started to produce a greater number of side shoots. The same authors also studied the inhibition of biosynthesis of ricinine in the castor bean plant and found that nicotinic acid did not depress the incorporation of aspartate nor did asparagine depress the incorporation of nicotinic acid. They suggested that asparagine, aspartate and nicotinic acid did not have an obligatory substrate -product relationship, but that parallel biosynthetic pathways for ricinine formation might exist. Such a relationship could exist in complete agreement with the operation of the pyridine nucleotide cycle. They also reported that nicotinonitrile and O-demethyl ricinine inhibited the conversion of nicotinic acid to ricinine.

C. Metabolism of Alkaloids

During the early part of this century, it was generally thought that alkaloids were byproducts produced by a number of irreversible

and physiologically useless reactions (16, 36), however, there are growing evidences in the literature (92, 127) that alkaloids are not final product of nitrogen metabolism.

Experimental evidence indicating that decomposition of poppy alkaloids could occur in the living plants was obtained as early as 1897 (113). Several other investigators (113, 128) have obtained similar non-isotopic results which support the view that alkaloids are not inactive metabolites.

The conversion of alkaloids to other organic compounds was first demonstrated, using isotopically labelled compound, by Tso and Jeffrey (129). They administered ^{15}N-labelled tobacco alkaloids to tobacco plants which degraded the labelled alkaloid and it was found that some of the products were used for resynthesis of the nicotine and anabasine. Leete and Bell (130) demonstrated that nicotine could act as a methyl group donor; 90 % of methyl-^{14}C-nicotine activity was found in the methyl groups of choline. Griffith et al. (131) showed that nicotinic acid was a metabolite of nicotine. They found that during the first four days of metabolism about 30 % of the nicotine was lost through metabolic processes, but subsequently little loss occured up to 14 days. Isolated nicotinic acid contained a significant quantity of isotope with dilution ranging from 18 to 88. Tso and Jeffery (132) have shown that the carbon-14 activity of the groups of compounds recovered from tobacco plants which had been fed with carbon-14 and nitrogen-15 labelled nicotine decreased in the following order with respect to carbon-14 activity: alkaloids, free amino acids, pigments, furfural (after hydrolysis), amino acids (after hydrolysis), sugars (after hydrolysis), free organic acids, free sugars, insoluble residue and organic acids (after hydrolysis). A different order was observed

in the isolated groups of compounds which contain nitrogen-15.

Waller and Nakazawa (124) reported a finding that was interpreted to indicate that the alkaloid had a "sparing" action on the vitamin. They found that ricinine was rapidly utilized by castor bean cotyledons in the dark; however, the amount of ricinine did not decrease when nicotinic acid was presented in the medium.

Waller et al. (115) demonstrated that ricinine-H^3 and ricinine -8-^{14}C were metabolized by the castor bean plant and that the extent of metabolism varied from 75 to 90 % after 20 weeks following administration of the alkaloid. In addition it was demonstrated that the alkaloid could be transported to the seeds since radioactive alkaloid administered to a young non-flowering castor bean plant was isolated from the seeds of mature plants.

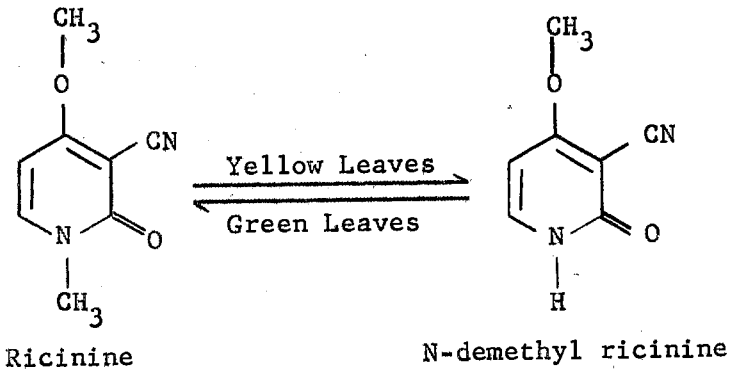
The conversion of ricinine to respiratory $^{14}CO_2$ in vivo indicating degradation of α-pyridone ring of ricinine was reported by Waller and Lee (92). They also demonstrated that yellow leaves, which contained a trace amount of the alkaloid, could metabolize ricinine-3,5-^{14}C to $^{14}CO_2$.

Recently a rapid interconversion of exogenous ricinine and N-demethyl ricinine in senescent and green castor bean plant leaves (Figure 33) was reported by Skursky et al. (93). They made the assumption that the interconversion might be involved in a salvage operation performed by the plant in order to utilize ricinine from the leaves which are being prepared for abscission. This assumption was supported by the fact that a rapid methylation of N-demethyl ricinine to give ricinine occurred in fresh green leaves.

Waller and Skursky (97) showed that a remarkably high proportion of the total administered radioactivity of ricinine-3,5-^{14}C was found

in the racemes of immature fruit after two days and more than 95 % of the radioactivity isolated was found in ricinine. Skursky et al. (93) recently identified N-demethyl ricinine, 3-cyano-4-methoxy-2-pyridone, as a ricinine metabolic product (Figure 33). They presented chromatographic, ultraviolet, infrared, mass spectral and nuclear magnetic data to support the structure of N-demethyl ricinine and discussed in detail the possible role of demethylation and methylation reaction in ricinine translocation during senescence.

Catabolism and translocation of some alkaloids have been known and some connections between the processes and physiological state of the plant have been made, however, little is known about the metabolic or physiological significance of these processes for the plants.



Ricinine N-demethyl ricinine

Figure 33. Interconversion of Ricinine and N-demethyl Ricinine in the Castor Bean Plant

CHAPTER VII

EXPERIMENTAL METHODS

A. Materials and Methods

1. Plants

The castor bean plants used were of the Cimarron variety grown on port clay loam at the Agronomy farm of the Oklahoma State University in Stillwater, Oklahoma. The plants of 4 to 6 months of age which were planted on May 30, 1970 were used for these experiments. The other group of the plants planted on June 30, 1970 were grown in pots with a mixture of clay loam soil and vermiculite at the green house of the Horticulture Department, Oklahoma State University, Stillwater, Oklahoma. The yellow leaves used were the senescent ones (from 70 to 95 % of the leaf was yellow) which were on the lower part of the stalk.

2. Labelled Compounds Used

Ricinine-3,5-^{14}C was synthesized on a micro scale from sodium cyanoacetate-2-^{14}C, according to the procedure described by Yang and Waller (34). The synthesized ricinine was purified by preparative thin layer chromatography using a solvent system consisting of chloroform and methanol (5 : 1, v/v). The purity was checked by ascending paper chromatography on Whatman No. 1 paper in two different solvent systems. The R_f values of ricinine in 85 % isopropanol and in

95 % ethanol-1 M ammonium acetate (7 : 3, v/v, pH 5.0) were 0.75 and 0.85 respectively. The constant specific activity of the ricinine used was 1.78×10^4 dpm/mg.

O-demethyl ricinine-3,5-^{14}C was isolated from the yellow castor bean plant leaves fed with ricinine correspondingly labelled by the procedure described in the following section. The constant specific radioactivity of this compound was 1.12×10^4 dpm/mg.

B. Metabolism

1. Administration of Labelled Compounds

The general method of injection used for the castor bean plants was identical to the method described in Part I. The yellow leaves were given injected with a 5 % methanol solution of ricinine-3,5-^{14}C (5 mg/ml) in the petioles near the leaves and veins.

2. Isolation and Purification

The fresh plant material was weighed, frozen with liquid nitrogen and homogenized with a Virtis "23" omnimixer in 80 % methanol. This extraction procedure with methanol was repeated until the remaining material was free of soluble pigments. The organic solvent was removed from the pooled extracts by evaporation at room temperature and reduced pressure. The remaining aqueous solution was freed of liquid and pigments by extraction with petroleum ether. The aqueous phase, which contained ricinine and the metabolites of interest, was evaporated to dryness. The petroleum ether extract and the plant debris were not further examined. The dry residue from the aqueous portion of the extract was extracted with boiling methanol, the volume was

reduced, and this solution was then used for preparative thin layer chromatography as described in Part I, except the solvent systems: I) chloroform and methanol (5 : 1, v/v) and II) ethanol : ammonium hydroxide : water (80 : 4 : 16, v/v/v). The solvent systems I and II were used for separation of the relatively non-polar compounds, ricinine and N-demethyl ricinine, and relatively polar unknown compounds respectively. The radioactive and/or UV quenching zone of interest on the thin layer chromatography plates was scraped off, put in a small scale column and extracted with a sufficient volume of methanol. The methanol extract was usually rechromatographed and further purified. The isolated compounds were crystallized in the cold methanol and sublimed. The purity of the isolated compounds were checked with ascending paper chromatography using an isopropyl alcohol-toluene-acetic acid-water (5 : 10 : 1 : 1) solvent system.

3. Measurement of Radioactivity

Methods for the measurement of the radioactivity on thin layer chromatography plates and of purified compounds were identical with the procedure described in Part I.

The respiratory $^{14}CO_2$ from the castor bean plant was measured (92) as follows: the plant was immediately placed in a closed respiratory chamber after administering ricinine-3,5-^{14}C into the petiol of the yellow leaves. The entering air was passed through with 1 N NaOH so that carbon dioxide-free air was present in the chamber. The outlet of the chamber was connected by small sections of rubber tubing to a series of four test tubes, 150 x 115 mm, as shown in Figure 3. The first tube was cooled to liquid nitrogen temperature, and was used for trapping the volatile compounds. The second and third tubes each

contained 10 ml of ethanolamine in methylcellosolve (1 : 1, v/v) and were used for collecting the respiratory CO_2. The final tube contained 10 ml of saturated barium hydroxide which was used as an indicator: the lack of precipitation in the saturated barium hydroxide indicated that all the carbon dioxide was quantitatively trapped by the ethanolamine in methylcellosolve. A portion of the CO_2 trapping solution, 3 ml, was added to the scintillation solution (10 ml) which was composed of methylcellosolve and toluene (1 : 2, v/v) with 5.5 grams of PPO per liter for determination of the radioactivity by liquid scintillation spectrometry.

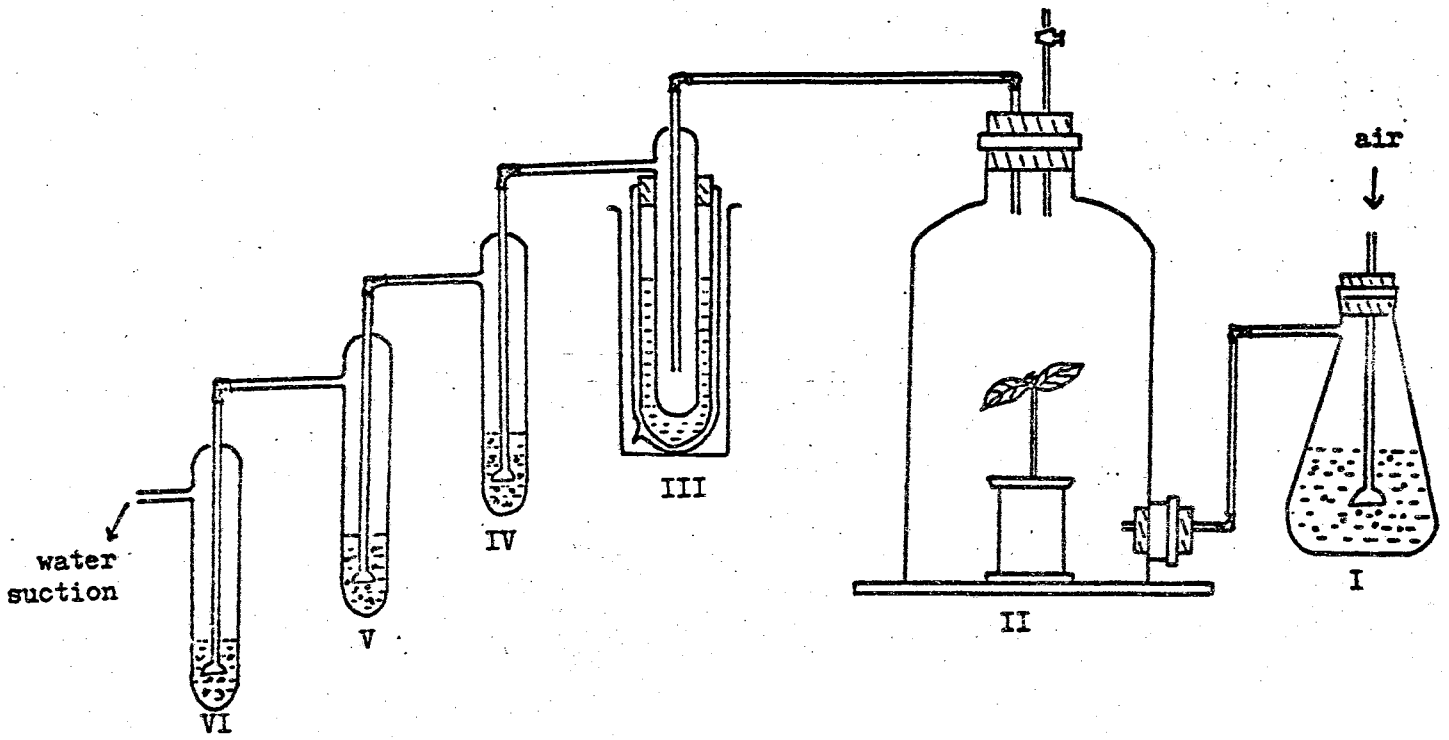


Figure 34. Respiratory Apparatus

I. NaOH (IN)
II. Respiratory Chamber
III. Absorption Trap (# 1)
IV. Absorption Trap (# 2) - 10 ml ethanolamine-methylcellosolve
V. Absorption Trap (# 3) - 10 ml ethanolamine-methylcellosolve
VI. Absorption Trap (# 4) - 10 ml saturated barium hydroxide

CHAPTER VIII

RESULTS AND DISCUSSION

A. Detection, Isolation and Identification of a New Ricinine Metabolite

1. Detection

During the course of quantitative studies on the catabolism of ricinine in senescent leaves of the castor bean plant following injection of ricinine-3,5-^{14}C, a radioactive zone was observed at the origin of the thin layer chromatography plate developed with the relatively non-polar solvent system, chloroform : methanol (5 : 1, v/v). Radioactivity in this non-migrating zone after development had been observed previously by Skursky (93); however, no attempts had been made to identify the compound(s) in the zone at that time. The thin layer chromatographic behavior of the compound(s) in the non-polar solvent system suggested that it might be a relatively more polar compound(s) than the N-demethyl ricinine which was recently identified as a ricinine metabolite in senescent leaves of the castor bean plant by Skursky *et al.* (93). It was thought that this polar compound(s) could be either a cleavage product of the α-pyridone ring, one or more pyridine nucleotides, or the O-demethyl form of ricinine.

2. Isolation and Purification

In order to positively identify this unknown compound(s), the silica gel zone at the origin (R_f value 0.0 - 0.05) was removed and extracted with methanol. The methanol extract was reduced in volume, applied on the thin layer chromatography plate and developed with a relatively polar solvent system, ethanol : water : ammonium hydroxide (80 : 16 : 4, v/v/v). A comparison of two thin layer chromatography plates developed with the relatively non-polar and polar solvent systems are shown in Figure 35. The thin layer chromatogram developed with the relatively polar solvent system showed two quenching zones at 254 nm. One of these zones was radioactive. The radioactive zone was eluted from the thin layer chromatogram with methanol and rechromatographed with various solvent systems as listed in Table XVI. For further purification, the unknown radioactive compound was dissolved in hot methanol and crystallized after letting stand overnight at 38°F after which it was further purified by sublimation. The following purification data were recorded with the non radioactive metabolite.

Number of Purification	Weight of product[a] (mg)	M.P./760 mm (uncorrected)
1. Crystallization 1	10.6	274 - 276°C
2. Crystallization 2	8.4	277 - 279°C
3. Sublimation	3.2	279°C

[a]Weight of compound was measured with $E = 7.32 \times 10^3$ l/mole. The extinction coefficient for O-demethyl ricinine was determined by the author in this study.

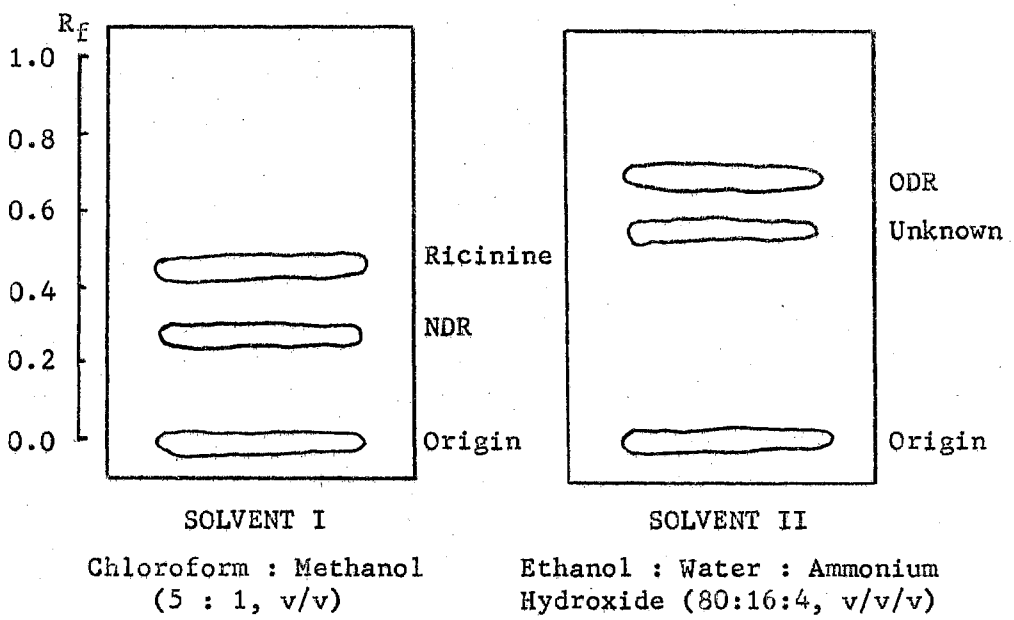


Figure 35. Schemetic Diagram of Thin Layer Chromatograms Developed with Relatively Non-polar and Polar Solvent Systems

NDR - N-demethyl ricinine ODR - O-demethyl ricinine

TABLE XVI

THIN LAYER CHROMATOGRAM R_f VALUES OF RICININE, N-DEMETHYL RICININE AND UNKNOWN METABOLITE

R_f value / Solvent system	Ricinine	N-demethyl ricinine	Unknown (O-demethyl ricinine)
Chloroform : Methanol (5 : 1, v/v)	0.42	0.27	0.0-0.03
Chloroform : Methanol (5 : 5, v/v)	0.58	0.56	0.35
Chloroform : Methanol (1 : 5, v/v)	0.48	0.50	0.68
Ethanol : Water : NH_4OH (80 : 16 : 4, v/v/v)	0.53	0.63	0.75

3. Identification

Mass Spectrometry: This new metabolite of ricinine formed white needles when crystallized from methanol and had a melting point of 279°C. The mass spectrum of the unknown compound was compared with mass spectrum of the authentic O-demethyl ricinine (ricininic acid) in Figure 36. The mass spectrum of the unknown compound was essentially identical with that of authentic O-demethyl ricinine. Both compounds had a molecular ion, M^+ 150, which was their base peak. The postulated route for the formation of the relatively abundant ions and a list of the metastable peaks and the transitions denoted by them are given in Figure 37 and Table XVII respectively.

Decomposition reactions that can account for at least some of the prominent features could have been predicted from earlier work with similar molecules such as ricinine and N-demethyl ricinine which contain the same functional groups. The molecular ion, M^+ 150, undergoes opening of the α-pyridone ring to form ion b, m/e 84, C_4H_6ON, which is the second most intense peak. It is of interest to compare the formation of ion m/e 84 with the ion m/e 82 which is the third most intense peak of ricinine (111) as shown in Figure 38. In molecular ions, M^+ 150 and M^+ 164, the only difference is the location of the hydrogen atom; for ion M^+ 150 the structure is a 4-OH, 2-pyridone and for ion M^+ 164 the structure is a 2-OH, 4-pyridone. The formation of ions m/e 84 and m/e 82 probably occur by analogous processes.

Ion b, m/e 84, can lose a hydrogen atom to form ion c, m/e 83, and further loss of CO results in ion e, m/e 55. The molecular ion loses CO and H to yield the 5 numbered heterocyclic ion d, m/e 121. The formation of a similar ion species was reported (133) with

∝-pyridone ring compounds. The ion d, m/e 55, can be further cleaved and lose either CH_2 or C_2H_3 to form ion f, m/e 41 and ion g, m/e 28 whose species are already known (133).

Infrared Spectroscopy: The infrared spectra of O-demethyl ricinine isolated from the castor bean plant and that of standard in a KBr pellet are shown in Figure 39. The infrared spectra of the O-demethyl ricinine isolated from the castor bean plant was found to be virtually superimposable on that of the standard. Both spectra have a very broad OH stretching absorption in the region of 3.0 to 4.0 microns, strong =C–O stretching at 62 microns and a sharp ketone absorption band from 7.7 to 9.5 microns. A very sharp absorption peak at 4.5 microns indicated –C≡N stretching which is observed not only with O-demethyl ricinine but also with N-demethyl ricinine (93) and ricinine (86).

Ultraviolet Spectrophotometry: Figure 40 shows the ultraviolet spectra of O-demethyl ricinine, N-demethyl ricinine, dihydroricininic acid, and ricinine in distilled water. Note that the ultraviolet spectra of O-demethyl ricinine is similar to that of dihydroricininic acid and the spectra of N-demethyl ricinine is similar to that of ricinine where the absorption peaks of O-demethyl ricinine and N-demethyl ricinine are shifted to the shorter wavelength region as compared with those of dihydroricininic acid and ricinine respectively.

Based on these data (chromatographic, ultraviolet, infrared, nuclear magnetic resonance and mass spectrometry) the metabolite was conclusively identified as O-demethyl ricinine, N-methyl-3-cyano-4 -hydroxy-2-pyridone.

After the unknown metabolite of ricinine had been confirmed as O-demethyl ricinine, the molecular extinction coefficient of the compound in water at 272 nm ($E = 7.32 \times 10^3$ 1 $mole^{-1}$) was determined and

this value used for further quantitative metabolism studies (Figure 41).

Conclusive Proof of Occurrence of O-demethyl Ricinine as a Natural Castor Bean Plant Metabolite: To make certain that the metabolite had actually been produced by the castor bean plant and was not an artifact as the results of microbial action, the following two experiments were conducted.

In the first experiment, ricinine (10 mg) was added to a fresh homogenate of two green leaves (10 g) and ricinine and its metabolites were isolated as described. The green leaves were chosen for this experiment since it was known that they did not have demethylating activity (93) even though they contained a significant amount of endogenous ricinine (1 % dry weight). The recovery of ricinine was good (94 %) and practically no metabolite of ricinine was detected as shown in Table XVIII.

In the second, a short term experiment under semisterile conditions as described by Skursky et al. (93) was carried out to eliminate the possible demethylation by microorganisms. Ricinine (10 mg) in a sterile solution was injected into two yellow leaves (15 g) which had been previously washed with 1 % aqueous sodium hypochlorite solution and rinsed with sterile water. The recovery and conversion data after three hours of injection is shown in Table XIX.

These two short term experiments provided convincing evidence that O-demethyl ricinine in the yellow leaves of the castor bean plant was a normal metabolite of ricinine.

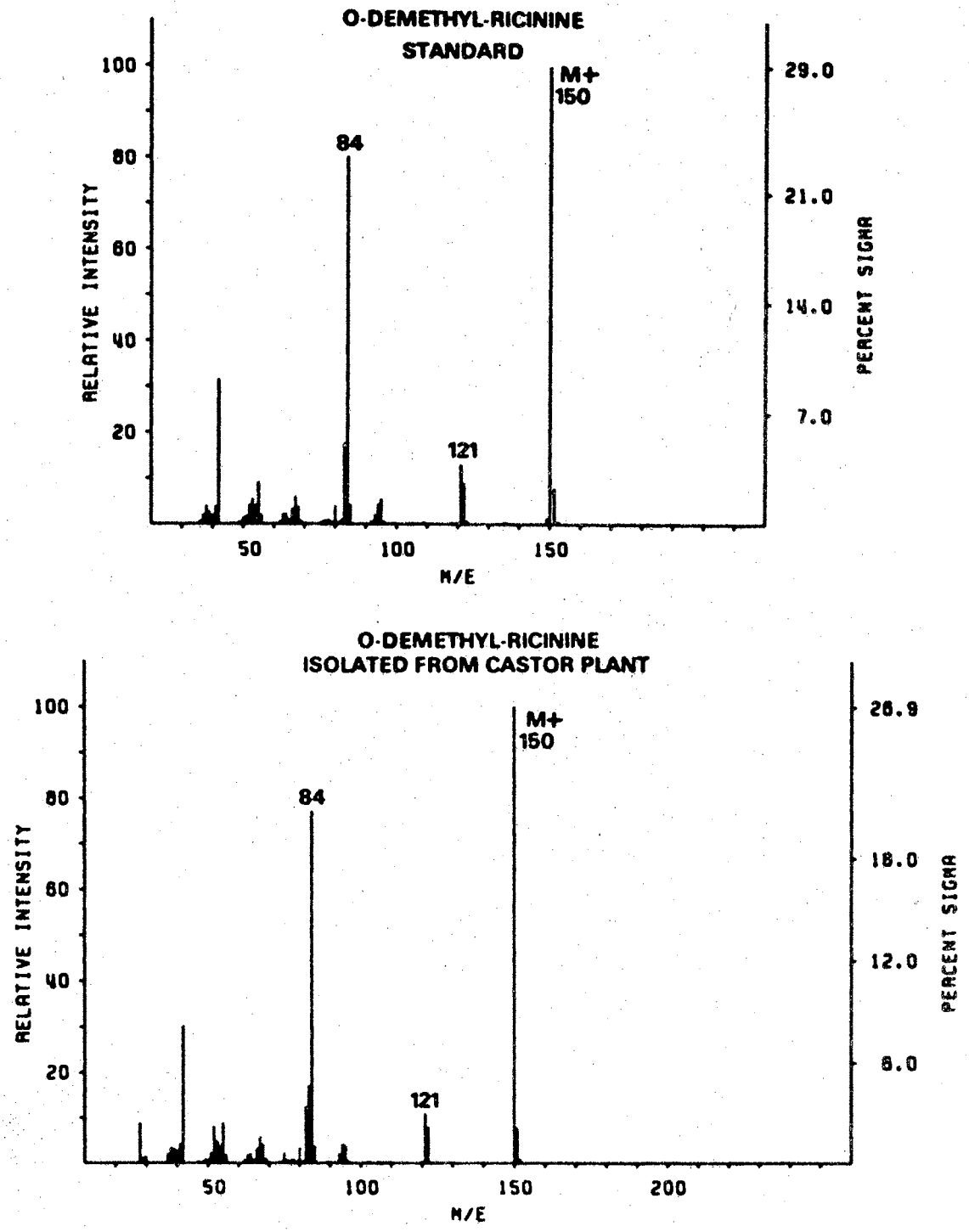


Figure 36. Mass Spectra of the Standard and Isolated O-demethyl Ricinine

TABLE XVII

METASTABLE PEAKS IN MASS SPECTRUM OF O-DEMETHYL RICININE

Apparent mass	Transition denoted	Probable neutral product
120.0	$(122)^+ \longrightarrow (121)^+ + 1$	H
99.2	$(150)^+ \longrightarrow (122)^+ + 28$	CO or CH_2N
47.2	$(95)^+ \longrightarrow (67)^+ + 28$	CO
36.4	$(85)^+ \longrightarrow (55)^+ + 28$	CO
21.0	$(84)^+ \longrightarrow (42)^+ + 42$	CH_2CO

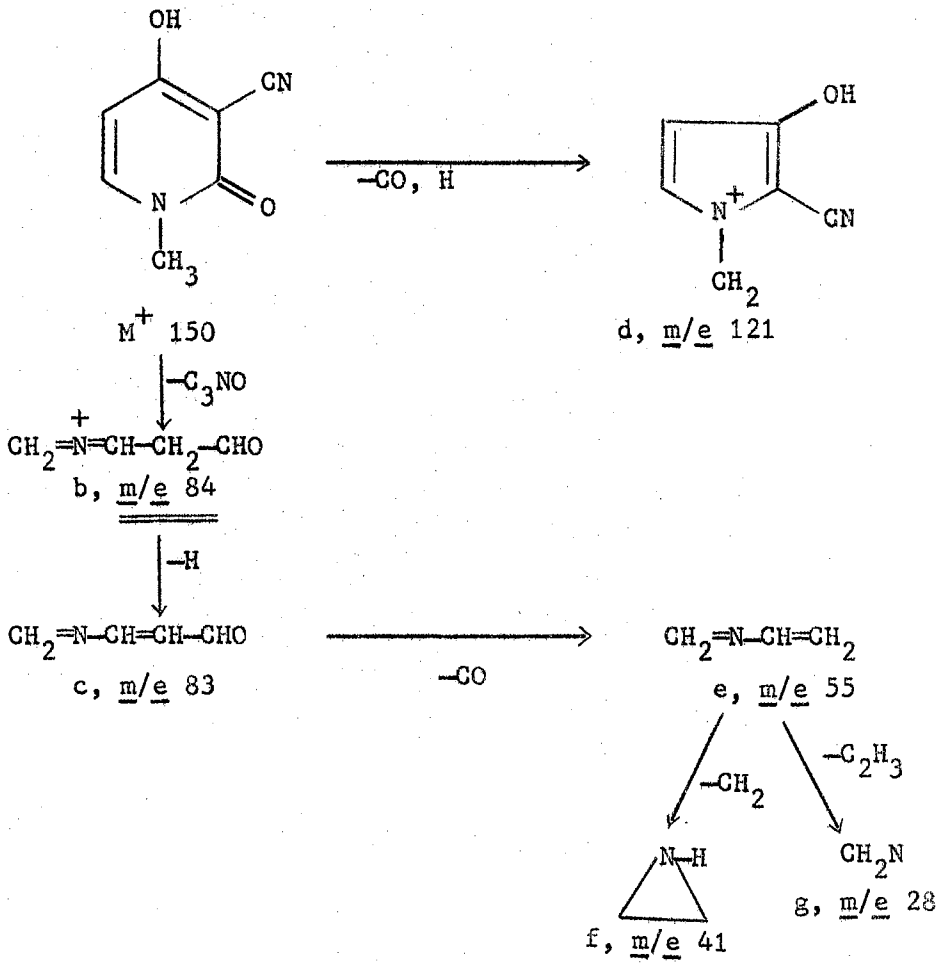


Figure 37. Proposed Partial Fragmentation of O-demethyl Ricinine

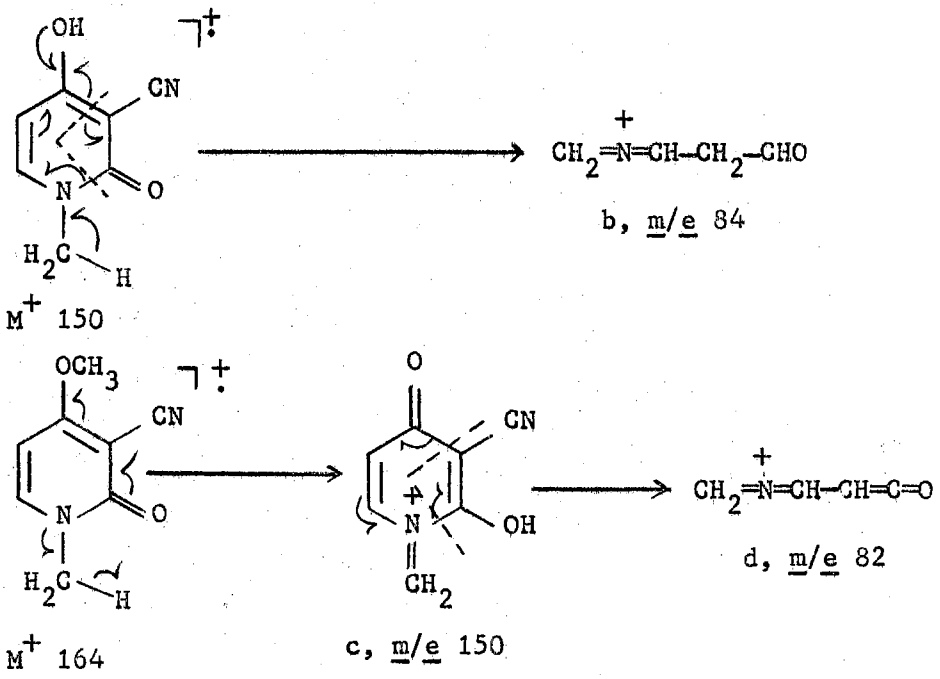


Figure 38. Comparison of the Formation of the Ion, m/e 84, from O-demethyl Ricinine and the Ion, m/e 82, from Ricinine

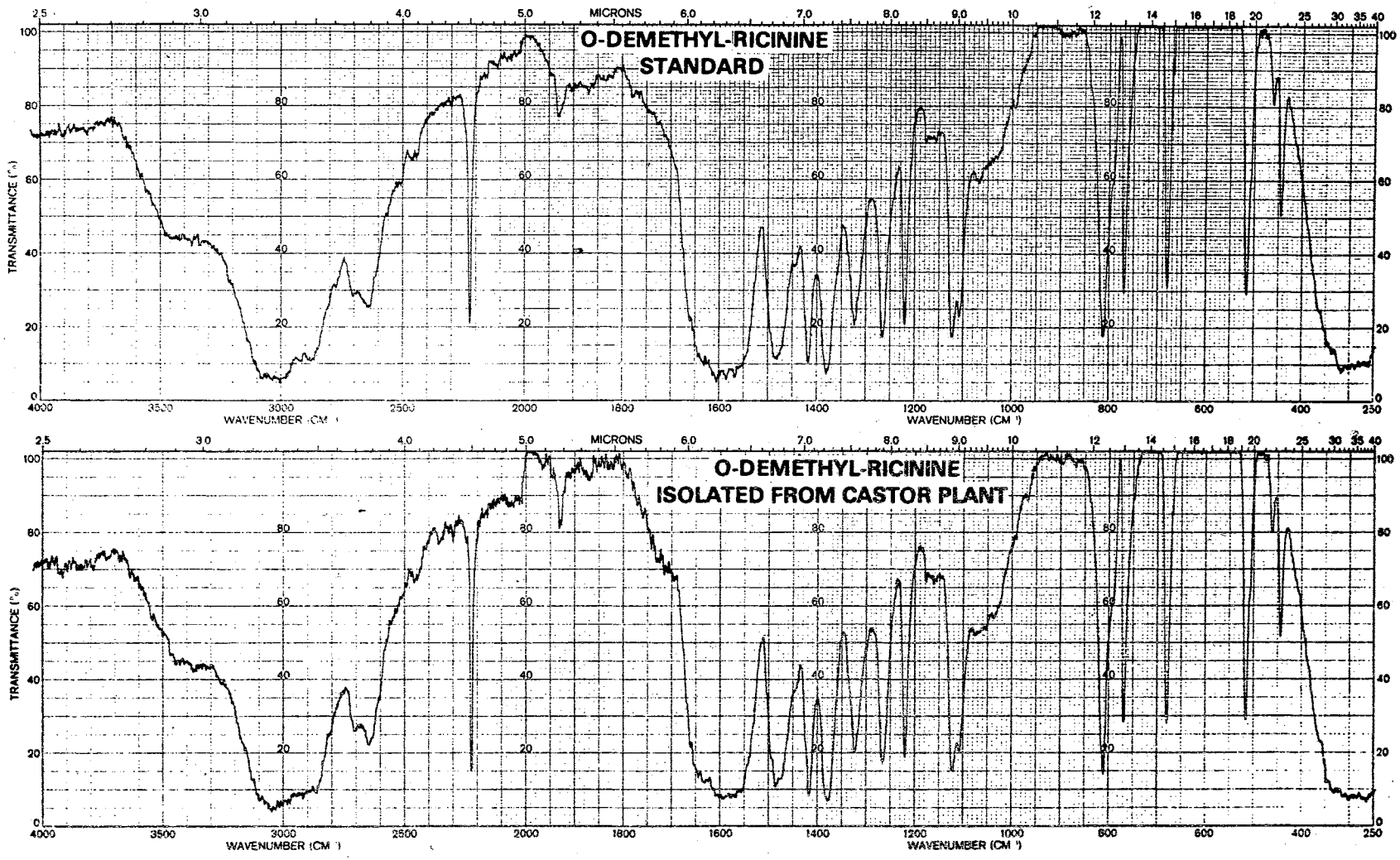


Figure 39. Infrared Spectra of Standard and Isolated O-demethyl ricinine

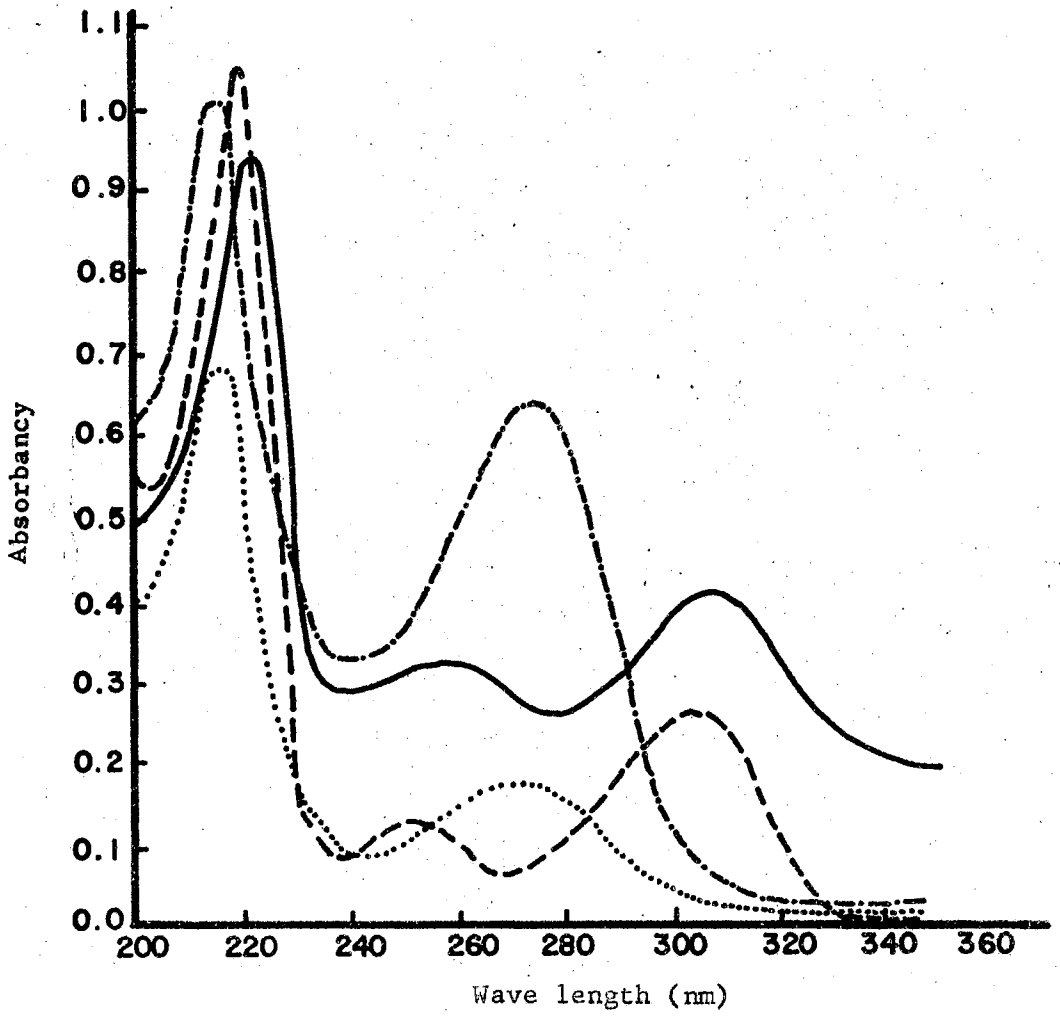


Figure 40. Ultraviolet Spectra of Ricinine, N-demethyl ricinine, O-demethyl ricinine and Dihydro Ricininic Acid

——— Ricinine ·—·—· Dihydro ricininic acid
···· O-demethyl ricinine ---- N-demethyl ricinine

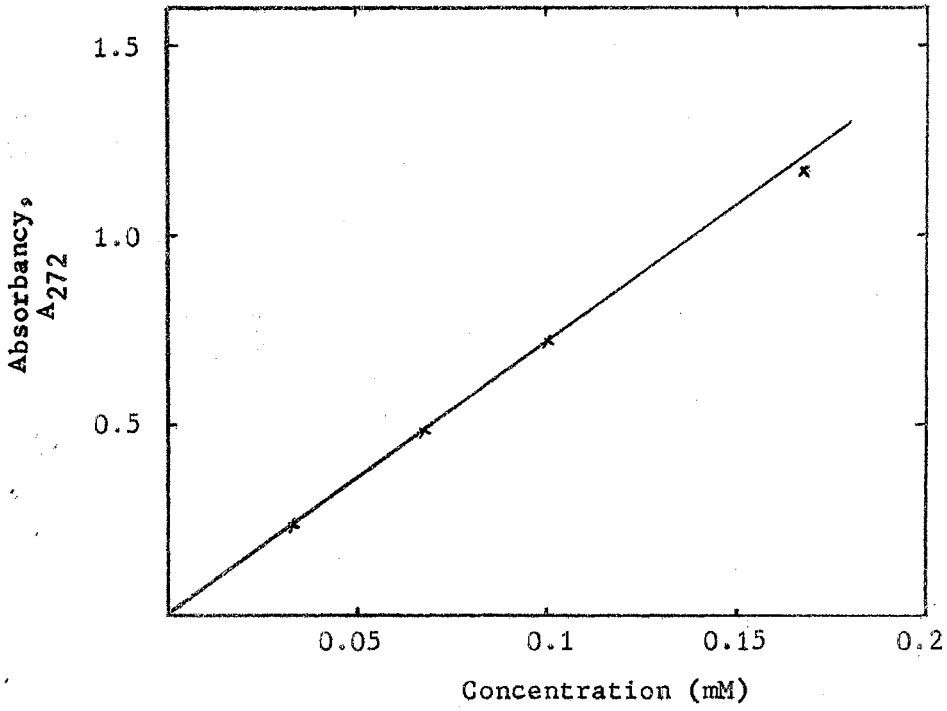


Figure 41. Absorbancy of O-demethyl ricinine in Water at 272 nm (Determined E = 7.32×10^3 liter per mole)

TABLE XVIII

RECOVERY OF RICININE FROM A HOMOGENATE OF THE GREEN LEAVES

Administered ricinine	Recovered ricinine	Recovery (uncorrected)	Recovery (corrected)[1]
mg	mg	%	%
10	21.3	213	94

No demethylated form of ricinine was detected.

[1]Recovery percentage was corrected based on the natural content of the ricinine (0.7 %) and of water (83 %) in the average green leaf.

TABLE XIX

FORMATION OF N-DEMETHYL RICININE AND O-DEMETHYL RICININE FROM RICININE BY SEMI-STERILIZED YELLOW LEAVES

Compound	Isolated	Recovery
	mg	%
Ricinine	7.6	76
N-demethyl ricinine	1.35	13.5
O-demethyl ricinine	0.2	2.0
Total	9.15	91.5

Duration of experiment was 3 hours. Percentage of the recovery was determined by dividing total weight of ricinine administered by the weight of compound isolated.

B. Metabolism and Translocation of Ricinine

1. Demethylation of Ricinine in Excised Senescent Leaves

The formation of N-demethyl ricinine, O-demethyl ricinine and carbon dioxide from different amounts of administered ricinine in excised senescent castor bean plant leaves is summarized in Table XX. A major drawback to studying the metabolism of ricinine has been the difficulty in synthesizing high specific activity labelled ricinine. Large amounts of ricinine were administered in previous work (93), but the metabolism of ricinine using different dosage levels had not been done. In this study, three levels of ricinine, 34.5, 84 and 143 µg/g fresh weight, were administered to senescent leaves. The percentage of N- and O-demethylated ricinine products at the three different levels of ricinine administered in the yellow leaves are shown in Figure 42. These results suggest that ricinine inhibited the O- and N-demethylating reactions. They also indicated that senescent leaves have a certain limitation of the rate of demethylation activity. The low extent of N-demethylating activity obtained after the administration of the high level of ricinine agreed with previous work (93). The results showed that majority of the administered ricinine could be converted to its demethylated forms at low dosage level which is comparable to the normal physiological levels. This conclusion can be justified by extrapolating the line shown in Figure 42 to low dosage level. The approximate disappearance rate of ricinine obtained by extrapolating the ricinine curve is 3.8 µg/g fresh weight/day.

The low yield of CO_2, 0.2 - 0.4 %, suggested that intensive degradation of the α-pyridone ring does not occur in the yellow leaves, therefore, these results confirmed earlier findings (92).

As shown in Table XXI, 38.7 % of the administered O-demethyl ricinine was methylated by excised green leaves in 2 days. This conversion percentage is higher than the reported value of 12.3 % obtained with use of whole plant (86). Skursky et al. obtained about 48.7 % of ricinine after administration of N-demethyl ricinine. These two experimental results show that the O-demethylation and O-methylation of ricinine is not as active as the N-demethylation and N-methylation of ricinine in the castor bean plant. Nowacki recently showed an interesting result that O-demethyl ricinine inhibited the biosynthesis of ricinine from nicotinic acid-7-^{14}C (126). A schemetic diagram summarizing the extent of the demethylation of ricinine and the methylation of the demethylated forms of ricinine is given in Figure 43.

Demethylation has been found to be connected with the process of senescence in the case of nicotine (134), hyoscyamine (135) and ricinine (93). Methylation is considered to be connected with detoxication (136). Mothes (137) has suggested that the methylation of a compound makes it less reactive, or "metabolically stabilized". In contrast, the demethylation of a compound makes it metabolically or physiologically active. Demethylation of nicotine to nornicotine in connection with the quality of tobacco has been intensively studied since nornicotine has been found to be less toxic than nicotine. N-demethyl ricinine has been found to be a normal constituent of mature seeds and disappears during germination (125). The site of the demethylation reaction has been found mostly to be in the leaves (93). Even though the demethylation processes of some alkaloids have been demonstrated and some of the methylated products have been identified, the physiological or metabolic significance of this process is totally

TABLE XX

FORMATION OF N-DEMETHYL RICININE, O-DEMETHYL RICININE AND CARBON DIOXIDE FROM RICININE IN EXCISED SENESCENT LEAVES

Isolated compound \ Exp. No.	I			II			III		
	dpm x 10^2		%	dpm x 10^2		%	dpm x 10^2		%
Ricinine	13.51	11.5	16.4	62.83	12.6	38.2	143.20	10.1	43.5
N-demethyl ricinine	32.20	10.7	39.1	47.31	11.4	28.7	71.81	8.4	21.8
O-demethyl ricinine	4.94	9.9	6.1	4.12	11.5	2.5	4.94	8.5	1.5
Carbon dioxide	0.18		0.2	0.29		0.2	1.32		0.4
Total	50.83		61.8	114.55		69.6	221.27		67.2

Percentage of incorporation was determined by dividing total radioactivity administered by the total amount recovered.

Total Administered Ricinine Radioactivity

Experiment I. 8.23×10^3 dpm (0.5 mg), 34.5 µg ricinine/g. fresh weight
Experiment II. 1.646×10^4 dpm (1 mg), 83.0 µg ricinine/g. fresh weight
Experiment III. 3.292×10^4 dpm (2 mg), 143.0 µg ricinine/g. fresh weight

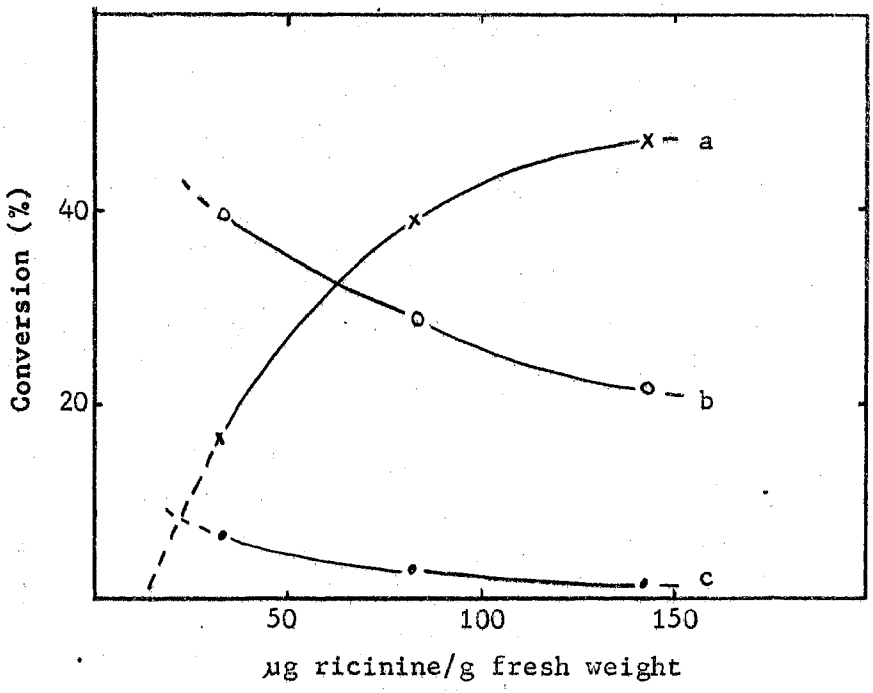


Figure-42. Formation of N-demethyl Ricinine and O-demethyl Ricinine from Ricinine-3, 5-^{14}C Administered in the Yellow Leaves after 48 Hours

Detailed legend is given in Table XX.

a - Ricinine b - N-demethyl ricinine
c - O-demethyl ricinine

unknown, much like the nature of the alkaloids themselves.

It is of interest that the demethylated form of ricinine is practically absent in the green leaves and that very small amounts of ricinine and N-demethyl ricinine are present in the yellow leaves as shown in Table XXII. The very small amounts of ricinine and N-demethyl ricinine found in the yellow leaves might be due to the incomplete process of senescence since naturally detached leaves were found to be void of both compounds. The fact that; a) absence of both ricinine and its metabolites in the yellow leaves, b) the methylation and demethylation reactions occur in the green leaves and yellow leaves respectively, and c) the -pyridone ring of ricinine is not intensively degraded in the yellow leaves, supported the conclusion that ricinine and/or N-demethyl ricinine or O-demethyl ricinine in the yellow leaves are translocated from the senescent tissue to other part of the plant, especially to the growing apex.

2. Demethylation and Translocation of Ricinine

Table XXIII shows the demethylation and translocation of ricinine-3,5-^{14}C administered to a yellow leaf attached to the lower part of the stem of a mature castor bean plant. The castor bean plant used in this experiment was 4 months of age, 60 cm high and grown in a pot in the green house. A plant possessing a similar appearance and similar conditions was selected for a duplicate experiment. The radioactivity of ricinine and the demethylated forms of it were found to be highest in the yellow leaf where ricinine was injected. Radioactive N-demethyl ricinine was found in all parts of the plant except in the root. The second highest total recovery of radioactive ricinine and N-demethyl ricinine was in the stems; however, the recovery of

TABLE XXI

METHYLATION OF O-DEMETHYL RICININE TO RICININE IN EXCISED GREEN LEAVES AFTER 48 HOURS

	dpm x 10^3	mg	dpm x 10^3/mg	%
Ricinine	7.67	3.45	2.223	38.7
O-demethyl ricinine	trace	-	-	-

The plants used were grown in the green house. O-demethyl ricinine (2 mg) with total radioactivity of 2.24 x 10^4 dpm was administered. Percentage of incorporation was determined by dividing the total radioactivity administered by the total amounts recovered.

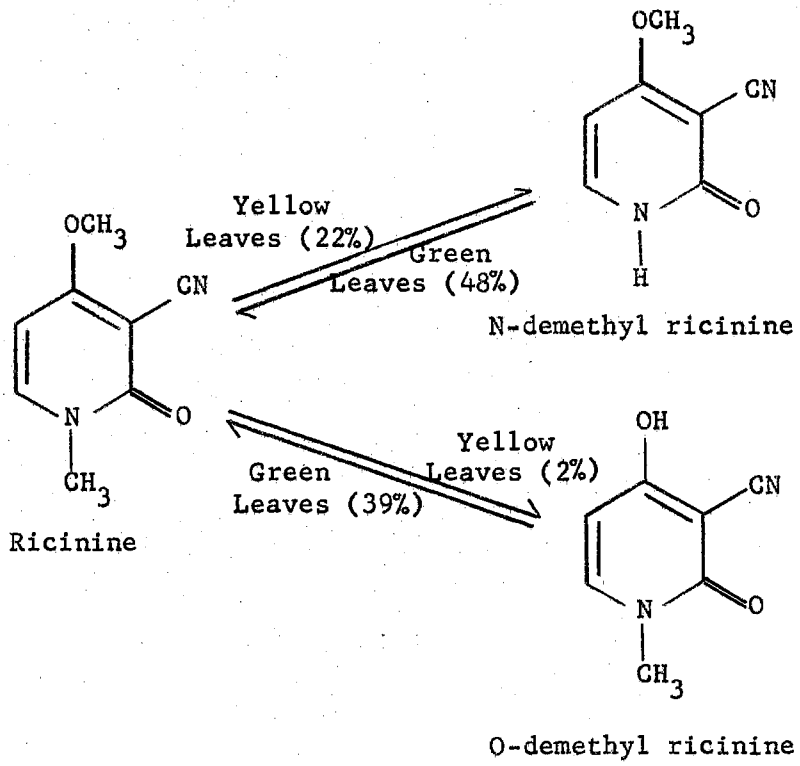


Figure 43. Methylation and Demethylation Reactions involving the Alkaloid Ricinine in the Castor Bean Plant Leaves

[1]Value of Skursky at a similar precursor level.

Conversion percentage are based on the results obtained when 1 mg of precursor was administered to one leaf of about 5 g.

TABLE XXII

CONTENTS OF RICININE, N-DEMETHYL RICININE AND O-DEMETHYL RICININE IN THE GREEN AND YELLOW LEAVES[1]

Leaves \ Isolated compound	Ricinine	N-demethyl ricinine	O-demethyl ricinine
Green (204 g)[2]	251 mg (0.84 %)[3]	-	-
Yellow (193 g)	2.25 mg (0.009 %)	0.075 mg (0.0003 %)	-

[1]Green leaves were fully developed the healthy leaves. Yellow leaves were 80 - 90 % senescent leaves.

[2]Fresh weight of the leaves.

[3]Dry weight was taken as 15 % of fresh weight.

TABLE XXIII

DISTRIBUTION OF RADIOACTIVITY IN THE CASTOR BEAN PLANTS FED WITH RICININE-3,5-^{14}C TO THE YELLOW LEAVES

Plant part \ Isolated compound	Ricinine	N-demethyl ricinine	O-demethyl ricinine	Total
	dpm x 10^2 (%)			
One yellow leaf (4 g)	64.42 (14.1)	31.60 (9.6)	6.26 (1.9)	84.28 (25.6)
Three green leaves (13 g)	8.88 (2.7)	1.65 (0.5)	-	10.53 (3.2)
Stems (18 g)	38.85 (11.8)	21.40 (6.5)	3.95 (1.2)	64.19 (19.5)
Growing apex (5 g)	17.45 (5.3)	3.62 (1.1)	-	21.07 (6.4)
Roots (15 g)	3.62 (1.1)	-	-	3.62 (1.1)
Total	115.22 (35.0)	58.27 (17.7)	10.21 (3.1)	183.69 (55.8)

The radioactivity was the average of two experiments. Duration of experiment was 48 hours. The plants used were grown in the green house. Ricinine-3,5-^{14}C (2mg) with a total radioactivity of 3.292×10^4 dpm was administered. Percentage of the incorporation was determined by dividing the total radioactivity administered by the total amounts recovered.

radioactive ricinine per gram of fresh plant weight and that of O-demethyl ricinine were high in the growing apex and stems respectively.

To gain a better understanding of the process of demethylation and translocation of ricinine-3,5-^{14}C administered to a yellow leaf, an experiment was conducted using castor bean plant cuttings with one yellow and one green leaf (Figure 44, A). The castor bean plants of 4 months of age grown in pots in the green house were selected; the distance between the yellow and green leaves on the cuttings varied from 15 to 20 cm but the plants were otherwise similar in appearance. The degree of senescence also varied; i.e., the extent of yellowing of the leaves was 80 - 90 %. A time course study on the accumulation of ricinine and its demethylated forms in the stems, and the yellow and green leaves following the administration of ricinine-3,5-^{14}C to a yellow leaf of these castor bean plant cuttings is shown in Table XXIV.

The recovery of radioactive ricinine and its demethylated forms in the green leaves were always very low compared to that found in the stems or the yellow leaves where ricinine was administered. This result suggested that the translocation of ricinine and/or its demethylated forms are reduced when the preferred translocation site organ is removed. The highest concentration of radioactive ricinine per gram of fresh plant weight was found in the growing apex except the yellow leaf where ricinine-3,5-^{14}C was administered. In the yellow leaves, radioactive ricinine was decreased and that of its demethylated forms were increased as the time elapsed (Figure 44, B).

Interpretations of the above two results were complicated by the fact that the radioactivity ratio between ricinine and its demethylated forms did not necessarily indicate the ratio of translocated forms

since the demethylated form of ricinine upon arrival of the green leaf from the yellow leaf may undergo methylation. The result showed that ricinine administered in the yellow leaves was translocated to healthy parts of the plant, especially the growing apex where ricinine is actively synthesized. The result supports the idea that ricinine translocation process might be a salvage operation performed by the plant in order to reutilize ricinine from the leaves which are being prepared for abscission. This is a broad concept of the traditional source-sink relationship (138); the translocation of ricinine is a reutilization of saving-type process. Another possible speculation for the phenomenon may be that the demethylation reactions, which is generally believed to make a compound more metabolically active, in the yellow leaves of the castor bean plants may represent a process whereby the vital precursors for the compounds in the pyridine nucleotide cycle are maintained within the yellowing leaves. This process might inhibit the progression of senescence.

The finding that both ricinine and its demethylated forms are present and the ratios between them in stems and yellow leaves are not strikingly different, indicates that there is no preferred form of translocation between them.

TABLE XXIV

DEMETHYLATION AND TRANSLOCATION OF RICININE-3,5-^{14}C FROM YELLOW LEAF TO GREEN LEAF OF THE CASTOR BEAN PLANT CUTTINGS

Exp. no.	Isolated compound \ Plant part	Green	Yellow	Stem	Total	
			dpm x 10^2			%
	Ricinine	2.6	129.3	47.3	179.2	54.4
I	N-demethyl ricinine	1.3	14.8	12.3	28.6	8.7
(12 hrs.)	O-demethyl ricinine	-	0.1	0.8	0.9	0.3
	Total	3.9	174.2	60.4	208.7	63.4
	Ricinine	4.5	90.8	56.9	152.2	46.2
II	N-demethyl ricinine	1.5	2.8	28.5	62.8	19.1
(24 hrs.)	O-demethyl ricinine	0.4	2.0	1.1	3.5	1.1
	Total	6.4	95.6	86.5	218.5	66.4
	Ricinine	8.6	78.4	36.8	123.8	37.6
III	N-demethyl ricinine	7.3	52.1	8.5	67.9	20.6
(36 hrs.)	O-demethyl ricinine	1.5	2.0	0.3	3.8	1.2
	Total	17.4	132.5	45.6	195.5	59.4

The plants used were grown in the green house. Ricinine-3,5-^{14}C (2 mg) with total radioactivity 3.292 x 10^4 dpm was administered. Percentage of incorporation was determined by dividing total radioactivity administered by the total amounts recovered.

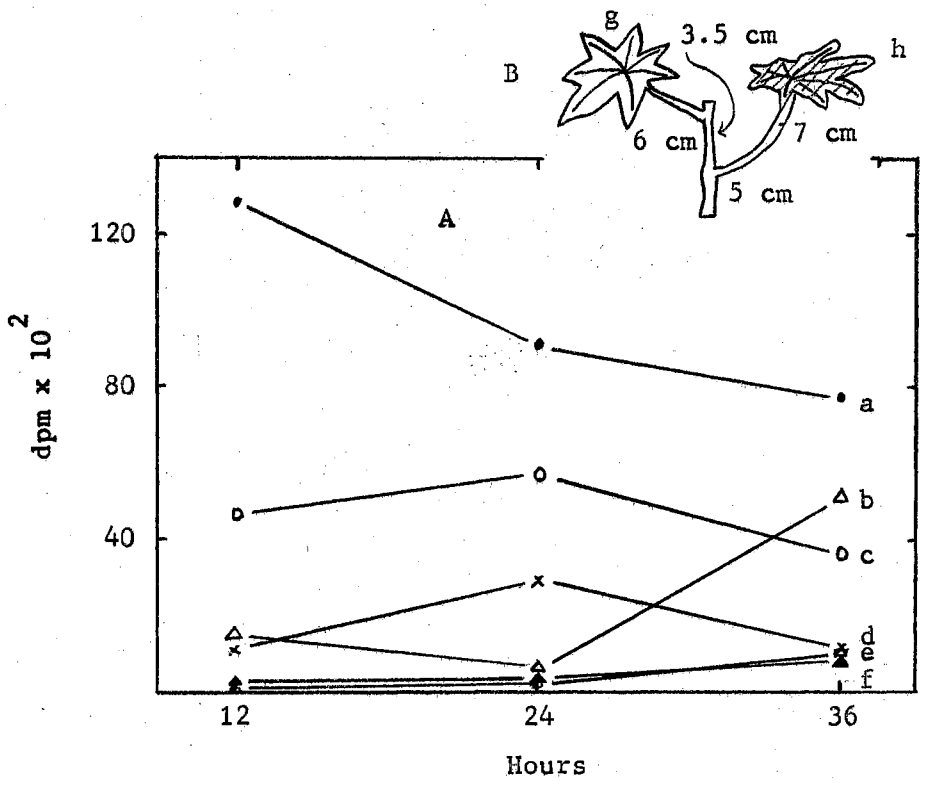


Figure 44. Demethylation and Translocation of Ricinine-3,5-^{14}C from the Yellow Leaf to Adjust Green Leaf of the Castor Bean Plant Cuttings (A), and Schemetic Representation of the Castor Bean Plant Cuttings with One Green and One Yellow Leaf (B)

a - Ricinine in the yellow leaves
b - Demethylated form of ricinine in the yellow leaves
c - Ricinine in the stems
d - Demethylated form of ricinine in the stems
e - Demethylated form of ricinine in the green leaves
f - Ricinine in the green leaves
g - Green leaf
h - Yellow leaf

CHAPTER IX

SUMMARY

In part one, the purpose of this study was to isolate and determine the structure of the insecticidal ester alkaloids from Tripterygium wilfordii Hook and their subcomponents by using modern micro analytical techniques. Another objective of this study was to determine the biosynthetic origin of the pyridine moiety of the ester alkaloids.

The confirmed molecular weight and formulae of the four ester alkaloids, wilforine (867, $C_{43}H_{49}O_{18}N$), wilforgine (857, $C_{41}H_{47}O_{19}N$), wilfordine (883, $C_{43}H_{49}O_{19}N$) and wilfortrine (873, $C_{41}H_{47}O_{20}N$) by using low and high resolution mass spectrometry were agreed with the data reported by Beroza based on elemental analysis in 1953. Four of the five acidic components of the ester alkaloids were identified as wilfordate, hydroxywilfordate, benzoate and acetate, and the structure of the polyhydroxy component was proposed.

Nicotinic acid-6-^{14}C and nicotinamide adenine dinucleotide-carbonyl-^{14}C were rapidly metabolized in T. wilfordii Hook with a resultant formation of all compounds in the pyridine nucleotide cycle, and the nicotinamide moiety of NAD was readily incorporated into the ester alkaloids with an efficiency of the same order of magnitude as nicotinic acid.

In part two, the purpose of the study was to identify the

possible new ricinine metabolites and to provide further knowledge on the demethylation and methylation reactions of the alkaloids in connection with the translocation within the castor bean plants.

A new ricinine metabolite was conclusively identified as O-demethyl ricinine, N-methyl-3-cyano-4-hydroxy-2-pyridone and the interconversion of ricinine and O-demethyl ricinine in the yellow and green leaves of the castor bean plants was demonstrated. The finding that both ricinine and its demethylated forms were present and the ratios between them in the stems and the yellow leaves were not strikingly different, indicates that there is no preferred form of translocation between them.

The result that ricinine administered in the yellow leaves was translocated to the healthy parts of the plant, especially the growing apex, supported the conclusion that translocation of ricinine may be a process performed by the plants in order to reutilize ricinine from the leaves which are being prepared for abscission.

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APPENDIX

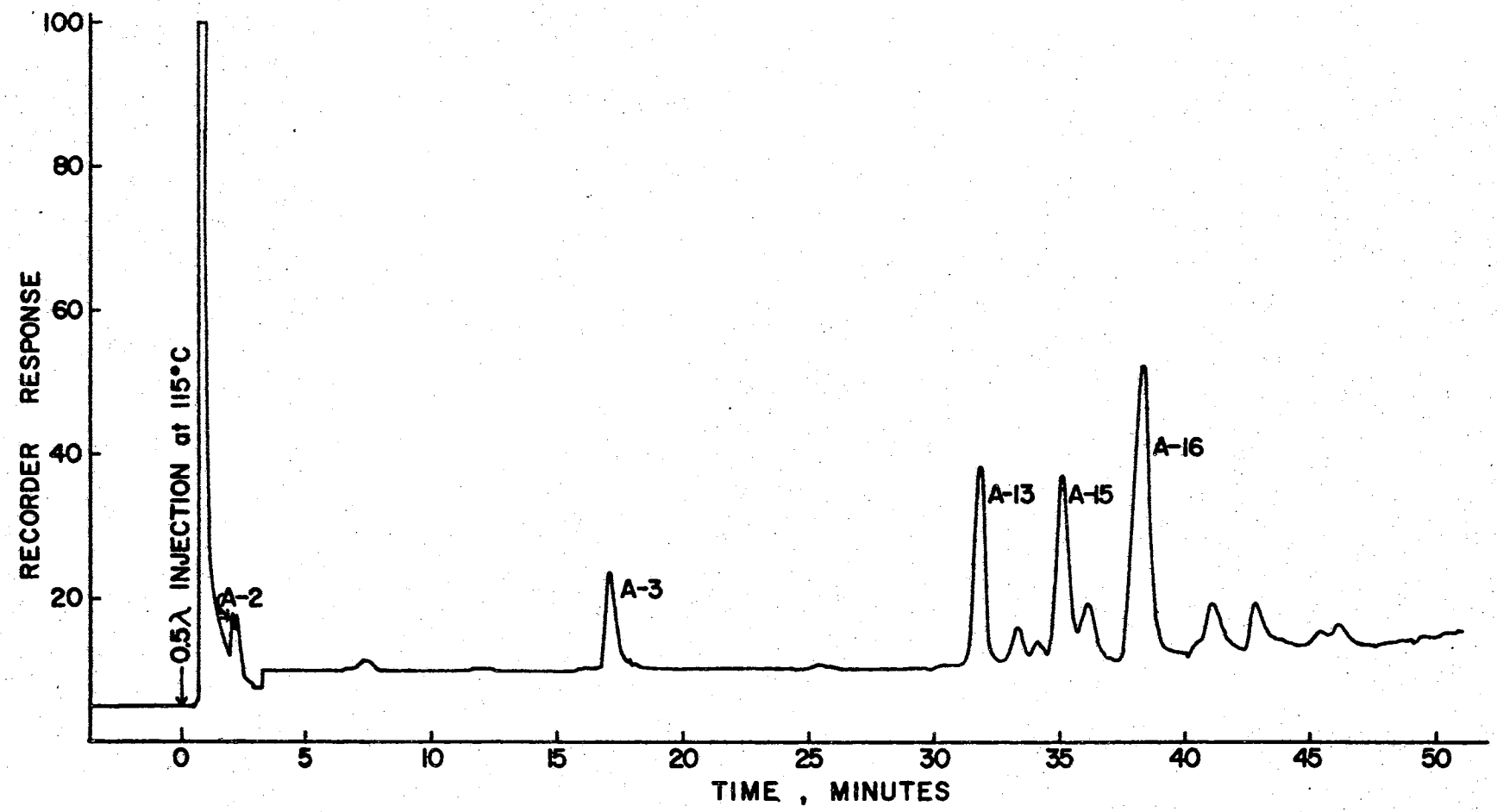

Figure 45. Gas Liquid Chromatographic Separation of Methyl Ester of Acidic Components from T. wilfordii Hook Alkaloids by Programming at 2°/min. (legene given in Fig. 15)

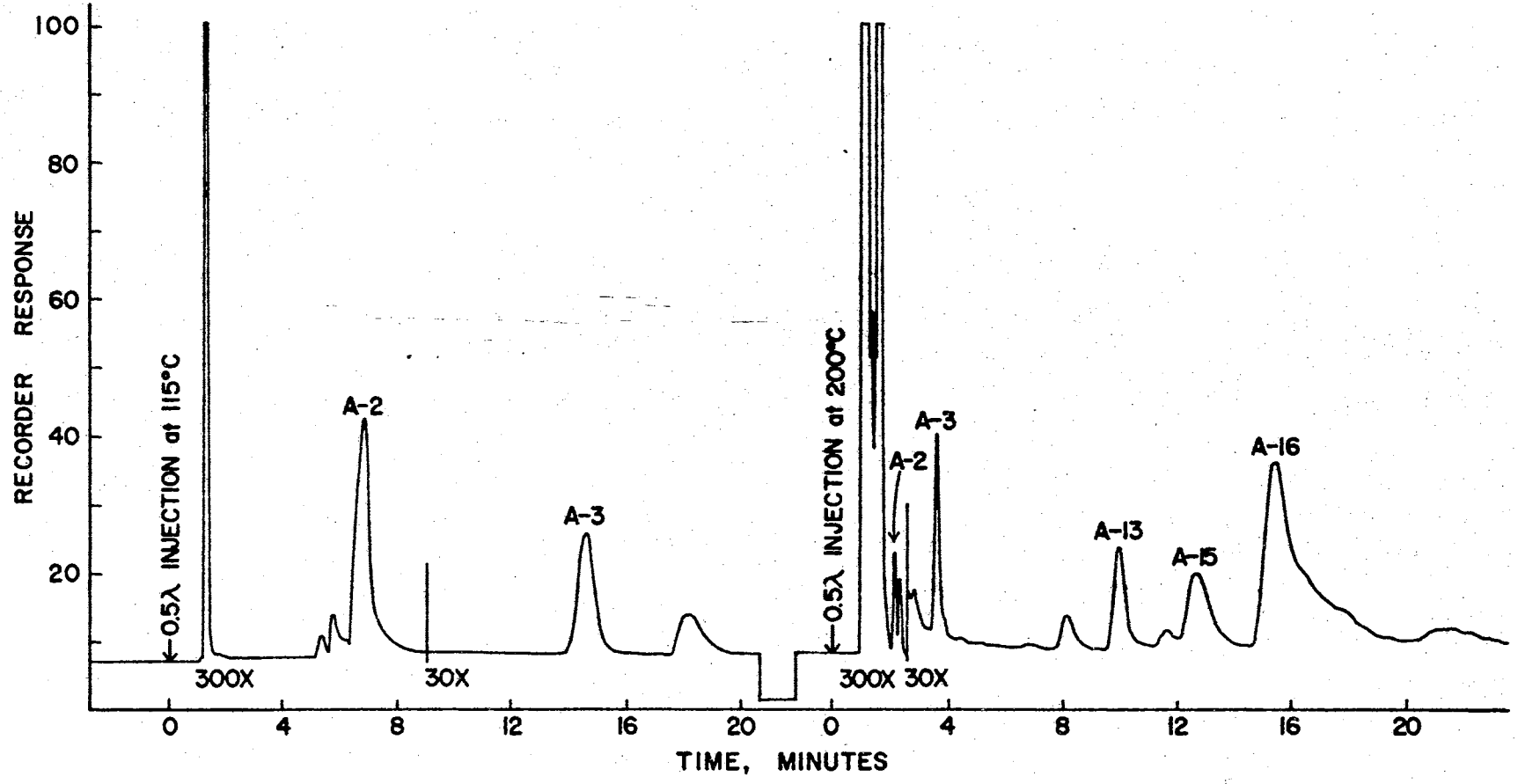

Figure 46. Gas Liquid Chromatographic Separation of Methyl Ester of Acidic Components from T. wilfordii Hook Alkaloids at Two Isothermal Temperatures (legene given in Fig.15)

TABLE XXV

HIGH RESOLUTION MASS SPECTRAL DATA OF WILFORINE

THIS IS AN EXPERIMENTAL VERSION OF CHAIN 3 OF THE STANDARD HIGH RESOLUTION DATA REDUCTION PROGRAM. PLEASE REPORT ANY APPARENT ERRORS TO NORMAN R. MANCUSO. CHANGES MADE IN THIS VERSION AND THEIR STATUS ARE AS FOLLOWS

1 SO CALLED 'GLITCHY' LINES ARE NOT SEARCHED FOR.(TEMPORARY)
2 DOUBLY CHARGED IONS ARE NOW LISTED ALONG WITH SINGLY CHARGED IONS AT THE DETERMINED MASS. THE ELEMENTAL COMPOSITION LISTED CORRESPONDS TO TWICE THE DETERMINED MASS AND A CODE (++) IDENTIFIES THESE IONS.(PERMANENT)
3 DOUBLY CHARGED IONS WHICH OCCUR AT NOMINAL MASSES ARE NOT PRESENTLY RECOGNIZED AS SUCH.(TEMPORARY)
4 ALL C-13 SPECIES CONTAINING UP TO 2 ATOMS OF C-13 ARE LISTED PROVIDED THAT SUFFICIENTLY* INTENSE C-12 IONS ARE PRESENT AT THE CORRESPONDING MASSES.(PERMANENT)(* THE INTENSITY OF THE C-12 ION MUST BE EQUAL OR GREATER THAN THE INTENSITY OF THE C-13 CONTAINING IONS.(TEMPORARY)
5 THE SULFUR/HALOGEN SEARCH IS PRESENTLY MEANINGLESS.(TEMPORARY)
6 THE ERROR LIMIT (TOLERANCE) IS NOW A FUNCTION OF MASS AND IS LISTED WITH THE FOUND COMPOSITIONS IN THE FIFTH COLUMN OF THE ELEMENTAL COMPOSITION TABLE.(PERMANENT)
7 A COMPLETE LIST OF THOSE 'MASSES' WHICH DO NOT CORRESPOND TO ANY COMBINATION OF THE ELEMENTS SPECIFIED (SEE BELOW) IS GIVEN ONLY WITH THE ELEMENTAL COMPOSITION TABLE. (I.E. NOT LISTED ON ELEMENT MAP).(TEMPORARY)
8 THE STANDARD FORM OF OUTPUT IS PRESENTLY THE ELEMENTAL COMPOSITION TABLE. A MAP MAY BE OBTAINED ONLY BY REPROCESSING OF THE MASS DECK WITH SENSE SWITCH ZERO IN THE UP POSITION.(TEMPORARY)
9 ONE MMU MAY BE ADDED TO THE TOLERANCE(SEE ITEM 6) BY PROCESSING THE DATA WITH SENSE SWITCH 2 ON. THIS CAN BE DONE FOR THE ENTIRE SPECTRUM OR FOR ANY GIVEN AREA(S) IN THE SPECTRUM.
10 IF LINES CORRESPONDING TO THE FOLLOWING NON-FLUOROCARBON SPECIES OCCUR. THEY WILL BE CONSIDERED STANDARD MASS LINES AND REMOVED
CO, N2, O2, CL, AR, HCL, CO2, CCL, F2S, CHCL2, XE, HG

A805-39-03 WILFORINE C.H. 01/14/71

TITLE	LIM	JH	HET	AT.WT.
C	100	2	0	12.000000
H	200	0	0	1.007825
N	1	1	1	14.003074
O	18	0	1	15.994915

NORMALIZATION FACTOR FOR COMPOUND LINES= 10.0000
THE MAXIMUM INTENSITY IS 310 (M/E = 78.0452)

HETEROATOM	SUM-SING.	SUM-MIX.	TOTAL	PERCENT
N	240	10	250	20.32
O	160	10	170	13.82

HETEROATOM	NUM-FOUND	SUM-INT	PERCENT
CL			.00
S	1	10	.81
BR			.00

TABLE XXV (Continued)

A805-39-03 WILFORINE C.H. 01/14/71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
0	73.03039	73.02895	1.4	1.7	3	.	5	.	2
10	74.01459	74.01564	-1.0		6	.	2	.	.
10	75.02319	75.02346	- .2		6	.	3	.	.
10	76.03129	76.03129	.0		6	.	4	.	.
300	77.03830	77.03912	- .7		6	.	5	.	.
30	79.04190	79.04219	- .2		5	.	5	1	.
0	81.03379	81.03403	- .1		5	.	5	.	1
0	83.04950	83.04969	- .1		5	.	7	.	1
0	84.01999	84.02112	-1.0		4	.	4	.	2
0	85.02879	85.02894	- .1		4	.	5	.	2
10	91.05499	91.05477	.2		7	.	7	.	.
10	92.05060	92.05003	.6		6	.	6	1	.
120	93.05740	93.05784	- .3		6	.	7	1	.
10	94.04099	94.04185	- .8		6	.	6	.	1
10	94.06049	94.06119	- .6		5	1	7	1	.
10	95.05079	95.04969	1.1		6	.	7	.	1
0	96.02849	96.02967	-1.1		1	.	6	1	4
0	97.03029	97.02895	1.3		5	.	5	.	2
0	98.03679	98.03678	.0		5	.	6	.	2
0	103.05539	103.05477	.6		8	.	7	.	.
0	104.02699	104.02621	.8		7	.	4	.	1
10	104.04969	104.05001	- .2		7	.	6	1	.
0	105.03370	105.03404	- .2		7	.	5	.	1
10	105.05689	105.05783	- .8		7	.	7	1	.
0	105.06890	105.07042	-1.4		8	.	9	.	.
20	106.06590	106.06568	.2		7	.	8	1	.
10	107.05020	107.04969	.5		7	.	7	.	1
10	107.07250	107.07082	1.7		4	.	11	.	3
10	107.07250	107.07349	- .9		7	.	9	1	.
0	108.05869	108.05751	1.2		7	.	8	.	1
0	110.03639	110.03677	- .3		6	.	6	.	2
0	111.04300	111.04460	-1.5		6	.	7	.	2
20	112.01549	112.01603	- .4		5	.	4	.	3
10	115.05389	115.05476	- .8		9	.	7	.	.
0	116.05039	116.05002	.4		8	.	6	1	.
10	117.05789	117.05784	.1		8	.	7	1	.
10	118.06540	118.06567	- .2		8	.	8	1	.
10	119.03480	119.03444	.4		4	.	7	.	4
10	120.04469	120.04492	- .1		7	.	6	1	1
0	121.02870	121.02895	- .2		7	.	5	.	2
0	121.05120	121.05009	1.1		4	.	9	.	4
0	121.05120	121.05276	-1.5		7	.	7	1	1
10	121.06460	121.06534	- .6		8	.	9	.	1
20	123.04150	123.04014	1.4		6	1	6	.	2
0	124.03220	124.03130	.9		10	.	4	.	.
0	124.05200	124.05243	- .3		7	.	8	.	2
0	125.05890	125.06025	-1.3		7	.	9	.	2
0	126.03099	126.03168	- .6		6	.	6	.	3
0	128.06390	128.06260	1.3		10	.	8	.	.
0	129.06969	129.07041	- .6		10	.	9	.	.
20	130.06549	130.06566	- .1		9	.	8	1	.
0	131.03539	131.03443	1.0		5	.	7	.	4
0	131.03539	131.03710	-1.6		8	.	5	1	1
0	131.04999	131.04969	.3		9	.	7	.	1
10	131.07290	131.07349	- .5		9	.	9	1	.
10	132.04469	132.04492	- .1		8	.	6	1	1
20	132.08020	132.07865	1.6		6	.	12	.	3
20	132.08020	132.08132	-1.0		9	.	10	1	.
10	133.05319	133.05276	.4		8	.	7	1	1
0	133.06479	133.06533	- .4		9	.	9	.	1

A805-39-03 WILFORINE C.H. 01/14/71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
20	133.08880	133.08914	- .2	1.7	9	.	11	1	.
20	134.05999	134.06057	- .5		8	.	8	1	1
10	134.09649	134.09696	- .4		9	.	12	1	.
0	135.04580	135.04461	1.2		8	.	7	.	2
10	136.05160	136.05243	- .7		8	.	8	.	2
30	137.04919	137.04767	1.5		7	.	7	1	2
0	138.05489	138.05549	- .5		7	.	8	1	2
0	140.02699	140.02621	.8		10	.	4	.	1
10	141.07189	141.07042	1.5		11	.	9	.	.
0	142.07719	142.07824	-1.0		11	.	10	.	.
10	145.06450	145.06534	- .7		10	.	9	.	1
10	146.06139	146.06058	.8		9	.	8	1	1
0	147.07989	147.08098	-1.0		10	.	11	.	1
10	148.04080	148.03986	.9		8	.	6	1	2
10	148.05199	148.05242	- .3		9	.	8	.	2
10	149.04889	149.04767	1.2		8	.	7	1	2
0	149.08269	149.08138	1.3		6	.	13	.	4
0	149.08269	149.08405	-1.3		9	.	11	1	1
70	150.05639	150.05550	.9	2.0	8	.	8	1	2
0	150.09260	150.09189	.7		9	.	12	1	1
0	151.03909	151.03951	- .3		8	.	7	.	3
10	151.06119	151.06065	.5		5	.	11	.	5
0	152.04929	152.04734	2.0		8	.	8	.	3
0	152.04929	152.05001	- .6		11	.	6	1	.
0	153.05499	153.05516	- .1		8	.	9	.	3
0	157.06590	157.06534	.6		11	.	9	.	1
10	158.05999	158.06057	- .5		10	.	8	1	1
10	158.07119	158.07315	-1.9		11	.	10	.	1
10	159.06929	159.06841	.9		10	.	9	1	1
10	159.08020	159.08099	- .7		11	.	11	.	1
10	160.04059	160.03985	.7		9	.	6	1	2
20	160.07559	160.07622	- .5		10	.	10	1	1
10	161.05929	161.06024	- .9		10	.	9	.	2
20	161.08260	161.08139	1.2		7	.	13	.	4
20	161.08260	161.08406	-1.4		10	.	11	1	1
10	162.05639	162.05550	.9		9	.	8	1	2
0	162.06759	162.06807	- .4		10	.	10	.	2
10	162.09160	162.09188	- .2		10	.	12	1	1
0	164.04899	164.04734	1.7		9	.	8	.	3
0	164.04899	164.05001	- .9		12	.	6	1	.
20	164.07100	164.07115	- .1		9	.	10	1	2
0	165.05419	165.05516	- .9		9	.	9	.	3
0	165.05419	165.05337	.8		11	1	6	1	.
0	165.05419	165.05477	- .5		3	2	9	1	5
0	166.06320	166.06300	.2		9	.	10	.	3
10	173.05839	173.06024	-1.8		11	.	9	.	2
10	174.05740	174.05551	1.9		10	.	8	1	2
10	174.06839	174.06808	.3		11	.	10	.	2
10	175.06320	175.06332	- .0		10	.	9	1	2
10	175.07419	175.07589	-1.6		11	.	11	.	2
40	176.07279	176.07115	1.6		10	.	10	1	2
0	177.05689	177.05517	1.7		10	.	9	.	3
0	177.05689	177.05783	- .8		13	.	7	1	.
10	177.07979	177.07897	.8		10	.	11	1	2
20	178.05209	178.05041	1.7		9	.	8	1	3
80	178.08680	178.08680	.0		10	.	12	1	2
0	179.07530	179.07350	1.8		13	.	9	1	.
10	179.09390	179.09195	2.0		7	.	15	.	5
10	179.09390	179.09462	- .6		10	.	13	1	2
0	186.06880	186.06808	.7		12	.	10	.	2

TABLE XXV (Continued)

A805-39-03 WILFORINE C.M. 01/14/71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
10	187.07639	187.07590	.5	2.0	12	.	11	.	2
10	188.07110	188.07115	- .0		11	.	10	1	2
0	189.05619	189.05517	1.0		11	.	9	.	3
0	189.05619	189.05783	-1.5		14	.	7	1	.
0	189.07970	189.07898	.7		11	.	11	1	2
0	190.08260	190.08412	-1.4		8	.	14	.	5
0	190.08260	190.08234	.3		10	1	11	1	2
10	191.07069	191.07081	- .0		11	.	11	.	3
10	192.06709	192.06606	1.0		10	.	10	1	3
0	192.10359	192.10245	1.1		11	.	14	1	2
10	193.07320	193.07122	2.0		7	.	13	.	6
10	193.07320	193.07369	- .6		10	.	11	1	3
0	200.08390	200.08373	.2	2.2	13	.	12	.	2
0	201.08880	201.08709	1.7		12	1	12	.	2
0	201.08880	201.08849	.3		4	2	15	.	7
0	202.06219	202.06298	- .7		12	.	10	.	3
10	204.06709	204.06606	1.0		11	.	10	1	3
120	206.07999	206.07904	1.0		8	.	14	.	6
120	206.07999	206.08170	-1.6		11	.	12	1	3
20	207.08690	207.08687	.0		8	.	15	.	6
0	215.07170	215.07082	.9		13	.	11	.	3
0	215.07170	215.07349	-1.7		16	.	9	1	.
10	216.08010	216.07865	1.5		13	.	12	.	3
10	216.08010	216.08132	-1.1		16	.	10	1	.
10	217.08670	217.08647	.2		13	.	13	.	3
0	220.09900	220.09737	1.6		12	.	14	1	3
0	233.07979	233.08137	-1.5		13	.	13	.	4
10	234.07709	234.07663	.5		12	.	12	1	4
0	234.11350	234.11302	.5		13	.	16	1	3
10	262.10670	262.10526	1.4	2.4	11	.	18	.	7
10	262.10670	262.10793	-1.1		14	.	16	1	4
0	572.21139	572.21400	-2.5	3.4	44	.	28	.	1
0	572.21139	572.21051	.9		26	.	36	.	14
0	572.21139	572.21317	-1.7		29	.	34	1	11
0	750.26519	750.26175	3.4	4.0	50	.	38	.	7
0	750.26519	750.26762	-2.3		43	.	42	.	12
0	750.26519	750.26443	.8		53	.	36	1	4
20	867.29730	867.29580	1.5	4.2	58	.	43	.	8
20	867.29730	867.29847	-1.1		61	.	41	1	5
20	867.29730	867.29497	2.3		43	.	49	1	18
10	868.30400	868.30362	.4		58	.	44	.	8
10	868.30400	868.30043	3.6		68	.	38	1	.
10	868.30400	868.30629	-2.2		61	.	42	1	5
10	868.30400	868.30280	1.2		43	.	50	1	18
10	868.30400	868.30502	- .9		50	1	47	.	13
10	868.30400	868.30183	2.2		60	1	41	1	5
10	868.30400	868.30770	-3.6		53	1	45	1	10
10	868.30400	868.30405	- .0		67	2	38	.	.
10	868.30400	868.30056	3.4		49	2	46	.	13
10	868.30400	868.30642	-2.3		42	2	50	.	16
10	868.30400	868.30324	.8		52	2	44	1	10

A805-39-03 WILFORINE C.M. 01/14/71

COMPOSITIONS OBTAINED USING EXPANDED ERROR LIMITS

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
0	71.01099	71.01329	-2.2	3.4	3	.	3	.	2
10	77.02830	77.02655	1.8		5	.	3	1	.
310	78.04520	78.04695	-1.7		6	.	6	.	.
0	86.03879	86.03678	2.0		4	.	6	.	2
120	122.03859	122.03678	1.8		7	.	6	.	2
10	123.02529	123.02347	1.8		10	.	3	.	.
0	127.03699	127.03951	-2.4		6	.	7	.	3
0	144.04779	144.04493	2.9		9	.	6	1	1
0	147.04269	147.04459	-1.8		9	.	7	.	2
10	149.05769	149.06024	-2.5		9	.	9	.	2
10	156.09160	156.09390	-2.2	4.0	12	.	12	.	.
0	163.07339	163.07589	-2.4		10	.	11	.	2
10	190.06049	190.06298	-2.4		11	.	10	.	3
10	194.08409	194.08171	2.4		10	.	12	1	3
10	203.06820	203.07082	-2.5	4.4	12	.	11	.	3
0	205.07629	205.07389	2.4		11	.	11	1	3
0	205.08419	205.08646	-2.2		12	.	13	.	3
20	218.08470	218.08172	3.0		12	.	12	1	3

VALID ELEMENTAL COMPOSITIONS COULD NOT BE FOUND FOR S LINES.

0 74.0073 0 75.0163 10 91.0474 10 96.0378 90

TOTAL IONIZATION OF SINGLY CHARGED SPECIES= 2240.

FRACTION OF UNASSIGNED IONS 0.049107

TABLE XXVI

HIGH RESOLUTION MASS SPECTRAL DATA OF WILFORGINE

A805-33-01 WILFORGINE C.H. 01-08-71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
0	70.53950++	70.53951	.0	1.7	7	.	11	1	2
0	75.01959	75.01900	.6		5	1	2	.	.
10	76.03180	76.03130	.5		6	.	4	.	.
0	78.03069	78.03168	- .9		2	.	6	.	3
0	78.03069	78.02990	.8		4	1	3	1	.
70	79.03819	79.03951	-1.2		2	.	7	.	3
0	82.02889	82.02927	- .3		4	.	4	1	1
10	83.05039	83.04969	.7		5	.	7	.	1
10	84.02129	84.02113	.2		4	.	4	.	2
0	84.05619	84.05750	-1.2		5	.	8	.	1
0	85.03050	85.02896	1.5		4	.	5	.	2
0	88.01669	88.01604	.7		3	.	4	.	3
0	94.05179	94.05041	1.4		2	.	8	1	3
10	94.06970	94.06931	.4		5	2	8	.	.
10	96.04389	96.04492	- .9		5	.	6	1	1
10	99.01149	99.01089	.6		7	.	1	1	.
0	102.03220	102.03170	.5		4	.	6	.	3
0	103.04480	103.04583	- .9		6	2	5	.	.
0	103.05450	103.05477	- .2		8	.	7	.	.
20	104.04800	104.04735	.7		5	.	8	.	3
150	105.03379	105.03403	- .1		7	.	5	.	1
10	105.04969	105.05069	- .9		3	1	8	.	3
10	105.04969	105.04890	.8		5	2	5	1	.
20	105.05859	105.05784	.8		7	.	7	1	.
0	106.02119	106.02213	- .8		2	1	5	.	4
0	106.02119	106.02034	.9		4	2	2	1	1
10	106.03840	106.03740	1.0		6	1	5	.	1
30	106.06580	106.06568	.1		7	.	8	1	.
10	107.04920	107.04969	- .4		7	.	7	.	1
10	107.07330	107.07349	- .1		7	.	9	1	.
0	108.04639	108.04493	1.5		6	.	6	1	1
0	108.05819	108.05751	.7		7	.	8	.	1
0	109.06550	109.05534	.2		7	.	9	.	1
0	110.03809	110.03678	1.3		6	.	6	.	2
10	111.04450	111.04460	- .0		6	.	7	.	2
30	112.01509	112.01603	- .8		5	.	4	.	3
10	113.01009	113.00861	1.5		1	.	5	.	6
10	113.01009	113.01128	-1.1		4	.	3	1	3
10	115.05439	115.05476	- .3		9	.	7	.	.
10	117.05829	117.05784	.5		8	.	7	1	.
10	118.06620	118.06568	.5		8	.	8	1	.
10	119.03690	119.03711	- .1		7	.	5	1	1
10	120.04510	120.04494	.2		7	.	6	1	1
40	122.03590	122.03678	- .8		7	.	6	.	2
0	123.01149	123.01089	.6		9	.	1	1	.
0	124.03999	124.03985	.1		6	.	6	1	2
0	124.05410	124.05243	1.7		7	.	8	.	2
0	125.05950	125.06025	- .7		7	.	9	.	2
0	128.06320	128.06260	.6		10	.	8	.	.
0	129.05890	129.05785	1.1		9	.	7	1	.
0	129.06940	129.07042	- .9		10	.	9	.	.
10	130.06660	130.06568	.9		9	.	8	1	.
0	131.00000	131.00072	- .6		7	.	1	1	2
0	131.03660	131.03711	- .4		8	.	5	1	1
10	131.07440	131.07350	.9		9	.	9	1	.
10	132.04560	132.04494	.9		8	.	6	1	1
0	132.06549	132.06605	- .5		5	.	10	1	3
10	132.08170	132.08133	.4		9	.	10	1	.
0	133.03689	133.03749	- .5		4	.	7	1	4
10	133.08979	133.08914	.7		9	.	11	1	.

A805-33-01 WILFORGINE C.H. 01-08-71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
20	134.06109	134.06058	.5	1.7	8	.	8	1	1
0	134.08310	134.08172	1.4		5	.	12	1	3
10	134.09760	134.09698	.6		9	.	12	1	.
10	136.05209	136.05242	- .2		8	.	8	.	2
10	136.05209	136.05204	.1		2	2	8	1	4
0	137.03109	137.03241	-1.2		3	.	7	1	5
0	138.05489	138.05549	- .5		7	.	8	1	2
0	142.07940	142.07825	1.2		11	.	10	.	.
0	144.04409	144.04492	- .7		9	.	6	1	1
0	144.05719	144.05750	- .2		10	.	8	.	1
10	145.06510	145.06534	- .1		10	.	9	.	1
0	147.04339	147.04459	-1.1		9	.	7	.	2
0	148.07779	148.07623	1.6		9	.	10	1	1
0	149.07189	149.07243	- .4		4	2	11	.	4
0	149.08250	149.08139	1.1		6	.	13	.	4
0	149.08250	149.08406	-1.5		9	.	11	1	1
0	150.03039	150.03168	-1.2	2.0	8	.	6	.	3
0	150.07820	150.07664	1.6		5	.	12	1	4
10	150.09349	150.09188	1.6		9	.	12	1	1
10	151.06259	151.06065	1.9		5	.	11	.	5
10	151.06259	151.06331	- .6		8	.	9	1	2
0	152.03069	152.03208	-1.3		4	.	8	.	6
0	152.05059	152.05002	.6		11	.	6	1	.
0	153.05499	153.05516	- .1		8	.	9	.	3
0	153.05499	153.05337	1.6		10	1	6	1	.
0	153.05499	153.05478	.2		2	2	9	1	5
0	156.09419	156.09390	.3		12	.	12	.	.
0	159.05429	159.05315	1.1		5	.	9	1	4
10	160.04160	160.03986	1.7		9	.	5	1	2
0	160.05709	160.05651	.6		5	1	9	1	4
10	160.07629	160.07523	.1		10	.	10	1	1
10	161.05939	161.06024	- .8		10	.	9	.	2
10	161.07040	161.06881	1.6		6	.	11	1	4
10	161.08230	161.08139	.9		7	.	13	.	4
10	161.08230	161.08406	-1.7		10	.	11	1	1
0	162.09219	162.09188	.3		10	.	12	1	1
0	163.04689	163.04806	-1.1		5	.	9	1	5
0	163.07489	163.07589	- .9		10	.	11	.	2
0	164.05139	164.05002	1.4		12	.	6	1	.
0	164.05139	164.05141	.0		4	1	9	1	5
10	164.07090	164.07115	- .2		9	.	10	1	2
0	165.05399	165.05516	-1.1		9	.	9	.	3
0	165.05399	165.05337	.6		11	1	6	1	.
0	165.05399	165.05477	- .7		3	2	9	1	5
0	174.61740++	174.61894	-1.4		21	.	33	.	4
10	174.65520++	174.65532	- .0		23	.	41	.	2
50	175.06520	175.06333	1.9		10	.	9	1	2
20	176.07069	176.07114	- .4		10	.	10	1	2
0	177.03959	177.03990	- .2		6	.	9	.	6
10	177.06530	177.06373	1.6		6	.	11	1	5
0	177.07470	177.07630	-1.5		7	.	13	.	5
0	177.07470	177.07451	.2		9	1	10	1	2
10	178.04950	178.04774	1.8		6	.	10	.	6
10	178.04950	178.05041	- .8		9	.	8	1	3
10	178.06680	178.06568	1.1		13	.	8	1	.
10	178.06680	178.06707	- .2		5	1	11	1	5
10	178.08559	178.08412	1.5		7	.	14	.	5
10	178.08559	178.08679	-1.1		10	.	12	1	2
0	179.07890	179.07937	- .4		6	.	13	1	5
10	179.09100	179.09195	- .9		7	.	15	.	5

TABLE XXVI (Continued)

A805-33-01 WILFORGINE C.H. 01-08-7

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	D
10	179.09100	179.09016	.8	2.0	9	1	12	1	2
0	185.05919	185.06024	-1.0		12	.	9	.	2
0	187.05489	187.05477	.1		15	.	7	.	.
0	187.07399	187.07589	-1.8		12	.	11	.	2
0	189.03819	189.03990	-1.6		7	.	9	.	6
10	189.07030	189.07042	- .0		15	.	9	.	.
10	190.06300	190.06300	.0		11	.	10	.	3
0	190.07989	190.07825	1.6		15	.	10	.	.
10	191.07240	191.07082	1.6		11	.	11	.	3
10	191.07240	191.07349	-1.0		14	.	9	1	.
0	193.07390	193.07390	.0		10	.	11	1	3
0	200.09199	200.09227	- .2	2.2	9	.	14	1	4
0	202.06360	202.06300	.6		12	.	10	.	3
0	202.06360	202.06567	-2.0		15	.	8	1	.
0	203.06960	203.07082	-1.1		12	.	11	.	3
10	204.06820	204.06607	2.1		11	.	10	1	3
30	206.07909	206.07904	.1		8	.	14	.	6
0	207.06530	207.06573	- .3		11	.	11	.	4
10	207.08600	207.08686	- .8		8	.	15	.	6
0	216.06650	216.06607	.4		12	.	10	1	3
0	216.07820	216.07864	- .3		13	.	12	.	3
0	217.08660	217.08647	.1		13	.	13	.	3
0	222.07259	222.07394	-1.3		8	.	14	.	7
0	233.08330	233.08139	1.9		13	.	13	.	4
0	233.08330	233.08406	- .7		16	.	11	1	1
0	234.10260	234.10446	-1.8		17	.	14	.	1
0	250.09830	250.09938	-1.0	2.4	17	.	14	.	2
0	250.10879	250.10793	.9		13	.	16	1	4
0	262.10889	262.10793	1.0		14	.	16	1	4
0	572.21089	572.21400	-3.0	3.4	44	.	28	.	1
0	572.21089	572.21051	.4		26	.	36	.	14
0	572.21089	572.21317	-2.2		29	.	34	1	11
0	740.25389	740.25040	3.5	3.8	59	.	32	.	.
0	740.25389	740.25626	-2.3		52	.	36	.	5
0	740.25389	740.25277	1.1		34	.	44	.	18
0	740.25389	740.25543	-1.4		37	.	42	1	15
0	784.25140	784.25198	- .5	4.0	46	.	40	.	12
0	784.25140	784.24879	2.6		56	.	34	1	4
0	784.25140	784.25465	-3.2		49	.	38	1	9
0	798.23539	798.23475	.6		64	.	30	.	.
0	798.23539	798.23711	-1.6		39	.	42	.	18
0	798.23539	798.23392	1.5		49	.	36	1	10
0	798.26260	798.26176	.8		54	.	38	.	7
0	798.26260	798.26443	-1.7		57	.	36	1	4
0	798.26260	798.26093	1.7		39	.	44	1	17
0	799.20669	799.20619	.5		63	.	27	.	1
0	799.20669	799.20855	-1.8		38	.	39	.	19
0	799.20569	799.20536	1.3		48	.	33	1	11
0	799.26010	799.26370	-3.5		61	.	35	.	2
0	799.26010	799.26020	- .0		43	.	43	.	15
0	799.26010	799.25701	3.1		53	.	37	1	7
0	799.26010	799.26267	-2.7		46	.	41	1	12
0	829.24979	829.25313	-3.2	4.1	65	.	33	.	1
0	829.24979	829.24963	.2		47	.	41	.	14
0	829.24979	829.24643	3.4		57	.	35	1	6
0	829.24979	829.25230	-2.4		50	.	39	1	11
0	829.28209	829.28014	2.0		55	.	41	.	8
0	829.28209	829.28601	-3.8		48	.	45	.	13
0	829.28209	829.28281	- .6		58	.	39	1	5
0	829.28209	829.27931	2.8		40	.	47	1	18

A805-33-01 WILFORGINE C.H. 01-08-71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	D
20	857.27490	857.27506	- .1	4.2	56	.	41	.	9
20	857.27490	857.27187	3.0		66	.	35	1	1
20	857.27490	857.27773	-2.7		59	.	39	1	6
20	857.27490	857.27424	.7		41	.	47	1	19
10	858.27849	858.27701	1.5		63	.	38	.	4
10	858.27849	858.27967	-1.1		66	.	36	1	1
10	858.27849	858.27618	2.3		48	.	44	1	14
10	858.27849	858.28204	-3.5		41	.	48	1	19
10	858.27849	858.27841	.1		65	1	41	.	9
10	858.27849	858.27521	3.3		65	1	35	1	1
10	858.27849	858.28108	-2.5		58	1	39	1	6
10	858.27849	858.27758	.9		40	1	47	1	19
10	858.27849	858.27980	-1.2		47	2	44	.	14
10	858.27849	858.27662	1.9		57	2	38	1	6
10	858.27849	858.28248	-3.9		50	2	42	1	11

TABLE XXVII

HIGH RESOLUTION MASS SPECTRAL DATA OF WILFORDINE

A805-27-03 WILFORDINE C.H. 01/14/71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
0	73.57900++	73.57907	.1	2.7	7	1	20	1	1
10	74.01459	74.01564	-1.0		6	.	2	.	.
10	74.01459	74.01525	- .6		.	2	2	1	2
0	75.01429	75.01452	- .1		4	2	1	.	.
10	75.02299	75.02346	- .4		6	.	3	.	.
10	75.02299	75.02307	- .0		.	2	3	1	2
10	76.03319	76.03130	1.9		6	.	4	.	.
20	77.02419	77.02387	.3		2	.	5	.	3
20	77.02419	77.02653	-2.2		5	.	3	1	.
100	77.04139	77.03912	2.3		6	.	5	.	.
380	78.04719	78.04695	.2		6	.	6	.	.
20	79.04410	79.04220	1.9		5	.	5	1	.
0	81.03499	81.03404	1.0		5	.	5	.	1
0	82.04349	82.04186	1.6		5	.	6	.	1
0	83.04940	83.04969	- .2		5	.	7	.	1
0	84.48910++	84.48874	.4		6	.	1	.	6
0	84.50780++	84.50695	1.0		7	.	5	.	5
0	84.50780++	84.50818	- .3		10	.	3	1	2
0	84.52320++	84.52504	-1.7		8	.	9	.	4
0	84.55130++	84.55093	.4		13	.	13	.	.
0	85.02789	85.02894	-1.0		4	.	5	.	2
0	86.03650	86.03678	- .2		4	.	6	.	2
0	86.55010++	86.55259	-2.4		8	.	15	1	3
0	89.03669	89.03911	-2.3		7	.	5	.	.
0	89.05859	89.06024	-1.6		4	.	9	.	2
0	89.09490	89.09664	-1.6		5	.	13	.	1
0	91.01879	91.01839	.4		6	.	3	.	1
0	91.02719	91.02694	.3		2	.	5	1	3
0	91.07370	91.07590	-2.1		4	.	11	.	2
0	92.04630	92.04734	- .9		3	.	8	.	3
10	93.05600	93.05517	.8		3	.	9	.	3
10	93.05600	93.05784	-1.7		6	.	7	1	.
0	95.03709	95.03443	2.7		2	.	7	.	4
0	95.03709	95.03710	.0		5	.	5	1	1
10	95.04749	95.04968	-2.1		6	.	7	.	1
10	96.02370	96.02113	2.6		5	.	4	.	2
10	97.02519	97.02448	.7		4	1	4	.	2
0	102.02949	102.03168	-2.1		4	.	6	.	3
0	103.05490	103.05478	.1		8	.	7	.	.
0	104.02779	104.02621	1.6		7	.	4	.	1
20	104.05050	104.05003	.5		7	.	6	1	.
520	105.03620	105.03404	2.2		7	.	5	.	1
10	105.05730	105.05517	2.1		4	.	9	.	3
10	105.05730	105.05784	- .4		7	.	7	1	.
70	106.03929	106.04185	-2.5		7	.	6	.	1
70	106.03929	106.03739	1.9		6	1	5	.	1
70	106.03929	106.04146	-2.1		1	2	6	1	3
30	106.06630	106.06568	.6		7	.	8	1	.
10	107.04889	107.04968	- .7		7	.	7	.	1
0	107.05859	107.05824	.4		3	.	9	1	3
10	107.07110	107.07082	.3		4	.	11	.	3
10	107.07110	107.07349	-2.3		7	.	9	1	.
10	107.07110	107.06903	2.1		6	1	8	1	.
0	108.05719	108.05750	- .2		7	.	8	.	1
0	109.06039	109.06086	- .4		6	1	8	.	1
0	110.03620	110.03678	- .5		6	.	6	.	2
0	111.04150	111.04014	1.4		5	1	6	.	2
0	111.04150	111.04421	-2.6		.	2	7	1	4
20	112.01479	112.01603	-1.1		5	.	4	.	3
0	115.03870	115.03952	- .7		5	.	7	.	3

A805-27-03 WILFORDINE C.H. 01/14/71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
10	117.05600	117.05517	.8	2.7	5	.	9	.	3
10	117.05600	117.05784	-1.7		8	.	7	1	.
0	118.06350	118.06300	.5		5	.	10	.	3
0	118.06350	118.06567	-2.1		8	.	8	1	.
10	119.03319	119.03442	-1.1		4	.	7	.	4
10	120.04279	120.04226	.5		4	.	8	.	4
10	120.04279	120.04492	-2.0		7	.	6	1	1
80	122.03650	122.03678	- .2		7	.	6	.	2
10	123.02119	123.02346	-2.2		10	.	3	.	.
0	123.02699	123.02934	-2.3		3	.	7	.	5
20	123.03840	123.04013	-1.6		6	1	6	.	2
10	124.02940	124.03130	-1.8		10	.	4	.	.
0	128.06300	128.06260	.4		10	.	8	.	.
0	129.06849	129.07041	-1.8		10	.	9	.	.
0	129.06849	129.06595	2.5		9	1	8	.	.
0	129.06849	129.07002	-1.4		4	2	9	1	2
20	130.06530	130.06300	2.3		6	.	10	.	3
20	130.06530	130.06567	- .3		9	.	8	1	.
120	131.00239	131.00072	1.7		7	.	1	1	2
10	131.04810	131.04969	-1.5		9	.	7	.	1
10	131.07150	131.07082	.7		6	.	11	.	3
10	131.07150	131.07349	-1.9		9	.	9	1	.
20	132.04440	132.04226	2.1		5	.	8	.	4
20	132.04440	132.04493	- .4		8	.	6	1	1
10	132.07960	132.07865	1.0		6	.	12	.	3
10	132.07960	132.08132	-1.6		9	.	10	1	.
40	133.05219	133.05008	2.1		5	.	9	.	4
40	133.05219	133.05275	- .5		8	.	7	1	1
30	134.05969	134.05791	1.8		5	.	10	.	4
30	134.05969	134.06057	- .8		8	.	8	1	1
10	135.04310	135.04460	-1.4		8	.	7	.	2
0	135.06229	135.06126	1.0		4	1	10	.	4
0	135.06229	135.06393	-1.5		7	1	8	1	1
10	136.05049	136.05242	-1.8		8	.	8	.	2
0	137.04810	137.04768	.4		7	.	7	1	2
10	137.05789	137.06024	-2.3		8	.	9	.	2
0	140.02650	140.02622	.3		10	.	4	.	1
10	141.07020	141.07042	- .1		11	.	9	.	.
0	142.07639	142.07824	-1.8		11	.	10	.	.
0	144.05719	144.05750	- .2		10	.	8	.	1
0	145.03969	145.03750	2.2		5	.	7	1	4
10	145.06329	145.06533	-1.9		10	.	9	.	1
0	146.05799	146.05791	.1		6	.	10	.	4
0	146.05799	146.06057	-2.5		9	.	6	1	1
0	146.07049	146.07315	-2.6		10	.	10	.	1
10	147.04259	147.04459	-1.9		9	.	7	.	2
10	147.06899	147.06841	.6		9	.	9	1	1
0	147.07929	147.08098	-1.6		10	.	11	.	1
0	148.01889	148.01872	.2		11	.	2	1	.
20	148.03989	148.03717	2.7		5	.	8	.	5
20	148.03989	148.03985	.0		8	.	6	1	2
10	148.07479	148.07356	1.2		6	.	12	.	4
10	148.07479	148.07622	-1.3		9	.	10	1	1
20	149.08090	149.08138	- .4		6	.	13	.	4
100	150.05459	150.05282	1.8	3.0	5	.	10	.	5
100	150.05459	150.05549	- .8		8	.	8	1	2
20	150.08989	150.08921	.7		6	.	14	.	4
20	150.08989	150.09187	-1.9		9	.	12	1	1
10	151.05779	151.06064	-2.8		5	.	11	.	5
10	151.05779	151.05617	1.6		4	1	10	.	5

TABLE XXVII (Continued)

A805-27-03 WILFORDINE C.H. 01/14/71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
10	151.05779	151.05884	-1.0	3.0	7	1	8	1	2
10	152.04929	152.04734	2.0		8	.	8	.	3
10	152.04929	152.05001	- .6		11	.	6	1	.
0	153.05699	153.05517	1.8		8	.	9	.	3
0	153.05699	153.05783	- .7		11	.	7	1	.
0	157.06409	157.06533	-1.1		11	.	9	.	1
20	158.07069	158.07315	-2.4		11	.	10	.	1
10	159.08100	159.08099	.0		11	.	11	.	1
10	160.03979	160.03717	2.6		6	.	8	.	5
10	160.03979	160.03984	- .0		9	.	6	1	2
10	161.05929	161.06024	- .9		10	.	9	.	2
10	162.05509	162.05282	2.3		6	.	10	.	5
10	162.05509	162.05549	- .3		9	.	8	1	2
10	163.07380	163.07590	-2.0		10	.	11	.	2
0	164.05489	164.05588	- .9		5	.	10	1	5
20	164.07320	164.07116	2.0		9	.	10	1	2
10	165.05579	165.05517	.6		9	.	9	.	3
10	165.05579	165.05783	-1.9		12	.	7	1	.
20	168.00529	168.00586	- .5		7	.	4	.	5
40	169.01279	169.01369	- .8		7	.	5	.	5
10	173.05899	173.06024	-1.2		11	.	9	.	2
10	174.05759	174.05550	2.1		10	.	8	1	2
10	174.06650	174.06808	-1.5		11	.	10	.	2
20	175.06030	175.06065	- .3		7	.	11	.	5
10	175.07020	175.07143	-1.1		10	1	10	.	2
10	176.00079	176.00162	2.4		8	.	2	1	4
90	176.06779	176.06846	- .6		7	.	12	.	5
20	177.00719	177.00620	1.0		8	.	3	1	4
10	177.06860	177.07042	-1.7		14	.	9	.	.
10	177.06860	177.06596	2.6		13	1	8	.	.
10	177.06860	177.06736	1.2		5	2	11	.	5
10	177.06860	177.07003	-1.3		8	2	9	1	2
40	178.04259	178.04186	.7		13	.	6	.	1
0	179.04440	179.04299	1.4		5	.	9	1	6
0	179.04440	179.04521	- .7		12	1	6	.	1
0	179.04440	179.04662	-2.1		4	2	9	.	6
10	184.00349	184.00079	2.7		7	.	4	.	6
10	184.00349	184.00346	.0		10	.	2	1	3
0	186.06520	186.06808	-2.8		12	.	10	.	2
10	187.07399	187.07589	-1.8		12	.	11	.	2
0	189.05509	189.05516	- .0		11	.	9	.	3
0	189.05509	189.05783	-2.6		14	.	7	1	.
0	190.06049	190.06298	-2.4		11	.	10	.	3
0	190.06049	190.05852	2.0		10	1	9	.	3
0	190.06049	190.06119	- .6		13	1	7	1	.
0	190.06049	190.06259	-2.0		5	2	10	1	5
10	191.07180	191.07082	1.0		11	.	11	.	3
10	191.07180	191.07349	-1.6		14	.	9	1	.
20	192.06619	192.06339	2.8		7	.	12	.	6
20	192.06619	192.06606	.1		10	.	10	1	3
50	194.08440	194.08172	2.7		10	.	12	1	3
10	195.08310	195.08099	2.1		14	.	11	.	1
0	200.08260	200.08373	-1.0	3.2	13	.	12	.	2
0	202.06279	202.06298	- .1		12	.	10	.	3
0	202.06279	202.06566	-2.8		15	.	8	1	.
10	203.06890	203.07082	-1.8		12	.	11	.	3
10	204.06639	204.06339	3.0		8	.	12	.	6
10	204.06639	204.06606	.3		11	.	10	1	3
0	205.08680	205.08647	.3		12	.	13	.	3
0	205.08680	205.08914	-2.2		15	.	11	1	.

A805-27-03 WILFORDINE C.H. 01/14/71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
20	206.08090	206.07904	1.9	3.2	8	.	14	.	6
20	206.08090	206.08171	- .7		11	.	12	1	3
0	207.06839	207.06573	2.7		11	.	11	.	4
0	207.06839	207.06840	.0		14	.	9	1	1
0	207.08890	207.08687	2.0		8	.	15	.	6
0	207.08890	207.08954	- .5		11	.	13	1	3
20	215.01790	215.01918	-1.2		8	.	7	.	7
0	215.07650	215.07669	- .1		6	.	15	.	8
0	215.07650	215.07350	3.0		16	.	9	1	.
0	215.07650	215.07937	-2.8		9	.	13	1	5
10	216.08540	216.08719	-1.7		9	.	14	1	5
10	217.09499	217.09501	.0		9	.	15	1	5
30	231.01239	231.01408	-1.6		8	.	7	.	8
110	232.00169	232.00079	.9		11	.	4	.	6
110	232.00169	232.00345	-1.7		14	.	2	1	3
110	233.00969	233.00861	1.1		11	.	5	.	6
110	233.00969	233.01128	-1.5		14	.	3	1	3
0	233.09209	233.08993	2.2		9	.	15	1	6
20	234.08600	234.08921	-3.1		13	.	14	.	4
20	250.10670	250.10526	1.4	3.4	10	.	18	.	7
20	250.10670	250.10793	-1.1		13	.	16	1	4
10	718.27149	718.27191	- .3	4.8	50	.	38	.	5
10	718.27149	718.27109	.4		35	.	44	1	15
10	839.29690	839.29418	2.7	5.1	49	.	45	1	12
10	839.29690	839.30005	-3.1		42	.	49	1	17
10	840.30370	840.30201	1.7		49	.	46	1	12
10	840.30370	840.30787	-4.1		42	.	50	1	17
10	840.30370	840.30341	.3		41	1	49	1	17
10	840.30370	840.30563	-1.8		48	2	46	.	12
10	883.28549	883.28400	1.5	5.2	50	.	45	1	14
10	883.28549	883.28987	-4.3		43	.	49	1	19

TABLE XXVIII

HIGH RESOLUTION MASS SPECTRAL DATA OF WILFORTRINE

A805-19-01 WILFORTRINE C.H. 1-08-71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
0	70.04419	70.04186	2.3	2.7	4	.	6	.	1
0	70.07790	70.07825	- .3		5	.	10	.	.
10	71.01569	71.01330	2.4		3	.	3	.	2
0	71.05170	71.04969	2.0		4	.	7	.	1
0	71.08840	71.08608	2.3		5	.	11	.	.
0	74.01339	74.01564	-2.2		6	.	2	.	.
10	74.03599	74.03677	- .7		3	.	6	.	2
0	75.02459	75.02347	1.1		6	.	3	.	.
0	75.04630	75.04461	1.7		3	.	7	.	2
0	76.03300	76.03130	1.7		6	.	4	.	.
280	77.03809	77.03911	- .9		6	.	5	.	.
30	78.03569	78.03437	1.3		5	.	4	1	.
10	78.04550	78.04695	-1.4		6	.	6	.	.
60	79.04220	79.03952	2.7		2	.	7	.	3
60	79.04220	79.04220	.0		5	.	5	1	.
10	79.05539	79.05477	.6		6	.	7	.	.
10	81.03550	81.03404	1.5		5	.	5	.	1
10	81.07220	81.07043	1.8		6	.	9	.	.
10	82.04230	82.04187	.4		5	.	6	.	1
0	82.07850	82.07825	.3		6	.	10	.	.
0	82.53820++	82.53825	.1		6	.	13	.	5
0	82.53820++	82.53948	-1.2		9	.	11	1	2
50	83.05020	83.04969	.5		5	.	7	.	1
10	83.08810	83.08608	2.0		6	.	11	.	.
10	84.02139	84.02113	.3		4	.	4	.	2
0	84.05649	84.05750	- .9		5	.	8	.	1
0	84.09180	84.09390	-2.0		6	.	12	.	.
0	84.09180	84.08943	2.4		5	1	11	.	.
30	85.02919	85.02895	.2		4	.	5	.	2
0	85.10270	85.10173	1.0		6	.	13	.	.
10	86.03620	86.03678	- .5		4	.	6	.	2
10	86.09690	86.09697	- .0		5	.	12	1	.
0	87.04469	87.04460	.1		4	.	7	.	2
0	89.03720	89.03912	-1.8		7	.	5	.	.
0	90.50130++	90.50063	.7		7	.	3	1	5
0	90.51240++	90.51447	-2.0		12	.	5	.	2
0	90.53810++	90.53705	1.2		9	.	11	1	3
0	90.54400++	90.54336	.8		10	.	13	.	3
0	90.54400++	90.54457	- .5		13	.	11	1	.
0	90.54680++	90.54751	- .6		6	.	15	1	5
0	90.55340++	90.55380	- .3		7	.	17	.	5
0	90.55340++	90.55513	-1.6		10	.	15	1	2
70	91.05389	91.05476	- .8		7	.	7	.	.
0	91.07800	91.07591	2.1		4	.	11	.	2
10	92.04999	92.04734	2.7		3	.	8	.	3
10	92.04999	92.05001	.0		6	.	6	1	.
0	92.05780	92.05812	- .2		6	1	7	.	.
0	92.48670++	92.48628	.6		6	.	1	.	7
0	92.50860++	92.50861	.0		3	.	7	1	8
0	92.52600++	92.52677	- .7		4	.	11	1	7
0	92.53460++	92.53459	.2		8	.	11	1	4
0	92.55630++	92.55868	-2.5		10	.	17	.	3
0	92.55630++	92.55664	- .3		9	1	16	.	3
0	92.55630++	92.55798	-1.6		12	1	14	1	.
0	92.55630++	92.55584	.5		11	2	13	1	.
0	92.59610++	92.59533	.8		12	.	25	.	1
0	92.60550++	92.60717	-1.6		12	.	27	1	.
0	92.61350++	92.61353	.0		13	.	29	.	.
30	93.05730	93.05517	2.1		3	.	9	.	3
30	93.05730	93.05784	- .4		6	.	7	1	.

A805-19-01 WILFORTRINE C.H. 1-08-71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
0	93.49290++	93.49394	- .9	2.7	6	.	3	.	7
0	93.49290++	93.49527	-2.3		9	.	1	1	4
0	93.52850++	93.52741	1.1		15	.	7	.	.
0	93.52850++	93.53032	-1.7		8	.	11	.	5
0	94.04200	94.04187	.1		6	.	6	.	1
0	95.02190	95.02186	.0		1	.	5	1	4
10	95.05160	95.04969	1.9		6	.	7	.	1
10	95.08750	95.08608	1.4		7	.	11	.	.
10	97.03089	97.02895	1.9		5	.	5	.	2
10	97.10340	97.10173	1.7		7	.	13	.	.
20	98.03660	98.03678	- .1		5	.	6	.	2
0	98.07090	98.07316	-2.2		6	.	10	.	1
10	98.07510	98.07317	1.9		6	.	10	.	1
0	99.04719	99.04460	2.6		5	.	7	.	2
0	100.05429	100.05243	1.9		5	.	8	.	2
0	101.05960	101.06025	- .6		5	.	9	.	2
0	102.03089	102.03168	- .7		4	.	6	.	3
10	103.04020	103.03952	.7		4	.	7	.	3
10	103.04020	103.04219	-1.9		7	.	5	1	.
0	103.05439	103.05476	- .3		8	.	7	.	.
40	104.04910	104.04735	1.8		4	.	8	.	3
40	104.04910	104.05002	- .8		7	.	6	1	.
10	105.03580	105.03404	1.8		7	.	5	.	1
20	105.04510	105.04260	2.5		3	.	7	1	3
20	105.05710	105.05517	1.9		4	.	9	.	3
20	105.05710	105.05784	- .6		7	.	7	1	.
0	105.07010	105.07042	- .2		8	.	9	.	.
0	106.02859	106.02661	2.0		3	.	6	.	4
0	106.02859	106.02927	- .6		6	.	4	1	1
0	106.04150	106.04186	- .3		7	.	6	.	1
0	106.04150	106.04148	.0		1	2	6	1	3
60	106.06430	106.06300	1.3		4	.	10	.	3
60	106.06430	106.06567	-1.3		7	.	8	1	.
10	107.05020	107.04969	.5		7	.	7	.	1
10	107.07400	107.07350	.5		7	.	9	1	.
0	108.05780	108.05752	.3		7	.	8	.	1
0	109.06550	109.06534	.2		7	.	9	.	1
0	109.10230	109.10173	.6		8	.	13	.	.
10	110.03699	110.03678	.2		6	.	6	.	2
10	111.04239	111.04459	-2.1		6	.	7	.	2
10	111.04239	111.04013	2.3		5	1	6	.	2
10	111.04239	111.04420	-1.7		.	2	7	1	4
0	111.11540	111.11737	-1.9		8	.	15	.	.
40	112.01629	112.01604	.3		5	.	4	.	3
40	112.01629	112.01871	-2.3		8	.	2	1	.
0	112.05420	112.05243	1.8		6	.	8	.	2
10	113.01919	113.01939	- .1		4	1	4	.	3
10	113.01919	113.01760	1.6		6	2	1	1	.
10	115.05420	115.05477	- .6		9	.	7	.	.
10	117.05639	117.05517	1.2		5	.	9	.	3
10	117.05639	117.05783	-1.3		8	.	7	1	.
0	118.06820	118.06568	2.5		8	.	8	1	.
0	118.06820	118.06930	-1.0		7	2	8	.	.
10	119.03660	119.03444	2.2		4	.	7	.	4
10	119.03660	119.03711	- .4		7	.	5	1	1
0	119.04899	119.04968	- .6		8	.	7	.	1
10	120.04580	120.04494	.9		7	.	6	1	1
0	120.05560	120.05751	-1.8		8	.	8	.	1
0	121.06360	121.06534	-1.6		8	.	9	.	1
0	122.03770	122.03678	.9		7	.	6	.	2

TABLE XXVIII (Continued)

A805-19-01 WILFORTRINE C.H.

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
10	123.04410	123.04460	- .4	2.7	7	.	7	.	2
10	124.05399	124.05243	1.6		7	.	8	.	2
0	125.00589	125.00860	-2.6		2	.	5	.	6
0	125.06250	125.06026	2.2		7	.	9	.	2
0	126.03499	126.03437	.6		9	.	4	1	.
0	128.06269	128.06260	.1		10	.	8	.	.
0	129.07310	129.07043	2.7		10	.	9	.	.
10	130.06619	130.06567	.5		9	.	8	1	.
0	131.03859	131.03711	1.5		8	.	5	1	1
0	131.05219	131.04969	2.5		9	.	7	.	1
10	131.07429	131.07349	.8		9	.	9	1	.
20	132.04569	132.04493	.8		8	.	6	1	1
0	132.08289	132.08132	1.6		9	.	10	1	.
50	133.05439	133.05276	1.6		8	.	7	1	1
0	133.06440	133.06534	- .8		9	.	9	.	1
20	134.06049	134.05791	2.6		5	.	10	.	4
20	134.06049	134.06057	- .0		8	.	8	1	1
10	136.05289	136.05243	.5		8	.	8	.	2
10	136.05289	136.05204	.9		2	2	8	1	4
0	139.02449	139.02426	.2		3	.	7	.	6
0	139.02449	139.02693	-2.3		6	.	5	1	3
0	139.04090	139.03952	1.4		7	.	7	.	3
0	139.04090	139.04219	-1.2		10	.	5	1	.
10	141.07180	141.07043	1.4		11	.	9	.	.
0	143.00269	143.00072	2.0		8	.	1	1	2
0	144.00860	144.00588	2.7		5	.	4	.	5
0	144.00860	144.00856	.0		8	.	2	1	2
0	145.01580	145.01370	2.1		5	.	5	.	5
0	145.01580	145.01637	- .5		8	.	3	1	2
10	145.06559	145.06534	.3		10	.	9	.	1
0	145.07170	145.07389	-2.1		6	.	11	1	3
0	146.06609	146.06868	-2.5		9	1	9	.	1
0	146.06609	146.06422	1.9		8	2	8	.	1
0	147.04619	147.04460	1.6		9	.	7	.	2
0	147.08209	147.08099	1.1		10	.	11	.	1
0	147.08209	147.08060	1.5		4	2	11	1	3
10	148.04239	148.03985	2.5		8	.	6	1	2
10	149.05939	149.06024	- .8		9	.	9	.	2
20	149.08400	149.08139	2.6		6	.	13	.	4
20	149.08400	149.08406	- .0		9	.	11	1	1
70	150.05769	150.05550	2.2	3.0	8	.	8	1	2
0	150.07900	150.07664	2.4		5	.	12	1	4
20	150.09390	150.09189	2.0		9	.	12	1	1
0	151.04099	151.03952	1.5		8	.	7	.	3
0	151.04099	151.04218	-1.1		11	.	5	1	.
10	151.06160	151.06065	1.0		5	.	11	.	5
10	151.06160	151.06332	-1.6		8	.	9	1	2
10	151.06160	151.05886	2.7		7	1	8	1	2
10	152.05139	152.05002	1.4		11	.	6	1	.
10	153.05679	153.05517	1.6		8	.	9	.	3
10	153.05679	153.05783	- .9		11	.	7	1	.
10	153.05679	153.05924	-2.4		3	1	10	1	5
10	153.05679	153.05478	2.0		2	2	9	1	5
0	153.07339	153.07042	3.0		12	.	9	.	.
0	153.07339	153.07629	-2.8		5	.	13	.	5
0	155.00249	155.00072	1.8		9	.	1	1	2
0	156.09349	156.09389	- .3		12	.	12	.	.
0	158.06139	158.06058	.8		10	.	8	1	1
10	158.07049	158.07315	-2.6		11	.	10	.	1
10	159.08129	159.08099	.3		11	.	11	.	1

A805-19-01 WILFORTRINE C.H. 1-08-71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
10	160.04010	160.03718	2.9	3.0	6	.	8	.	5
10	160.04010	160.03986	.2		9	.	6	1	2
10	161.05869	161.06024	-1.5		10	.	9	.	2
10	162.06199	162.06360	-1.5		9	1	9	.	2
10	162.06199	162.05913	2.9		8	2	8	.	2
0	162.07030	162.06808	2.2		10	.	10	.	2
0	163.07769	163.07590	1.8		10	.	11	.	2
0	165.04479	165.04259	2.2		8	.	7	1	3
0	165.06079	165.05784	3.0		12	.	7	1	.
0	165.06079	165.06371	-2.8		5	.	11	1	5
0	166.06590	166.06300	2.9		9	.	10	.	3
0	166.06590	166.06568	.2		12	.	8	1	.
0	166.06590	166.06707	-1.1		4	1	11	1	5
0	167.07989	167.07937	.5		5	.	13	1	5
10	168.00469	168.00586	-1.1		7	.	4	.	5
0	173.06090	173.06026	.6		11	.	9	.	2
10	174.06989	174.06808	1.8		11	.	10	.	2
60	175.06469	175.06332	1.4		10	.	9	1	2
0	175.07489	175.07589	- .9		11	.	11	.	2
0	175.07489	175.07550	- .5		5	2	11	1	4
30	176.07259	176.07115	1.4		10	.	10	1	2
0	177.06290	177.06372	- .7		8	.	11	1	5
10	177.07740	177.07630	1.1		7	.	13	.	5
10	177.07740	177.07897	-1.5		10	.	11	1	2
10	177.07740	177.07451	2.9		9	1	10	1	2
20	178.04999	178.04774	2.3		6	.	10	.	6
20	178.04999	178.05040	- .3		9	.	8	1	3
0	179.07049	179.07081	- .2		10	.	11	.	3
0	179.07049	179.07348	-2.9		13	.	9	1	.
0	180.04310	180.04226	.8		9	.	8	.	4
0	180.04310	180.04493	-1.7		12	.	6	1	1
0	181.05039	181.05008	.3		9	.	9	.	4
0	181.05039	181.05275	-2.3		12	.	7	1	1
0	182.02979	182.03006	- .2		4	.	8	1	7
0	182.04419	182.04265	1.5		5	.	10	.	7
0	182.04419	182.04532	-1.0		8	.	8	1	4
20	182.08289	182.08171	1.2		9	.	12	1	3
0	182.11219	182.10955	2.6		14	.	14	.	.
10	182.15520	182.15449	.7		11	.	20	1	1
0	182.16970	182.16707	2.6		12	.	22	.	1
0	182.18850	182.19087	-2.3		12	.	24	1	.
0	183.08820	183.08687	1.3		6	.	15	.	6
0	183.08820	183.08954	-1.2		9	.	13	1	3
10	187.07800	187.07591	2.1		12	.	11	.	2
0	189.01909	189.01678	.3		10	.	5	.	4
0	189.01909	189.02145	-2.3		15	.	3	1	1
0	190.06300	190.06300	.0		11	.	10	.	3
0	190.06300	190.06567	-2.6		14	.	8	1	.
10	191.07520	191.07350	1.7		14	.	9	1	.
0	192.00430	192.00587	-1.5		9	.	4	.	5
0	195.08660	195.08686	- .2		7	.	15	.	6
0	195.05660	195.08954	-2.8		10	.	13	1	3
0	195.08660	195.08508	1.5		9	1	12	1	3
10	203.06870	203.07082	-2.0	3.2	12	.	11	.	3
10	204.06800	204.06607	1.9		11	.	10	1	3
0	205.08750	205.08647	1.0		12	.	13	.	3
0	205.08750	205.08914	-1.5		15	.	11	1	.
10	206.08010	206.07904	1.1		8	.	14	.	6
10	206.08010	206.08171	-1.5		11	.	12	1	3
0	216.07670	216.07864	-1.8		13	.	12	.	3

TABLE XXVIII (Continued)

A805-19-01 WILFORTRINE C.H. 1-08-71

INT	DETM.	CALC.	DIFF	TOL	C	C'	H	N	O
0	217.08320	217.08200	1.2	3.2	12	1	12	.	3
0	217.08320	217.08467	-1.4		15	1	10	1	.
0	217.08320	217.08340	- .1		4	2	15	.	8
0	217.08320	217.08021	3.0		14	2	9	1	.
0	217.08320	217.08608	-2.8		7	2	13	1	5
0	222.07170	222.07395	-2.2		8	.	14	.	7
0	233.08409	233.08138	2.7		13	.	13	.	4
0	233.08409	233.08406	.0		16	.	11	1	1
0	234.00889	234.00972	- .7		3	.	8	1	11
10	250.10789	250.10525	2.6	3.4	10	.	18	.	7
10	250.10789	250.10792	.0		13	.	16	1	4
0	718.27370	718.27193	1.8	4.8	50	.	38	.	5
0	718.27370	718.27780	-4.0		43	.	42	.	10
0	718.27370	718.27460	- .8		53	.	36	1	2
0	718.27370	718.27110	2.6		35	.	44	1	15
0	718.27370	718.27697	-3.2		28	.	48	1	20
10	829.27649	829.27427	2.2	5.1	62	.	37	.	3
10	829.27649	829.28013	-3.5		55	.	41	.	8
10	829.27649	829.27693	- .3		65	.	35	1	.
10	829.27649	829.27930	-2.7		40	.	47	1	18
0	830.28400	830.28210	1.9		62	.	38	.	3
0	830.28400	830.28797	-3.9		55	.	42	.	8
0	830.28400	830.28477	- .7		65	.	36	1	.
0	830.28400	830.28714	-3.0		40	.	48	1	18
0	830.28400	830.28350	.5		54	1	41	.	8
0	830.28400	830.28617	-2.1		57	1	39	1	5
0	830.28400	830.28268	1.3		39	1	47	1	18
0	830.28400	830.28840	-4.3		64	2	36	.	.
0	830.28400	830.28490	- .8		46	2	44	.	13
0	830.28400	830.28171	2.3		56	2	38	1	5
0	830.28400	830.28758	-3.5		49	2	42	1	10
10	873.26629	873.26410	2.2	5.2	63	.	37	.	5
10	873.26629	873.26996	-3.6		56	.	41	.	10
10	873.26629	873.26676	- .4		66	.	35	1	2
10	873.26629	873.26913	-2.7		41	.	47	1	20

VITA

Hee Joung Lee

Candidate for the Degree of

Doctor of Philosophy

Thesis: I. STRUCTURE AND BIOSYNTHESIS OF PYRIDINE ALKALOIDS FROM TRIPTERYGIUM WILFORDII HOOK

II. METABOLISM OF RICININE IN RICINUS COMMUNIS L.

Major Filed: Biochemistry

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