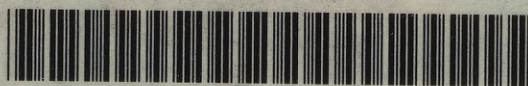


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A COMPARISON OF THE ALKALOIDS
OF THE FACIATED AND NON-FACIATED
STEMS IN TWO SPECIES OF SOPHORA

PEDRO ISLAS CHAVEZ



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APPROVED BY SUPERVISORY COMMITTEE:

Presented to the Faculty of the University of Illinois at Urbana-Champaign

The Graduate College

in partial fulfillment of

the requirements for

the degree of

MASTER OF SCIENCE

6

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THE UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN

August, 1961

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by

Pedro Islas Chavez, B.A., M.A.

DEDICATION
TO MY LOVING WIFE, MARY

DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT AUSTIN

August, 1981

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DEDICATION:

TO MY LOVING WIFE, MARY

Dr. Tom Henry of the Department of Botany, University of Texas, Austin and Dr. W.J. Miller of Northeast Louisiana University have supplied material data or pure sample compounds.

Finally, my deepest thanks to the Ford Foundation for its financial support from 1965-1974.

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ABSTRACT

Fasciations are flattened, ribbon shaped deformities of the stem tip or other parts of the plant which arise unpredictably and sporatically on higher plants. These growths which proliferate rapidly have been compared to cancers or monstrosities of the plant. The causes of fasciations are still under investigation and may involve a hormonal imbalance in the plant due to genetic, environmental, biological and chemical factors. At the present time, experimental methods have been developed to induce some forms of fasciations.

The effects of fasciations on the secondary plant constituents are of pharmaceutical and medicinal interest since these growths may alter the chemical composition of the plants. These changes may produce novel drugs at the site of fasciation. Alternatively, fasciations may lead to higher yields of useful natural products from medicinal plants.

Two plants from the same genus, *Sophora secundiflora* and *S. tomentosa*, were observed to possess a limited number of fasciated stems. Since pharmaceutically active alkaloids have been reported in the genus, this group of compounds was used in a chemical comparison to detect variability between the stem types.

Classical (liquid, column, preparative and thin-layer chromatography techniques) and more recent (gas chromatography, high pressure liquid chromatography, combined gas chromatography-mass spectrometry) methods were developed to separate, isolate and purify eleven

alkaloids present in both stem types. Structural characterization of these eleven alkaloids was accomplished utilizing chemical and physical tests, melting points and mass, ultra-violet, infrared and nuclear magnetic resonance spectral data.

Compounds previously reported from *S. secundiflora* by several authors are epi-lupinine, sparteine, β -isosparteine, cytosine, N-methylcytosine, 11-allyl cytosine, lupanine, Δ 5,6-dehydrolupanine, rhombifoline, anagyrine and thermopsine. In our analysis of the normal and fasciated stem of *S. secundiflora* all but three of these compounds were encountered: 11-allyl cytosine, β -isosparteine and thermopsine. Two quinolizidine alkaloids were detected in the stems which had not been reported previously in this plant, N-formyl cytosine and N-acetyl cytosine.

Compounds previously reported from *S. tomentosa* are N-methylcytosine, matrine, matrine N-oxide, baptifoline, N-acetyl cytosine and anagyrine. In our analysis matrine, cytosine, N-acetyl cytosine, anagyrine and N-methylcytosine were detected by a combination of gc-ms and other techniques. Also detected by this method were epi-lupinine and N-formyl cytosine, two previously unreported alkaloids from this species of *Sophora*.

The differences observed between the two stem types of the same species were of quantitative nature. Overall the amount of the major alkaloid, cytosine in *S. secundiflora* and matrine in *S. tomentosa*, was reduced in the fasciated stem when compared to the non-fasciated stem and an internal standard. Other alkaloid consti-

tuents were either increased, reduced or remained the same. Noteworthy of these alkaloids with higher concentrations in the fasciated stems were sparteine in *S. secundiflora* (considered to be a precursor to cytisine) and the cytisine group of compounds (considered to be a biogenetic branch from matrine) in *S. tomentosa*.

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INTRODUCTION

In fasciations of the plant stem the cylindrical apical growth typical of the vascular plants is replaced by a ribbon or fanshaped growth with multiple centers. Well known examples of fasciated type growth are the cockscomb (*Celosia cristae*) and the crested succulents such as the large fanshaped "cancered" *Carnegiea gigantea*. The exact cause of fasciation is unknown and may involve a multitude of factors. The causes implicated involve genetic, environmental, physiological and biological factors. Each of these factors may act on the hormonal system of the plant producing the observed effects. In a few instances, fasciations have been induced chemically or by radiation.

A survey of the literature of the plant kingdom revealed that the occurrence of fasciations is widespread. According to White (1), fasciations are reported to exist in at least 107 of the 303 families into which Engler divided the living vascular plants. This number may, however, be misleading since the chances of finding a fasciation in a particular genus of a particular family are extremely slim. The chances become miniscule if one tries to find a fasciation on a particular plant in a particular population at a particular time. The chance discovery of fasciations on two species of *Sophora* by Dr. Gerald Sullivan in Texas made it possible for this

investigator to conduct a chemical comparison which could not be attempted before between healthy and fasciated stems on the same plant.

An interesting aspect of fasciations is the sudden rapid

changes which occur in the plant to produce this growth. Should

quantitative or qualitative changes occur in the secondary natural

products of the fasciated plant, fasciation might serve as a source

of new drugs. One class of drugs which may be useful for this com-

parison is the alkaloids. Alkaloids are defined as heterocyclic

basic compounds which usually exhibit a physiological effect on man

(2). Quinolizidine alkaloids have been known in *Sophora* since before

the turn of the century but plants of this genus are not used as a

source for any commercially available drugs at the present time. The

purpose of this dissertation is to compare naturally grown fasciated

and non-fasciated stems of *Sophora secundiflora* and *Sophora tomentosa*

for their alkaloid contents. To assist in this endeavor, an intro-

duction to the taxonomy and alkaloid chemical constituents of the

genus is presented.

TAXONOMY

Kingdom: Plantae
Division: Anthophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Rosales
Family: Leguminosae (Papilionaceae)
Genus: *Sophora*
Species: *S. tomentosa* L. subsp. *occidentalis* (L.) Brummitt
S. secundiflora (Gomez Ortega) Lagasca ex DC.

Introduction to the Leguminosae

According to Turner (3), the family Leguminosae is composed of trees, shrubs and herbs with mostly alternate compound leaves and stipules. This family is one of the largest families of flowering plants containing on a world wide basis some 13,000 species distributed into about 550 genera. In Texas there are approximately 391 legume taxa in 59 genera (3). The family is economically important since it includes important food crops (peas, beans, groundnut, soybeans), forage crops (clover, alfalfa), ornamentals (broom, acacia, sweet peas) and timber crops. The family is also a source of dyes, such as indigo (4).

Some authors contend that the family should be divided into three separate families or subfamilies. A description of these subfamilies follows. The characteristics of the subfamily Mimosoideae are pinnately twice compound leaves, small radially symmetrical flowers on spikes, heads or congested racemes with sepals united their full length, petals are separate or coalescent, valvate in bud, stamens 4 to numerous, usually greatly exceeding the petals in length. The characteristics of the subfamily Caesalpinoideae are one or twice

pinnately compound or simple leaves, weakly bilaterally symmetrical often large and showy flowers with sepals often separate to the top of the floral cup with 5 petals which are equal in length, never valvate in bud, stamens 5 to 10 usually shorter than the petals or only as long. The characteristics of the subfamily Papilionoideae are once pinnately compound or simple but never pinnately twice compound leaves, strongly bilaterally symmetrical often showy flowers, with sepals united forming a distinct calyx tube prolonged beyond the floral cup, petals usually imbricated in the bud with the banner external. The genus *Sophora* is taxonomically placed in the Papilionoideae (5).

Taxonomy of Sophora

Members of the genus *Sophora* can be distinguished from other genera of the Papilionoideae by the following characteristics as outlined by Hutchinson (6):

Trees, shrubs, or rarely perennial herbs; leaves imparipinnate, leaflets numerous and small or few and larger; stipels setaceous or often absent; flowers white, yellow or rarely blue-violet, in terminal racemes or leafy panicles; bracts linear, minute or absent; bracteoles often absent; calyx-teeth short; vexillum broadly obovate or orbicular, erect or spreading, usually shorter (rarely longer) than the keel; wings oblong, oblique, keel oblong, almost straight, the petals overlapping or connate dorsally; stamens free, or rarely connate at the base into a ring; anthers versatile; ovary shortly

stipitate, ovules numerous; style incurved, stigma minute, terminal; fruit moniliform, terete or slightly compressed, fleshy to wood, not opening or very tardily 2-valved; seeds obovoid or globose, not strophiolate; cotyledons thick.

Rudd (7) divided the genus *Sophora* into five sections: *Sophora*, *Oresbios*, *Aigialodes*, *Calia* and *Styphnolobium*. The section *Aigialodes* is composed of plants with stipules linear-deltoid, caducous or lacking; inflorescences racemose, terminal, calyx truncate or subtruncate; corolla white or yellow, the keel petals usually somewhat connate; fruit torulose. Type species, *Sophora tomentosa* L. The section *Calia* is composed of shrubs or small trees; leaflets coriaceous, stipules minute, linear or deltoid, sometimes lacking, inflorescence racemose, terminal or occasionally axillary; calyx hypanoid, with well developed teeth, the vexillar teeth usually connate in part; corolla blue or violet, or white with purplish marking; fruit terete or laterally compressed. Type species. *Sophora secundiflora* (Gomez Ortega) Lagasca ex DC.

Synonymy

Sophora tomentosa L. is subdivided into several subspecies. According to Rudd (7) the Texas plant belongs to *Sophora tomentosa* L. subsp. *occidentalis* (L.) Brummit. The synonymy of this subspecies is listed below:

Sophora tomentosa L. subsp. *occidentalis* (L.) Brummitt

Sophora occidentalis L.

Sophora havanensis Jacq.

Sophora tomentosa L. subsp. *havanensis* (Jacq.) Yakovl.

Sophora tomentosa L. subsp. *longiflora* Yakovl.

Conversely, the following names have been applied to

Sophora secundiflora:

Sophora secundiflora (Gomez Ortega) Lagasca ex DC.

Broussonetia secundiflora Gomez Ortega

Virgilia secundiflora (Gomez Ortega) Cab

Calia erythrosperma Berland, in Mier Teran

Agastianis secundiflora (Gomez Ortega) Rag.

Dermatophyllum speciosum Scheele

Sophora speciosa (Scheele) Benth.

Sophora sempervirens Engelm.

Cladrastis secundiflora (Gomez Ortega) Lagasca ex DC. f.

xanthosperma Rehd.

Calia secundiflora (Gomez Ortega) Yakovl.

Calia secundiflora (Gomez Ortega) Yakovl. subsp.

secundiflora f. *xanthosperma* (Rehder) Yakovl.

Calia secundiflora (Gomez Ortega) Yakovl. subsp. *albofiliole*
Yakovl.

Sophora secundiflora is characterized by the features of the section *Calia* listed above and is an evergreen shrub 5-35 dm. tall, with usually dense dark-green foliage; leaflets very firm, 5-11

(to 13) per leaf often 17 mm. broad to broader and glabrous above; racemes 5-15 cm. long, 5-10 cm. broad, bluish-purple and very showy, the individual flowers 1-2 cm. long, the banner external to the others in bud; fruit woody between the seeds; seeds red (5).

Sophora tomentosa L. is characterized by the features of the section Aigialodes and is a round shrub about 1 m. (in the Texas latitude, often freezing back in winter and behaving as subshrubs) foliage densely pubescent, grayish, flowers in terminal racemes, bright yellow (5).

Range

Sophora secundiflora is frequent in brushy vegetation at the southern edge of the Edward Plateau (N. to Travis Co.) and caliche cuestras in the Rio Grande Plains, scattered in the western part of the Edward Plateau and Trans Pecos mountains, there is an isolated record from Cameron Co. Flowers in the spring. Range: Texas, New Mexico in the mountains, to San Luis Potosi.

Sophora tomentosa is infrequent near and along the coast in S. Texas as far north as Aransas Co., flowers March to October. This plant is also widely distributed along warm coastal areas of the world (5).

INTRODUCTION TO FASCIATION

Fasciations may occur on various parts of the plant: the main stem, under ground stem, branches, leaves, inflorescences, flowers, fruits, roots (1) and are defined as a "multiple forking of an axis; ribbon or fangrowth of an axis" (8). The normal non-fasciated plant possess a cylindrical or polygonal stem whereas the fasciated stem is dilated: (a) into a flat ribbon with prominent ridges (linear fasciation); (b) into three or four flattened lobes (triradiate or quadriradiate fasciation); or, (c) into a ring, that is, a hollow stem with an internal cavity which is lined by an internal epidermis (ring fasciation) (9).

In fasciations there is a divergence from the normal cylindrical apical growth exhibited by the Angiosperm stem to a flattened stem with multiple centers of growth. Fasciations in *Sophora secundiflora* were first described by Vasey (10) in 1887; the extremity of the twigs, or racemes, become flattened and enlarged, gradually expanding and dividing toward the apex--sometimes in a few, often into a large number of segments--the surface studded with small scales and mostly dormant buds. Sometimes, however, these buds develop into more or less perfect flowers.

Plate 1 shows a landscape photograph of the affected plant on the University of Texas campus. Plate 2 is a closer view of the same fasciated stem.

Fasciations of *S. tomentosa* have not been reported in the literature. In general, the fasciations of *S. tomentosa* are less fan-shaped and more clubshaped than those of *S. secundiflora*. The spacing between the dormant buds is greater in *S. tomentosa* than in *S. secundiflora*, as is evident in Plate 3.

Causes of Fasciations

The scientific study of fasciations has been of scholarly merit for many centuries. Even the great taxonomist Linneaus encountered and described the phenomenon. At the turn of this century, Wordsdell (8) studied fasciation and other plant monstrosities. He noted similarities between fasciation in vascular plants, and the typical patterns which occur in the lower evolutionary forms such as the Cryptograms (mosses, ferns, algae). A second observation made by Wordsdell was that fasciations were a pathological condition stimulated by the presence of superabundant nutriment at the growing tips. A discussion of this second observation follows. Wordsdell used experiments with the scarlet runner bean *Phaseolus multiflorus* as proof of his theory. Newly, sprouted beans were induced to fasciate by selectively excising the main axis of the plumule (root) of the seedling. The shoots on the axile of the cotyledon subsequently flattened and became fanshaped. Wordsdell interpreted this experiment as indicating that once the roots were severed, the nutriment for the stems was increased tremendously resulting in a hypernutritive microenvironment at the growth tip. This increased nourishment led to an abnormal expression of the meristem resulting in fasciation. He concluded that mechanical factors were the prime inducers of fasciations (8).

At the present time, all evidence points to fasciation being caused by hormonal variation which may be induced by a variety of factors. The genetic machinery to produce fasciation is, therefore, present in all Angiosperms. Once a hormonal imbalance occurs, the growing tip may respond in one of three ways to cause fasciation. The meristem may either (a) develop a number of buds in close proximity to each other which exert such a mechanical pressure upon each other that they become grafted to form a single shoot, first proposed by Linnaeus; (b) initiate growth and expansion of some shoot in one plane, first proposed by Moquin-Tandon; or (c) continued growth but without differentiation into tissue parts, first proposed by Wordsdell (8). Several reported observations favor the theory proposed by Moquin-Tandon (9).

Several factors singly or in combination have been implicated as inducing fasciation. These include, genetical, environmental, physiological, chemical and biological causes.

Genetic

Plants which breed true for fasciations have been known for centuries. Two common examples are the cockscomb and mummy pea. The common cockscomb thrives only in cultivation and is believed to have been selected by the ancient Indians specifically for its monstrous form because of religious, magical or superstitious reasons. The selection process involved reduction of the breeding group and interbreeding. This process produced recessives for fasciations, color and other traits (11). Almost magically, the ancient Indians fortuitously chose the correct plant to use in their selection scheme. The more common weedy member

of the genus *Celosia* (*C. argentea*) is an octaploid species. The Indians were able to find an endemic race of *C. argentea* which was quadraploid. It was this race of *C. argentea* which was selected by the Indians to give rise to *C. cristata*, a quadraploid. Remnants of the quadraploid types of *C. argentea* were recently discovered and crossed with the crested *C. cristata* and genetically proved the origin of the fasciated *C. cristata* (12).

The mummy or crown pea is a true breeding form of ring fasciation in which the flowers are bunched at the top of the stem and arranged almost in a false umbel. The upper part of the stem is more or less widened in section. In the normal condition, the flowers are axillary, that is, distributed along the main stem and the stem progressively tapers to the tip. The genetics of this type of pea have been known since the time of Mendel. Indeed, one of the original seven traits used by Mendel involved differences in the position of the flowers. When mummy peas were crossed with regular peas, the first filial generation was totally normal, indicating that fasciation was recessive. Crosses between the progeny of this cross resulted in 651 cases normal and 207 terminal, roughly the expected Mendelian ratio of 3 to 1 for a recessive trait (13).

The genetic transmittance of fasciations may have had an important role in the development of important food crops. The wild type tomato has a fruit which is the size of a cherry or currant and has only two locules. The commercial varieties of tomatoes are appreciably larger in size and multiloculed. The commercial varieties

developed from the 2 loculed varieties by fasciations of the fruit followed by increase in size and weight. The commercial varieties Marglobe, Rutgers and Pritchard average from 5.38 to 6.02 locules, while Oxheart and Beefstake average 11.12 to 13.49 locules. Fasciation may have also been important in the selection and development of

Also of commercial importance, are fasciations which may have arisen by somatic mutation and are maintained by vegetative means.

- Somatic mutations arise sporadically in nature and are propagated vegetatively by grafting, budding or rooting of the particular part of the plant, if the mutation has some benefits over the parent types. Varieties of navel oranges arose as sport buds on citrus trees and are maintained by horticultural means and not necessarily by seed propagation (14). This vegetative reproduction holds true for some forms of fasciations especially in the succulents such as members of the genus *Euphorbia*, *Crassula*, *Opuntia*, *Mamillaria*, *Sedum*, etc. Two forms which are well known in collections are *Euphorbia lactea* var. *cristata* and *Opuntia serpentina*, the boxing glove cactus. In most instances the genetics of these plants are not known (1).

Environmental Causes

At the environmental level, one is concerned with the effects of external stimuli on the plant by factors normally experienced by the plants in nature. These factors may be increased water, harsh winters, shading, etc. In most instances, the environmental stimuli

may increase the ratio of fasciations which are normally seen in the population. An unusual aspect of these environmental causes is that the factors which may affect one plant may not affect its neighbor one foot away. In winter peas the frequencies in the number of fasciations increases with age. This increase is believed to be prompted by the seasonal shortening of the days in the fall and winter (15). Age may also be a factor in perennial plant fasciations, since *Delphinium hybridum* which lives an average of 4 years, *Hesperis matronalis* which averages 3 years and *Pyrethrum roseum* which averages 5-7 years have maximum fasciations during the third, second and second-third years respectively. In *Hesperis matronalis* each plant may develop 35-70% fasciated shoots (16). Other factors which may affect frequency of fasciations include optimum conditions of water and fertilizer (17,18), unfavorable weather (high precipitation, low temperatures, snow cover) (19), or other meteorological conditions (20), boron deficiency and external factors such as daylength, intensity of illumination and temperature (21).

Physiological Basis

On a physiological basis, the plant may be exposed to a regimented stimuli such as mechanical injury, transplantation to a harsher environment, daylength variability and temperature variability. Transplantation of plants from one environment to a harsher one may increase the number of plants with fasciation. Along this context Shavrov (22) noted that the severe condition in the Polar-Alpine

Botanical Gardens of the Academy of Science USSR at Eirovsk stimulated the appearance of fasciations in introduced southern plants. Fasciation in cacti are economically important since the plants demand a high price among collectors. Synder and Weber (23) relate the work of Wolthuy as a typical example of some of the experiments attempted in order to induce fasciation: "He subjected young plants of the genus *Echinopsis* to the following stimuli: cutting away the top; sticking rusty nails into the plant; lacerating the plant all over with knives; striking heavy blows with a steel brush; similar blows on decapitated plants; pouring salt soda and other material into wounds; injecting lactic acid, oxalic acid, formic acid, various other chemicals, and pure water into the plant; planting in various types of soil with different degrees of moisture. He produced not a single crest." Kewen (24) produced a crest on *Opuntia cylindrica* by drawfing the plant with weekly applications of an arsenic solution followed by forced growth through applying nitrate of soda. The crest revert to normal growth after 4 years.

Radiation and Chemical

When seeds are exposed to radiation, one of the common manifestations is fasciations. Kundu and Rao (9) treated jute seeds with X-rays. The seeds developed plants with fasciated stems, some of which attained a width of 2-4 inches. Kamra (25) incorporated Sr-90 and Cs-137 on the crucifer *Arabidopsis thaliana*, and found that gross physiological changes (loss of apical dominance, dwarfism, gigantism

and fasciation) and inheritable mutations were evident in the treated plant and their progeny. Radiation, however, was not a factor in cacti (23).

Chemicals may also induce changes in the plant which result in fasciation. *Rudbeckia hirta* L. and *R. amplexicaule* under the influence of carbendazim, diethyl sulfate or colchicine developed vegetative cones which resulted in increased numbers of floral shoots. Frequently these led to fasciations. According to the author, most of these changes including fasciations were retained in subsequent generations with both vegetative and sexual reproductions (26). Ethephon and amorphactin IT 3456 applied as a spray to "Carignone" grapes, resulted in the veins of the leaves and clusters of the grapes developing fasciations, though vine vigor was normal (27). Dostal (28) induced ring fasciations in *Bryophyllum rosei* with triiodobenzoic acid.

Biological Causes

The fact that one form of fasciation may be associated with a bacterium was reported independently in 1936 by Lacey and Tilford. Lacey (29,30) isolated a bacterium, *Bacterium fascians* as the causative agent of the fasciations of sweet peas, "cauliflower" strawberry and the leafy gall of various plants. Tilford (31) examined fasciations in the sweet pea and isolated a bacteria which he considered a new species, *Phytomonas fascians*. This bacterium formed yellow colonies, was non-mobile at its early stages and was gram-positive. These facts prompted Dowson (32) to change the name of the bacterium to *Corynebacterium fascians*. The taxonomy of this bacteria is controversial (33).

Corynebacterium fascians was implicated in the formation of galls and fasciations on a wide variety of hosts (29,30,34) and showed some degree of host specificity. Jacob and Mohanty (35) inoculated sweet peas and grains grown under sterile conditions with 21 different strains of *C. fascians* and obtained only 67% infection of the peas. Non-infection was attributed to possible contamination of the seedling by naturally occurring antibiotic-producing bacteria which destroyed the *C. fascians*.

One way the bacteria was transmitted was investigated by individuals working with "cauliflower" strawberries. The authors Pitcher and Cross (36,37) demonstrated that the nematode *Asphelenchoides fragariae* (Ritzema Bos) was partially responsible for the disease. Sterile grown strawberry plants injected with *C. fascians*, *A. fragariae*, or mixtures of the *C. fascians* and *A. fragariae* developed "cauliflowers" only in the mixtures. The authors concluded "a cauliflower may thus be considered primarily as a hyperplastic bacterial symptom, modified in detail by the activity of the eelworm, which in all but small seedling strawberries, is also essential as a vector of the bacterium." Therefore, in one instance, a vector for bacterial transmission was established.

The bacteria acted in part by upsetting the hormonal balance of the plant; two of which have been implicated, the auxins and the cytokinetins. Lacey (38) attributed the action of the bacteria as a destruction of plant auxins resulting in uncontrolled growth such as galls and fasciations. More recently, Thimann and Sach (39) noted the

similarities between sweet peas with bacterially induced fasciations and sweet peas treated with the purine based cytokinetins. In both plants there was an initiation of dormant buds, thickening of the stem, etc. These similarities, plus limited chemical tests, allowed the authors to conclude that *C. fascians* acted by production of cytokinetins. Klamt et al. (40) extracted $N^6(\Delta^2$ isopentenyl) adenine from broth cultures of *C. fascians* in the concentration of 10 ug/1 of culture broth. Others (41,42) have questioned the presence of cytokinetins in the cell broth at the concentration stated, claiming that the observed purine base is actually part of the breakdown of the t-RNA of the bacteria extracted by an acid step in the workup of the cytokinetins by Klamt et al. Still others (43-46) have questioned whether $N^6(\Delta^2$ isopentyl) adenine is sufficiently potent enough as a plant hormone to elicit the observed results. These individuals isolated six cytokinetins from the bacterial cultures, with most of these new cytokinetins being more potent as plant hormones than $N^6(\Delta^2$ isopentyl) adenine.

The discovery of cytokinetins as the causative agent in fasciations of the sweet pea supports Wordsdell's (8) original contention that a hypernutritive environment is responsible for fasciations. The ability of cytokinetins to draw nutriment toward the tissue which produces these compounds is a well documented phenomenon. Thimann and Sach (39) used the senescence test in their detection of microgram quantities of cytokinetins. In this test a drop of the test solution was placed atop a dying leaf and this leaf placed into

the dark and allowed to wither and turn yellow. The area where the cytokinetins were present, however, remained green due to the nutrient pulling properties of the cytokinetin. Cells possessing the hormone literally strip adjacent cells of their nutrients. Undoubtedly, bacteria infected tissues must also be nutrient rich. Teleologically the bacteria and the nematode benefit from this cytokinetin production to which the plant reacts by fasciation and gall formation.



Plate 1. Fasciation of *Sophora secundiflora*, on plant



Plate 2. Fasciation of *Sophora secundiflora*, close up

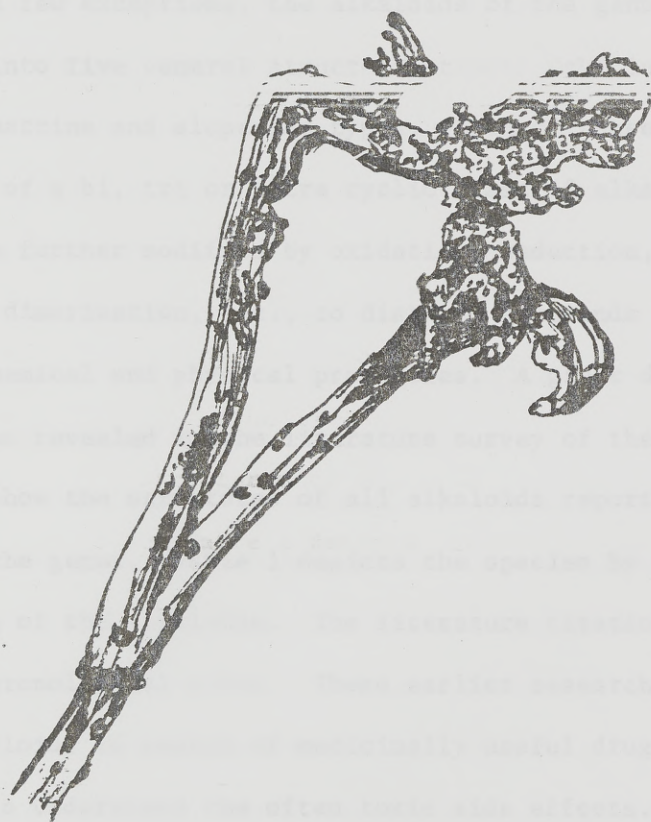


Plate 3. Fasciation of *Sophora tomentosa*, close up

INTRODUCTION TO THE ALKALOIDS OF SOPHORA

With few exceptions, the alkaloids of the genus *Sophora* can be grouped into five general structural types: the lupinine, cytisine, sparteine, matrine and aloperine types. Each of these types is representative of a bi, tri or tetra cyclic class of alkaloids. The class may be further modified by oxidation, reduction, epimerization, alkylation, dimerization, etc., to distinct compounds with specific spectral, chemical and physical properties. A great diversity of alkaloids was revealed in the literature survey of the genus. Figures 1, 2 and 3 show the structures of all alkaloids reported to be present in the genus. Table 1 depicts the species by species distribution of the alkaloids. The literature citations are (47-115) listed in chronological order. These earlier researchers examined *Sophora* alkaloids in search of medicinally useful drugs from the plants and to understand the often toxic side effects. As is often the case with other groups of plants, the genus *Sophora* produced members which were beneficial, as well as, harmful to man.

Pharmacological Importance of the Alkaloids

The search for pharmacoactive compounds has led to the discovery of several potentially useful drugs. The search for the active constituents of the Chinese drugs "Tuh-seng," the dried root of *Sophora flavescens* (64,66) and "Shan-Dou-Gen" from *Sophora*

subprostrats (109) led directly to the isolation of matrine (26)* and matrine N-oxide (28). When matrine and matrine N-oxide were tested against Ehrlich ascites tumors and Sarcoma-180 tumors in mice, matrine and to a lesser degree its N-oxide *in vivo* and *in vitro* were effective against the Ehrlich ascites. Both compounds showed good activity against the Sarcoma-180 solid tumor in mice. The chemotherapeutic index of matrine N-oxide for Sarcoma-180 solid tumors was 7-8 times larger than that of mitamycin-C (110). A second group of compounds which is of pharmacological importance is the sparteine series also found in species of *Sophora*. Sparteine (12) as the sulfate salt has been employed as an oxytocic agent (116).

Additional investigations of the alkaloids of *Sophora* have been conducted in search of specific toxins. In Dzheiranchel, Russia, *Sophora alopecuroides* is considered to be a deadly plant poison when ingested (117). In the United States, *S. secundiflora*, the mescal bean, is considered toxic if one or more of the reddish-yellow seeds from the plant is chewed and ingested. Ingestion of the seeds results in symptoms similar to nicotine intoxication. The major constituents, cytisine (4) is absorbed through the oral cavity, mucous membrane and the gastrointestinal tract. Symptoms occur within one hour and may include nausea, vomiting, excessive thirst,

*The underlined number in parenthesis refers to the specific chemical structure shown in Figure 2.

increased body temperature, mydriasis, muscle fasciculation, convulsion, paralysis, coma and death due to respiratory failure (118). *Sophora secundiflora* is also reputed to have hallucinogenic properties.

The search for drugs which can induce "legal highs" has prompted a compendia of literature by both the lay individual and the scholar in recent years. Schultes (119) lists *S. secundiflora* seeds as a bean utilized by at least twelve tribes of Indians in Northern Mexico, New Mexico and Texas as part of a religious dance ceremony known as the Red Bean, Wichita, Deer or Whistle Dance. Ingestion of an infusion of the seeds elicited oracular, divinatory and hallucinogenic experiences in the participants. The high toxicity of the *S. secundiflora* seeds often resulted in death from overdoses and prompted the Indians to change to less toxic hallucinogenic agents, such as peyote. In an attempt to explain the hallucinogenic responses, Keller (104) examined *S. secundiflora* seeds and isolated sparteine, cytisine and N-methylcytisine (5). Izaddoost et al. (105) isolated two additional quinolizidine alkaloids, some amino acids and lipids from the seeds. They contended that the toxic activity of the seeds may be attributed in part to either the quinolizidine alkaloids, the amino acids or the synergic effect of both. Hatfield et al. (106) searched for hallucinogenic agents by spraying thin-layer chromatographs of the seed extract with an indole selective reagent, p-dimethylamino-benzaldehyde, since hallucinogenic compounds in general possess the

indole nucleus. No indole-like compounds were detected by the authors. This fact prompted their re-examination of the evidence for hallucinations attributed to *S. secundiflora*. The authors concluded that the responses could not be attributed to drugs in the mescal bean proper but may be caused by exhaustion, culturally based anxiety and self hypnosis experienced by the participants in the Red Bean Dance which resulted in hallucination. In essence, the individual saw what he was expected to see.

Two other authors reported rhombifoline (6), β -isosparteine (14), 11-allyl cytisine (9) and lupanine (19) (107) from young seeds of *S. secundiflora*.

Taxonomic Importance of the Alkaloids

The distribution of the quinolizidine alkaloids may be of taxonomic importance at the family level since they are restricted to the Papilionideae. Even in this subfamily, this group of alkaloids are encountered only in the three most primitive tribes of the Papilionoideae: the Sophoreae, Papilionoideae and Genisteae. The other seven tribes of the Papilionoideae are either alkaloid free or contain alkaloids of different chemical structures. Matrine alkaloids are restricted to the Sophoreae, the l-sparteine derivatives are encountered only in the Genisteae and the pentacyclic ormosia alkaloids are found in the Sophoreae (*Ormosia*) and in the Podalvrieae (*Pipthanthus*) (120).

The alkaloidal constituents of *Sophora* have been utilized to a limited degree at the generic and species levels. The main stumbling block has been the lack of chemical studies upon which to establish a solid foundation. Indeed, the analysis of a single species *S. alopecuroides* has transcended fifty years of chemical study resulting in the isolation of some 14 different compounds. Before taxonomic correlations may be meaningful, a total chemical survey of *Sophora* is necessary. In addition, an understanding of the diurnal, seasonal, physiological, populational and geographical variation of the alkaloids in *Sophora* as well as other closely related taxa is required.

One study attempted to utilize the alkaloid chemical constituents at the generic level. Izaddoost (121) divided the genus into plants without alkaloids (*S. japonica*, *S. affinis*), plants with matrine exclusively (*S. spyostrata*, *S. pachycarpa*, *S. angostifolia*, *S. lupinoides*), plants with cytisine and matrine (*S. japonica*, *S. allopecuroides*, *S. flavescens*, *S. microphylla*, *S. longesmem*, *S. tomentosa*, *S. tetraptera*, *S. macrocarpa*, *S. stenophylla*, *S. chrysophylla*) and plants with cytisine exclusively (*S. nuteliana*, *S. masafeurana*, *S. moorcroftiana*, *S. leachiana*, *S. mollis*, *S. arizonica*, *S. formosa*, *S. gypsophylla* and *S. secundiflora*). Izaddoost (121) concluded that the most primitive members of the genus were those members which contain the cytisine series exclusively, based on the observation "of the universal occurrences of cytisine within the tribe Sophoreae. Those species containing both the cytisine and matrine alkaloids form a second subgenera which may be considered more advanced."

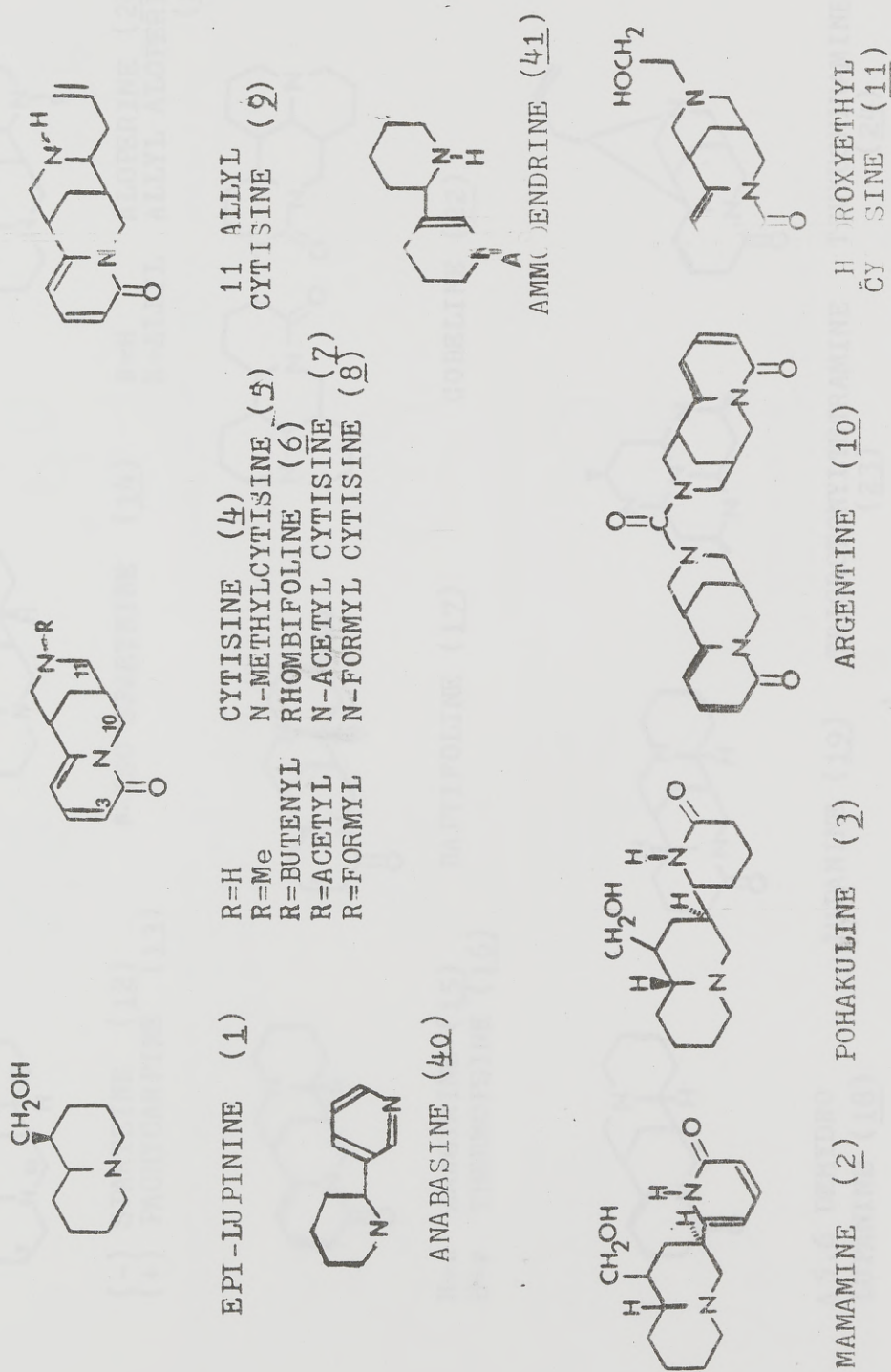
There are two problems in the analysis conducted by Izaddoost (105,121). The analytical techniques employed were not sensitive enough to detect alkaloids in two species of *Sophora* even though others have completely characterized alkaloids in *S. japonica* (78) and *S. griffithii* (73-77). Secondly, the method used by the author to differentiate the matrine series was by the use of the ferric chloride-hydrogen peroxide color test, also known as the Van der Moer test, which is selective for the pyridone ring found in cytisine, N-methylcytisine and anagyrine (15). A negative ferric chloride-hydrogen peroxide test, but a positive Dragendorff test, places the plant into the matrine series. The presence of sparteine, lupanine, Δ 5,6-dehydrolupanine (18), epi-lupanine (1) and β -isosparteine, in the plant would give a positive matrine spot from the typical cytisine-anagyrine-like compounds. In addition, matrine alkaloids such as sophoramine (38), also contain a pyridone ring and may react with ferric chloride-hydrogen peroxide. In conclusion, the survey of Izaddoost is a start in the right direction, but requires more detailed planning to make it a viable, useful approach. For these reasons, the survey of Izaddoost was not included in Table 1.

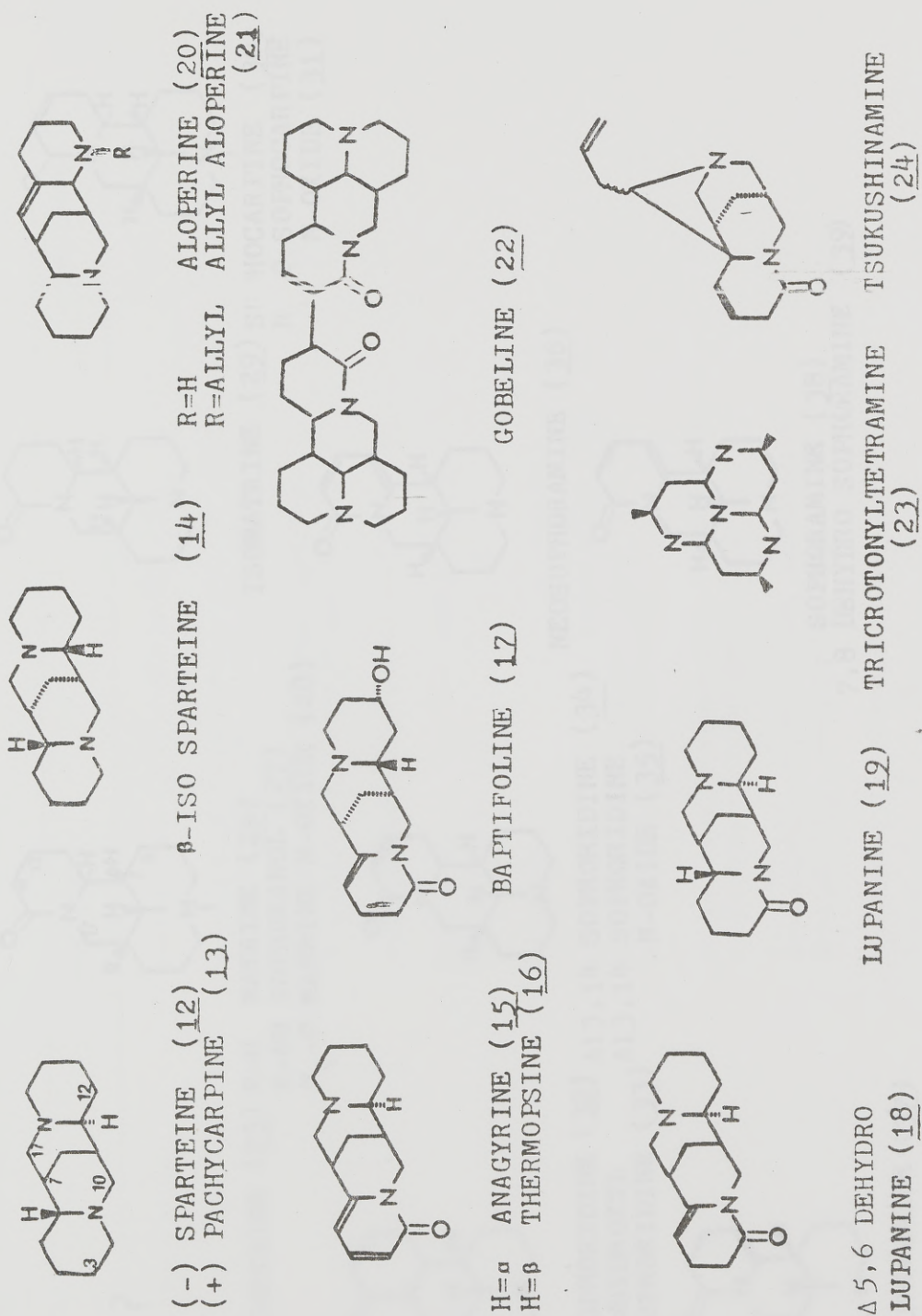
Briggs and co-workers found that the difference in alkaloid composition in chemical components could distinguish two different species of *Sophora* in New Zealand. The seeds of *S. microphylla* contain N-methylcytisine, cytisine, matrine, sophochrysine (25) and an unknown base (84) whereas the alkaloids of the seed of *S. tetraptera* contained matrine, N-methylcytisine and an alkaloid tentatively

identified as sophoramine (111). Once Briggs and co-workers had established the chemical identity of *S. microphylla* and *S. tetraptera*, they utilized a chemo-taxonomic approach to taxonomically differentiate the endemic island species of *Sophora*. *Sophora chrysophylla* is endemic to Hawaii resembling the New Zealand species, *S. microphylla*.

matrine and sophochrysine suggested to the authors that this species was unrelated to *S. microphylla* and, therefore, merited recognition as a new species (61). In a second situation of endemic species identity, a species of *Sophora* from Chatham Island believed to be either a separate species (*S. chathamica*) or a variety of *S. microphylla* was examined. The similarity in the alkaloid content of matrine, N-methylcytisine and sophochrysine supported the view that the island plant was identical with *S. microphylla* (60). In a third situation, plants which occur in a restricted area around Anawhata, New Zealand and believed to be either a variety of *S. microphylla* or a new species were examined. The new plant had a composition of 71% N-methylcytisine, 7% cytisine and 3% matrine but no detectable sophochrysine which suggested to the authors that the plant was indeed a new species (85).

Using an approach similar to Briggs and co-workers, Urzua and Cassels (112) attempted to identify species of *Sophora tetraptera* sensu Reiche from Chile with *S. tetraptera* from New Zealand. The fact that cytisine occurred in large quantities in the Chilean species favored segregation of the Chilean and New Zealand species of *Sophora*.

Figure 1. Epi-lupinine, Cytisine and Related Types of Alkaloids found in *S. tora*.

Figure 2. Sparteine, Aloperine and Other Types of Alkaloids Found in *Sophora*.

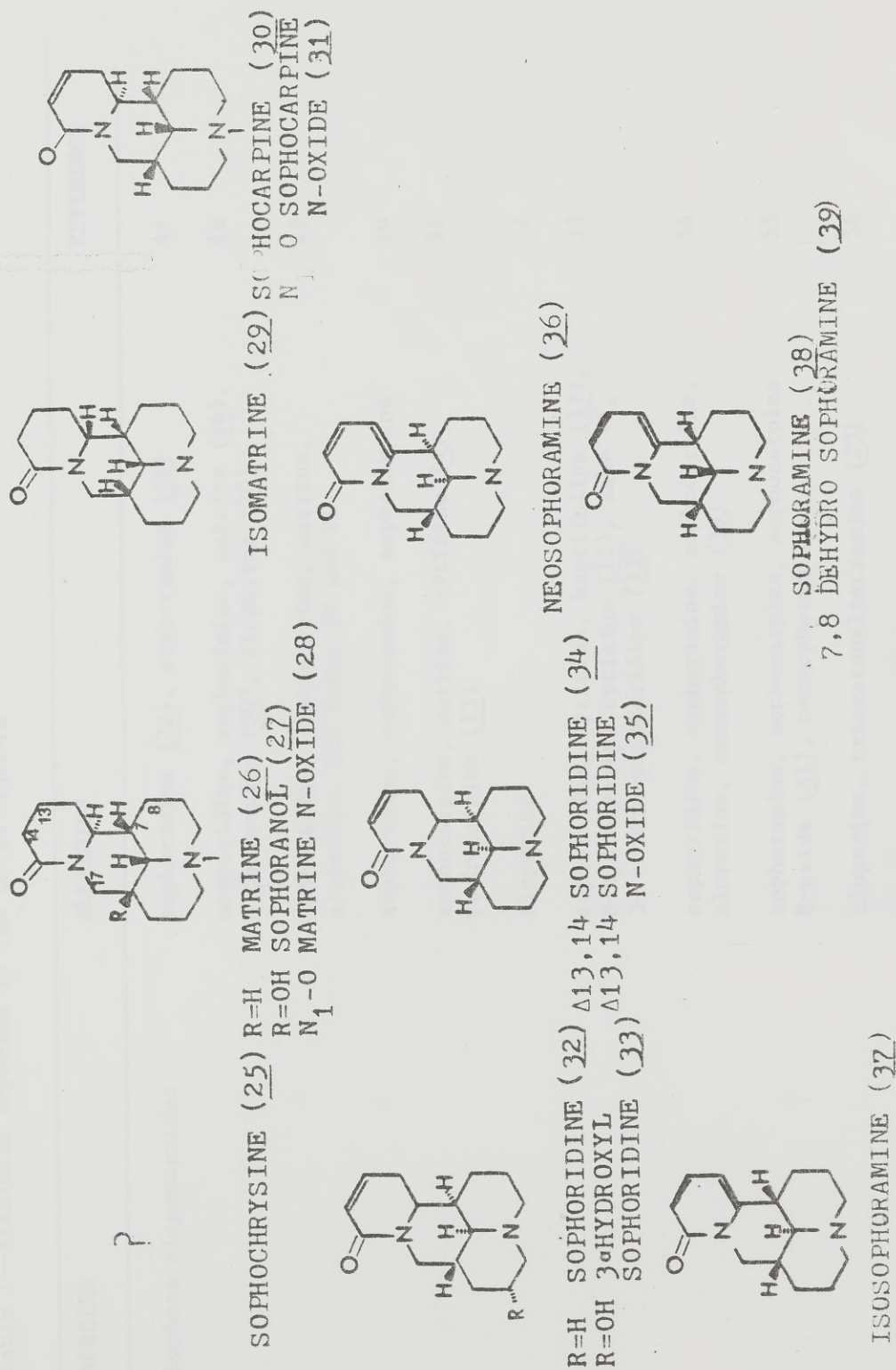


Figure 3. Matrine Types of Alkaloids Found in Sophora.

Table 1--Alkaloids Reported in the Genus *Sophora*

SPECIES	ALKALOIDS	REFERENCE
<i>Sophora alopecuroides</i>	sophoridine (32), sophoramine (38)	47
	sophoridine, sophoramine, matrine (26), sophocarpine (30), aloperine (20)	48
	sophoridine, sophocarpine, matrine, aloperine, New Bases IV and VI	49
	sophoridine, sophoramine, sophocarpine	50
	sophocarpine, matrine, cytosine (4), pachycarpine (13)	51
	aloperine	52
	sophoridine, cytosine, baptifoline (17), N-hydroxyethyl cytosine (11), Base VI, 3- -hydroxysophoridine (33)	53
	sophoridine, sophoramine, sophocarpine, aloperine, neosophoramine (36)	54
	sophoramine, sophocarpine, sophocarpine N-oxide (31), neosophoramine	55
	aloperine, tricrotonyltetramine (23)	56
	aloperine, N-allyl aloperine (21)	57

Table 1--Alkaloids Reported in the Genus *Sophora*

SPECIES	ALKALOIDS	REFERENCE
<i>S. alopecuroides</i> (cont.)	13,14 dehydrosophoridine N-oxide (<u>35</u>)	58
	13,14 dehydrosophoridine (<u>34</u>)	59
<i>S. chathamica</i>	matrine, N-methylcytisine (<u>5</u>), sophochryrine (<u>25</u>)	60
<i>S. chrysophylla</i>	cytisine, anagryne (<u>15</u>), sophochryrine	61
	cytisine, matrine, maminine (<u>2</u>), pohakuline (<u>3</u>)	62
<i>S. denudata</i>	cytisine, N-methylcytisine (<u>5</u>), matrine, matrine N-oxide (<u>28</u>), baptifoline, anagryne	63
<i>S. flavescens</i>	matrine	64
	matrine	65
	matrine N-oxide	66
	matrine, matrine N-oxide, baptifoline, sophoranol (<u>27</u>), anagryne, N-methylcytisine	67

Table 1--Alkaloids Reported in the Genus *Sophora*

SPECIES	ALKALOIDS	REFERENCE
<i>S. flavescens</i> (cont.)	matrine, matrine N-oxide, baptifoline, sophoranol, anagyrene, N-methylcytisine, sophocarpine	68
	isomatrine (29)	69
	sophocarpine N-oxide, 7,8 dehydro- sophoramine (39)	70
	sophocarpine isomer	71
	cytisine, rhombifoline (6), N-formyl cytisine (8), anagyrene, baptifoline, ammodendrine (42), tsukushinamine (24)	72
<i>S. franchetiana</i>	cytisine, pachycarpine	73
	cytisine, pachycarpine	74
	cytisine, pachycarpine, matrine, sophoramine, N-methylcytisine, Alkaloid A	75
	cytisine, N-methylcytisine, matrine, unknown alkaloid	76
<i>S. griffithii</i>	cytisine, N-methylcytisine, matrine,	77

Table 1--Alkaloids Reported in the Genus *Sophora*

SPECIES	ALKALOIDS	REFERENCE
<i>S. griffithii</i> (cont.)	argentine (10), sophocarpine	77
<i>S. japonica</i>	cytisine, N-methylcytisine, matrine sophocarpine, 4 unknown alkaloids	78
<i>S. linearifolia</i>	pachycarpine	79
	pachycarpine, matrine, matrine N-oxide	80
<i>S. lupinoides</i>	matrine N-oxide, sparteine (12), sophocarpine	81
<i>S. macrocarpa</i>	matrine	82
	matrine, baptifoline	83
<i>S. microphylla</i>	cytisine, N-methylcytisine, matrine, sophochryesine, unknown base	84
	cytisine, N-methylcytisine, matrine	85
	matrine, anagyrine, cytisine, sophochryesine, N-methylcytisine	86

Table 1--Alkaloids Reported in the Genus *Sophora*

SPECIES	ALKALOIDS	REFERENCE
<i>S. moorcroftiana</i>	matrine, matrine N-oxide, sophocarpine	87
<i>S. pachycarpa</i>	pachycarpine	88
	sophocarpine, sophocarpidine=matrine	89
	sophocarpidine=matrine, pachycarpine, sophocarpine	90
	sophocarpine, sophoramine, matrine	91
	pachycarpidine=matrine N-oxide, pachycarpine	92
	goebeline (22), matrine, sophocarpine, pachycarpine, compound I	93
	pachycarpine, sophocarpine, matrine N-oxide	94
	pachycarpine, isosophoramine (37)	95
	goebeline, pachycarpine, matrine, anabesine (40), sophocarpine	96
	goebeline	97
	cytisine, matrine N-oxide	98

Table 1--Alkaloids Reported in the Genus *Sophora*

SPECIES	ALKALOIDS	REFERENCE
<i>S. pachycarpa</i> (cont)	sophocarpine, matrine N-oxide, matrine sophoramine	99
	pachycarpine	100
	pachycarpine	101
<i>S. prodan<i>i</i></i>	cytisine	102
	sparteine	103
<i>S. secundiflora</i>	cytisine, N-methylcytisine, sparteine	104
	cytisine, N-methylcytisine, anagyrine, thermopsine (16)	105
	cytisine, N-methylcytisine, sparteine, anagyrine, epi-lupanine (1), Δ 5,6 dehydro- lupanine (18)	106
	rhombifoline, 11-allyl cytisine (9), β -isosparteine (14), lupanine (19)	107
<i>S. spyostriata</i>	matrine N-oxide, matrine	108

Table 1--Alkaloids Reported in the Genus *Sophora*

SPECIES	ALKALOIDS[	REFERENCE
<i>S. subprostrata</i>	N-methylcytisine, anagyrine, matrine, matrine N-oxide	109
	matrine, matrine N-oxide	110
<i>S. tetraptera</i>	N-methylcytisine, matrine, unknown base	111
	cytisine, N-methylcytisine, matrine, unknown alkaloid	112
<i>S. tomentosa</i>	cytisine, N-methylcytisine, matrine unknown alkaloid	113
	cytisine, N-methylcytisine, matrine, matrine N-oxide, baptifoline, N-acetyl cytisine (7), anagyrine	114
	N-acetyl cytisine	115

EXPERIMENTAL

Source of Plant Material

The primary plant material used for this investigation consisted of normal and fasciated stems from *Sophora secundiflora* (Gomez Ortega) Lagasca ex DC collected on the campus of the University of Texas at Austin, Texas. The plants were growing adjacent to several dormitories on the campus and were presumed planted as ornamental plants. Seeds from *S. secundiflora* were also collected from seed pods found on Mount Bonnell in northwest Austin, Travis County, Texas. These collections were made by Dr. Gerald Sullivan and Pedro Chavez.

Collections of *Sophora tomentosa* L. subsp. *occidentalis* (L.) Brummitt were obtained by Dr. Gerald Sullivan from an area adjacent to the Marine Science Institute, Port Aransas, Nueces County, Texas. The woody shrubs were collected in the fall of 1978. Voucher specimens are on file for both *S. secundiflora* and *S. tomentosa* at the Department of Botany Herbarium, the University of Texas at Austin.

Preparation of the Plant Material

The aerial portions of *S. secundiflora* were allowed to dry at room temperature for a period of at least two months; with repeated turnings. Seeds from *S. secundiflora* used for extraction and for identification of the individual alkaloids were obtained by

mechanically crushing the outer seed pod with pliers. The shell material was discarded leaving reddish-yellow seeds. The seed material was extracted without further drying since the seeds were encased in a hard seed coat which resisted drying.

The aerial portions of *S. tomentosa* were placed into a drying oven set at 40° and dried approximately two months. After that time, the material was brittle and could be easily manipulated.

After drying, the crude plant material was powdered. In the case of the stems, the brittle leaves and petioles were manually removed and discarded; the stems were then cut into approximately 1 cm pieces using small pruning shears. The cut pieces were further ground in an Arthur A. Thomas mill to a 20 mesh consistency. After grinding, 144.4 g of fasciated stems of *S. secundiflora* were obtained. The normal stems of the plant yielded only 80.0 g after grinding. The seed material was also reduced to a fine 20 mesh powder in the same mill but only after the seeds were broken mechanically. This breakage was accomplished by placing the shelled seeds within a cotton towel and striking with a hammer. The seed fragments were subsequently ground in the mill to a 20 mesh powder.

The fasciated stems of *S. tomentosa* presented a slightly different problem since only 14.5 g of fasciated plant stem was obtained after grinding. The normal stem yielded 20 g of ground material. The stem material from *S. tomentosa* was obtained from a number of plants and combined to yield the total weight for the semi-quantitative comparison. Unlike *S. secundiflora*, few seeds

were available of *S. tomentosa* and the remaining stem parts (i.e., stems, leaves, etc.) was also ground to a 20 mesh powder resulting in a total yield of 100 g of plant material. This aerial portion was stored and utilized in a later study to assist in the identification of the alkaloids in *S. tomentosa*.

Extraction of the Plant Material of *S. secundiflora*

The processing of the plant material should be a selective, controlled procedure which insures total extraction of all potential drugs. Therefore, a traditional multi-solvent extraction of the material was performed initially. The plant material was weighed and introduced directly into a Soxhlet apparatus cellulose extraction thimble. This thimble was placed into a continuously recycling Soxhlet apparatus and extracted with a series of increasingly polar solvents for 72-80 hours. This sequence included petroleum ether, diethyl ether, chloroform and methanol, in that order.

Extraction Method A: after the first solvent extraction with petroleum ether, the marc was dried, weighed and extracted with diethyl ether for 72-80 hours. The marc was again dried after the second extraction and subsequently just wetted with an alkaline solution of 95% ethanol-diethyl ether-28% ammonium hydroxide (40:80:32) and allowed to air-dry overnight. The basification of the marc converted bases to free bases which allowed extraction with an organic solvent. The basic marc was extracted with chloroform for 72-80 hours. Finally, the marc was dried, weighed and extracted with methanol.

The solvents were removed by air evaporation or by rotary evaporation under vacuum. In each instance a gummy residue remained which was subsequently weighed and placed in a refrigerator. In order to determine whether the alkaloids would be extracted in the petroleum ether, diethyl ether, chloroform or methanol 25 g of fasciated and 25 g of normal stems of *S. secundiflora* were extracted in the same manner. These crude extracts were spotted on a silica gel tlc plate and developed in a solvent system of chloroform-methanol (1:5). After drying and spraying with Dragendorff reagent, reddish-orange spots were noted in the chloroform and methanol extracts. The petroleum ether and the diethyl ether had no or very weak positive spots. The alkaloids therefore were localized in the more polar solvents.

Extraction Method B: for the semi-quantitative study and in instances where only a small amount of material was available, 3-20 g of either *S. secundiflora* or *S. tomentosa* were first basified with a solution of 95% ethanol-diethyl ether-28% ammonium hydroxide (40:80:32) and air dried for 5-20 hours. The plant material was subsequently introduced into a cellulose thimble, placed into a Soxhlet extraction apparatus and continuously extracted with methanol for 24 hours. The methanol was subsequently removed by rotary evaporation.

Purification of the Alkaloid Fraction

Traditionally, alkaloids have been purified by partitioning the plant extract between an acid solution such as sulfuric acid and an organic solvent and discarding the organic solvent. The acid solution is subsequently made basic with base to pH 10-11 and partitioned again with an organic solvent. The alkaloid bases will be retained in the organic layer.

The chloroform extracts from the 25 g extracted above resulted in 0.78 g of chloroform extract of normal stem and 0.91 g of chloroform extract of fasciated stem. These extracts were dissolved in 25 ml of 0.5 N sulfuric acid and partitioned with three 25 ml portions of chloroform. The aqueous layer was subsequently emptied into a beaker and the pH adjusted to 10-11 with 28% ammonium hydroxide as indicated by pH paper. The basic solution was reintroduced into the separatory funnel and partitioned a total of 3 times with 25 ml portions of chloroform. The extracts were combined and the solvent removed by rotary evaporation. The resulting extract resulted in 0.38 g of fasciated stem and 0.30 g of normal stem extracts. The extraction scheme is depicted in Figure 4.

Each of the extracts was placed into a 25 ml volumetric flask and brought to volume with chloroform. From this 25 ml solution, 5 μ l was spotted on tlc plates and developed with a variety of solvents so that a chromatographic profile could be developed for alkaloids present in both fasciated and normal stems.

A comparison of the profiles of *S. secundiflora* indicated some quantitative and possibly qualitative differences existed between the fasciated and the normal stems. The postulation that quantitative or qualitative differences do occur between fasciated and normal material was strongly supported by visually comparing the intensity of these reddish-orange spots on the tlc plate. A gc profile was also obtained by injection of 1.8 ul of each of these extracts using gc System A. This gc System is described on page 58 and the results are presented in Figure 5.

Thin-Layer Chromatography

Several investigators have reported successful tlc separation of quinolizidine alkaloids utilizing a variety of polar and non-polar solvents (57,105,106,122,123). For our general purposes these reported systems were satisfactory to determine the number of alkaloids in a plant, or to check the purity of a particular fraction. The adsorbent of choice was silica gel G on glass plates. These were obtained commercially from Analteck, Inc. as 0.25 mm coated Uniplates with or without fluorescent indicators.

In most instances, the plates were marked 1.5 cm from the bottom of the plate with a row of evenly spaced dots made with a dissecting needle. Ten cm up from this row of dots a line was inscribed into the silica. The normal procedure of spotting samples on the plate involved the one to one spotting of sample material on

each of the dots at the origin. Once spotted, the plate was immersed into the appropriate solvent system.

Solvent System

Three solvent systems were especially useful for the separation form-methanol (4:1), solvent system B, diethylamine-cyclohexane (3:7) and solvent system C, chloroform-methanol-28% ammonium hydroxide (90:9:1). The developed plates were air dried for 10-20 min and then sprayed with Dragendorff reagent. Due to an apparent interference between diethylamine and the Dragendorff reagent, the plates developed in the diethylamine system were allowed to dry for 18 hours before being sprayed for the detection of the alkaloids.

Detection of the Alkaloids

The Dragendorff reagent used was prepared according to Stahl (122). A stock solution was prepared by boiling 10.4 g bismuth subcarbonate and 20 g sodium iodide for a few minutes with 100 ml glacial acetic acid. After 2 hours, the precipitate was removed using a sintered glass funnel. To 80 ml of the clear, red brown filtrate was added 32 ml ethyl acetate; the stock solution was stored in a brown bottle. For spraying, 10 ml of the stock solution was mixed with 25 ml glacial acetic acid and 60 ml ethyl acetate. This solution was placed into an atomizer and sprayed directly on the plates. A reddish-orange spot was considered a positive indication for alkaloids.

A second spray reagent was made according to Izzadoost et al. (105) and was used to detect compounds with a pyridone nucleus. This reagent was prepared as a 1% ferric chloride solution in chloroform-acetone (4:1). Spraying of this solution on the plate resulted in the appropriate compound developing a reddish-orange coloration on a yellow background. After drying, the plates were sprayed with 5% hydrogen peroxide solution. A color change from reddish-orange to blue indicated that these compounds possessed the pyridone ring as part of their structure.

Attempted Purification of Major Constitutents

Since the amount of fasciated and non-fasciated material was limited and since the major constitutents appeared to be present also in the seed, initial experimentation was conducted with the seeds of *S. secundiflora*. A tlc of the chloroform extract of *S. secundiflora* indicated that there was one major constituent in the extract of both fasciated and normal stems, as well as the seeds. This compound constituted over 60% of the alkaloid extract. At times the entire chloroform extract developed octagonal white crystalline plates which coated the vessel when the solvent was removed. Since this crude extract behaved as if it were a pure compound with minor "impurities," attempts were made to purify this compound by recrystallization of the entire alkaloid extract. Several solvents were tried in hopes of crystallizing the main compound from solution. These solvents included hexane, cyclohexane, petroleum ether, chloroform,

dichlormethane, acetone and methanol. Crystals could be obtained from cyclohexane and acetone; the better crystals were obtained with cyclohexane.

To obtain crystals, cyclohexane was added to the crude material in an Erlenmeyer flask and heated over a steam bath. The material was not totally soluble in cyclohexane even after extensive heating. When the solvent reached a rolling boil, the entire solution was filtered through filter paper into a second heated flask. The hot solution in the second flask was allowed to cool to room temperature. Upon standing for a period of 1 to 2 days, white needles formed in the cooled solution. These crystals were filtered from the solution by gravity filtration, washed with cyclohexane and air dried on filter paper. The resulting crystals were recrystallized a total of 3 times with the hot cyclohexane procedure. After the last recrystallization, a solution of the crystals was spotted on tlc and also injected into the gc using gc system A. Both methods indicated the presence of a single compound.

A second manner of purifying this compound after the first recrystallization with cyclohexane as described above was by sublimation. Approximately 100 mg of the material was dissolved in a minimum amount of methanol. The solution was coated on approximately 10 g celite and the celite allowed to dry into a powder overnight. Portions of the dried powder were inserted into the closed end of a sublimation tube. A vacuum pump hose was placed at the open end of the tube and the tube inserted into a thermal gradient sublimation

system. The vacuum was carefully applied and heat was increased gradually to 110° over a period of 4 hours. White crystalline needles formed on the walls of the glass tube. When the maximum amount of sublimation had occurred, the tube was removed from the instrument, allowed to air cool and cut with a triangular file at points near the crystals. The crystals were subsequently scraped from the tube walls with a spatula and placed in a vial. Examination of a solution of the crystals by tlc and gc proved that the crystals were chromatographically pure. This compound was designated as compound 1.

Material insoluble in hot cyclohexane and all washes recovered from the recrystallization of the above compound (compound 1) were combined. Examination of this combined fraction by tlc in solvent system A revealed the presence of at least 10 Dragendorff positive compounds; compound 1 still constituted over half of this fraction. At this point, experiments were performed to determine if selective fractions could be obtained by partitioning the plant extracts between chloroform and various pH buffers.

Experimentation With Different pH Systems

An examination of traditional purification procedures for alkaloids revealed that the alkaloids are first partitioned between an acidic aqueous media and an immiscible organic solvent, usually chloroform. The aqueous layer which contained the salts of the alkaloids was subsequently made basic to a pH of 10-11 and partitioned a second

time with chloroform. The free bases are extracted in the organic layer (2,124). Two references had suggested that the quinolizidine alkaloids could be selectively fractionated due to their differences in ionization by partitioning with different pH buffers and organic solvents (53,125).

Experimentation with different pH system was therefore a feasible way of fractionating alkaloids. The goal of the experiment was to find a quick and simple system to remove the major compound from the extract and to fractionate the plant alkaloids into distinct groups of compounds.

The initial experiment to fractionate the alkaloids into different components by pH variation was performed by crudely increasing the pH of the acidic aqueous media with base. The purified alkaloid extract was first dissolved in 0.5N sulfuric acid (final pH 1.56) and base, 10% ammonium hydroxide, added until a pH of 2.3 was indicated on a pH meter. At this pH, the aqueous liquid was introduced into a separatory funnel and partitioned three times with chloroform. The aqueous fraction was reintroduced into a beaker and the pH adjusted to a pH of 3 with base. The partitioning of the plant material was repeated. This procedure was repeated for pH of 4, 6 and 10.5. The chloroform from each of the pH partitions was dried with magnesium sulfate, filtered and removed by rotary evaporation. The resulting yellowish syrups were examined by tlc and later by gc. The tlc of the material indicated that the pH 4 and pH 6 materials contained basically the same compounds. The pH 2.3 and

pH 3 extracts contained some of the compound present in pH 4 and 6 extracts but also several different compounds. Unexpectedly, the pH 10.5 partition contained the major constituent, compound 1, plus some minor compounds. The critical points of the fractionation experiment were therefore at pH 3, 6 and 11 since partitioning with chloroform at these pHs resulted in a definite fractionation of the alkaloids. The chloroform extract at pH 6 of the purified extract essentially removed the major compound from the other 9 compounds.

This experimentation with different buffer and pH systems resulted in a separation of the less basic alkaloids in a single step. The entire crude extract was rigorously shaken and dissolved in a mixture of 50 ml chloroform and 50 ml pH 6 buffer (0.2 M sodium phosphate monobasic/0.2 M sodium phosphate dibasic). The entire mixture was then introduced into a separatory funnel, allowed to separate and the aqueous layer extracted a total of three times with chloroform. The chloroform solution was dried over magnesium sulfate, filtered and the solvent was allowed to evaporate. This dark yellow material was labeled the pH 6 extract. A visual comparison between the pH 6 extract and the original extract can be made by comparing Figure 5 of the original fasciated and non-fasciated purified alkaloid extracts and Figure 6 of the respective pH 6 extracts.

The aqueous buffer layer from the pH 6 extract was made basic with 28% ammonium hydroxide to a pH of 10-11 and extracted three times

with chloroform. The chloroform solution was dried, filtered and the solvent evaporated. This clear amorphous syrup was labeled the pH 11 extract. The extraction scheme for the pH partitioning and subsequent isolation of the compounds is presented in Figure 7.

Isolation of *S. secundiflora* fasciated stem

For purposes of identification of the alkaloids of *S. secundiflora*, 44.4 g of fasciated stems were extracted by extraction method A, resulting in 0.588 g of alkaloid extract which yielded 0.332 g in the pH 11 extract and 0.136 g in the pH 6 extract.

Isolation of Compounds from the pH 11 Extract

The pH 11 extract as indicated above contained the major compound (compound 1) plus minor quantities of three other alkaloids as evident from tlc data. In solvent system A, faint spots were evident at Rf 0.01, 0.3 and 0.5. In solvent B, however, Dragendorff positive spots were evident at Rf 0.89, 0.67, 0.3 and 0.01. The tlc data indicated that these compounds could be separated by preparative tlc plates in solvent system B.

Preparative plates for chromatography were prepared using silica gel impregnated with a fluorescence indicator, (Silica Gel PF₂₅₄, E. Merck AG). To 50 g of the white powder was added 110 ml of distilled water in a 500 ml Erlenmeyer flask. This mixture was stirred until a thick consistent slurry was obtained which was subsequently poured into a Desaga tlc spreader. The glass plates were covered to a thickness of 0.75 mm, allowed to air dry for at least

five hours and activated in an oven set at 110° for 2 hours. After cooling, the plates were spotted with the appropriate sample solution and developed in solvent system B. After air drying, the plates were visualized with a UV lamp, Mineral Light, at 254 nm. Dark bands were visible against a yellow green background. The dark bands of silica were scrapped from the plate at R_f 0.89, 0.67, 0.3 and 0.01 with a razor blade, placed in individual beakers with methanol and stirred rigorously. Each solution was filtered and the solvent removed by rotary evaporation. A yellow oil resulted after removal of the solvent.

The compound at R_f 0.3 gave a positive test with a ferric chloride spray and proved to be identical to compound 1 and was purified by sublimation. The oil resulting from the bands at 0.89 (compound 2) and 0.67 (compound 3) were further purified by preparative tlc using solvent system B. Compound 2 afforded 2 mg of a tlc pure material which could not be crystallized. A single peak was evident on gc. Compound 3 was further purified by preparative tlc and afforded 1 mg of a tlc pure oily material which showed a single peak on analytical gc. Neither compound 2 or 3 gave a positive ferric chloride test. The material at R_f 0.01 could not be removed from the silica with methanol, water or ethanol elution. No further attempts were made to identify the compound.

Isolation of Compounds from the pH 6 Extract

The pH 6 extract contained at least 8 Dragendorff positive compounds in the yellow syrup. A preparative method using column chromatography should separate these compounds. Since tlc system C separated the individual fractions into discrete spots, a column using chloroform, methanol and a small amount of base as a modifier would be a logical choice for a column system.

The column was prepared in the following manner: Silica Gel 60-200 mesh (Baker Biosil G) was weighed in a large beaker in an amount that equaled 100 times the weight of the crude material to be separated. To this beaker enough solvent was added of chloroform-28% ammonium hydroxide (500:1) to make a viscous slurry. This slurry was carefully placed in batches into a (2 cm id X 36 cm) glass chromatographic column. After repeated tapping of the column to remove air bubbles, the stopcock at the bottom of the column was opened to allow solvent to pour from the column. A layer of sand was placed atop the column. A solution of the extract was carefully placed on this sand and the stopcock opened to allow the sample to percolate to the surface of the silica. The material was eluted with chloroform - 28% ammonium hydroxide (500:1) for approximately 150 ml. The solvent was changed to chloroform-methanol - 28% ammonium hydroxide (100:4:.2) for 400 ml and then (10:1:.02) for 500 ml. The column was then stripped with methanol. One hundred-twenty fractions of 8-10 ml were collected with the use of a fixed volume collector (Model LVMI,

Gilson Medic). Selected fractions were concentrated and monitored by means of tlc and gc. Fractions with similar compounds were combined.

From this column two major compounds were isolated in fractions 31-40 and 61-68. The compound isolated from fractions 31-40 (compound 4) was rechromatographed on preparative plates using solvent system A until a single compound was evident on a tlc plate. Ten mg of an oil was obtained which was chromatographically pure. The compound isolated from fractions 61-68 (compound 5) proved to be chromatographically pure by tlc and gc. An attempt to sublime the material at a temperature of 126° afforded white needles.

All remaining fractions plus the methanol used to strip the column were combined and labeled fraction 6B. At this point, detection of the alkaloids was becoming a problem since there was such a small amount of material. Chromatography using preparative hplc which used a uv detector could possibly prove useful for the isolation of these alkaloids.

HPLC of Fraction 6B

The use of preparative high pressure liquid chromatography is usually prohibitive due to the high cost of solvents, instrumentation and packing material. In an attempt to circumvent these restrictions, hplc techniques were applied using normal silica gel (Bio sil) as the adsorbent. A mixture of chloroform-methanol (9:1) was used as the initial solvent and a flow rate of 2 ml/min was used for elution.

The column (8 mm X 500 mm) was first stoppered with the proper fitting at one end and packed with dry silica gel (Bio sil G) in small increments with continuous packing and tapping of the column. Care was taken so as not to allow formation of air pockets. The
The column was attached to the hplc instrument and eluted with solvent for 10 hours.

Semi-preparative high pressure liquid chromatography (hplc) was achieved with a Tracor HPLC with a Tracor model 950 pump and a 670 A variable-wave length detector set at 310 nm. The unit was fitted with Rheodyne Model 7125 injector with a 500 ul fixed volume loop.

By use of this procedure, one fraction initially appeared to be chromatographically pure by tlc. An examination of the material using gc and gc-ms, revealed the presence of two compounds in roughly a 10:1 ratio. The main compound was isolated using preparative tlc with solvent system C and labeled compound 6. The examination of this compound using tlc and gc-ms revealed this compound to be pure. The second compound in this mixture, compound 7, was isolated contaminated with compound 6. The identity of compound 7 was achieved by synthesis of the compound and comparison of gc-ms, tlc and other properties with the natural alkaloid.

Other fractions from the hplc were combined into fraction gc. This fraction, a total of 10 mg, contained a mixture of three compounds, labeled compounds 8, 9, and 10. These compounds had Rfs

of 0.57, 0.55 and 0.76, respectively, in solvent system C. The compound with R_f 0.55 gave a positive ferric chloride reaction indicating the presence of a pyridone ring on the molecule. These three compounds could not be resolved by hplc and the separation and identity of these compounds would have to be achieved by gc-ms. The total scheme for the resolution of the 10 compounds of *S. secundiflora* is depicted in Figure .

Gas Chromatography of Quinolizidine Alkaloids

The development of a gc-ms system for the analysis of quinolizidine alkaloids is important for this research since the technique is critical for the analysis of small quantities of alkaloids. The major stumbling block encountered with this research was the limited amount of plant material available for analysis. To adequately resolve the problem, quantitative and qualitative analysis had to be accomplished on 1-5 g of plant material. After extraction of this plant material, mere milligrams of total extract were left to analyze.

The use of gas liquid chromatography for the separation and identification of quinolizidine alkaloids has facilitated the analysis of complex mixtures of these compounds. The first separation system utilized isothermic gc conditions. Lloyds et al. (126) used a 2-3% SE-30 on Chromosorb W at a temperature of 204° to separate 10 quinolizidine alkaloids. Cranmer (125) and Cranmer and Mabry (127) used 3% SE 60 on

Chromosorb W at 220° and a 5% DC 560 on Chromosorb W at 223°. The time required for the analysis by these earlier systems was prohibitive and required modification.

The use of temperature programmed systems for the analysis of the quinolizidine alkaloids was developed by Cho and Martin. Cho and Martin (128) used a 10% QF 1 on Chromosorb W and a temperature program from 140° to 230° at a rate of 2° per minute after an initial delay of 5 minutes at the first temperature. These investigators (128) used gc in both a quantitative and an analytical capacity. Fractions obtained from a gc preparative column were also spotted on tlc, analyzed by ms and compared with reference compounds. By use of this combined technique of preparative gc, ms and tlc, these same investigators (128) were able to obtain the "resolution and unambiguous identification of microgram amounts of 22 lupin alkaloids." A second system using temperature programs for quinolizidine alkaloids was developed by Hatfield et al. (106) and Keller (107). These investigators used a 3% OV-17 column on Gas Chrom Q and a temperature program from 125° to 265° at a rate of 5° per minute. The development of direct gc-ms analysis on an analytical column was first used by these investigators in the identification of quinolizidine alkaloids.

GC Column Packing Material

In considering a suitable packing for gc, factors such as flow rate, temperature of the column and time of analysis were considered important. Also important is instrumentation. Limitation of

an instrument were imposed by the single availability of a Varian Aerigraph GC which could only be maintained at an isothermal mode. After experimentation with several packings, temperatures and flow rates, a 3% QF 1 Column on Gas Chrom Q at 220° with a flow rate of 40 ml/min was used for the analysis of the alkaloids. Injection of the alkaloids of *S. secundiflora* resulted in retention times which ranged from 1 second to 120 minutes. The time requirement for each sample made this system unusable.

The QF-1 column could separate key compounds in this researcher's mixture. A temperature program, however was believed necessary to separate the compounds. The availability of a Finnigan GC which could be programmable facilitated the analysis of the compounds. After experimentation with the instrumentation, a temperature program was established from 140° to 230° at a rate of 5° per minute. The temperature was held at the initial temperature for 2 minutes and at the later temperature for 10 minutes. The injector temperature was set at 250° and the detector at 270°. The flow rate established was 40 ml helium/min. The column was preconditioned at 230° before use for 48 hours. This procedure was designated as gc system A.

A second system which was also useful for the analysis of the alkaloids of *Sophora* was a 3% OV-17 on Chrombsorb WHP. The temperature program for this system was from 140° to 265° at 4° per minute. The flow rate, injector and detector temperature was 40 ml nitrogen/min, 270° and 290° respectively. This procedure was designated as gc system B. The column was preconditioned at 270° before

use. The columns were attached to a Finnigan Model 9610 gc equipped with a fid detector.

Analysis of Fraction 6C

Fraction 6c was analyzed using gc System A and B and comparisons of gc retention times and ms fragmentation patterns were made with literature citations and authentic compounds. Fraction 6c was also subjected to tlc systems A, B, and C and compared to authentic compounds when available.

Semi-Quantitative Analysis

Two studies were conducted in order to compare quantitatively the fasciated and non-fasciated stem material. The first study involved only *S. secundiflora* plant material. The study utilized the same chloroform extract from extraction method A which was used initially for the quantitative study on tlc. The alkaloid extract from the fasciated stem and that from the normal stem were processed to obtain the corresponding alkaloid extracts. The extracts were introduced into a 25 ml volumetric flask and 1.8 ul of each solution injected into the gc system A. The resulting chromatographs are presented in Figure 5. A semi-quantitative analysis was conducted on this fraction using a comparison of peak heights.

Since in this analysis it appeared that some of the compounds with longer retention times were missing from the fasciated stems, a second analysis was conducted on this same extract. The solvent was removed from 20 ml of both the fasciated and the non-fasciated

stem resulting in a residue. This residue was partitioned between 25 ml of pH 6 phosphate buffer and chloroform. After removal of the chloroform, the chloroform residue was introduced into a 5 ml volumetric flask and 1.8 ul of the solution subjected to gc system A. The results are shown on Figure 7.

The second study utilized plant material from both *S. secundiflora* and *S. tomentosa*. The purpose of this semi-quantitative study was to compare the alkaloid constituents of the same plant part (stems) under the same conditions. To accomplish this study, 10.0 g of ground plant material was extracted in a soxhlet extraction apparatus after basification with a solution (5 ml) of 95% ethanol-diethyl ether - 28% ammonium hydroxide (40:80:32). To guarantee that the extraction was as complete as possible within 24 hours, a large volume of methanol was used in the distillation flask relative to the sample chamber. The net result was a turn over rate of 3-4 times per hour. After extraction, the methanol was processed in the usual manner to obtain the crude alkaloid fraction.

The crude alkaloid fraction was utilized directly for this study. The crude fraction was first transferred into a 25 ml volumetric flask. From this solution was obtained 100 ul by use of a fixed volume pipette (Precision Pipettes, Medical Laboratory Automation, Inc., Mount Vernon, N.Y.). This 100 ul sample plus 25 ul of the internal standard oxosparteine (Light and Co., Ltd., Colnbrook, England) was placed into a conical shaped vial and shaken vigorously. Two to 4 ul of this solution was injected into the Finnigan GC-MS and

the area under the curve tabulated automatically for the different constituents. The reported values are percent of the internal standard.

The components of the alkaloid fractions were analyzed by *gas chromatography* (gc-ms) on a Finnigan 4023 Automated GC-MS equipped with an Incos Data System. The effluent from the gc system A or B entered the mass spectrometer through a glass jet separator maintained at 250°. The ion source temperature was 200° and the ionizing voltage was 70 ev. Spectra were recorded every two seconds and numbered above the peak.

Examination of *S. tomentosa*

After air drying and processing the fasciated stems of *S. tomentosa*, a total of 14.5 g of powdered plant material was available for analysis. The non-fasciated stem tips amounted to 10 g after processing. Since the amount of plant material was miniscule, a slightly different approach was undertaken with these alkaloids. The approach believed to be better was to use the fasciated material for the semi-quantitative study. For the identification of the alkaloids, the remaining aerial portion of the plants were extracted and processed in the usual manner. Attempts to partition the alkaloids using different pH systems were not successful. The crude alkaloid extract was chromatographed on tlc and showed one major spot at Rf 0.83 in solvent system B and 0.64 in solvent system C. The

major compound did not give a positive Van der Moer reaction and therefore did not possess a pyridone ring in the molecule.

Column chromatography was attempted in order to isolate the compound. This resulted in a chromatographically pure compound which could not be crystallized from a variety of solvents and solvent mixtures. The material was however crystallized using gradient sublimation at a temperature of 90° and labeled compound 11.

Other fractions from the column were injected into the gc-ms and compared to standard compounds. The compounds gave characteristic gc retention times and mass spectral fragmentation patterns which were sufficient to identify the individual chemical components. One compound (compound 12), however, could not be unequivocally identified by this method and was not pursued further.

Reference Compounds

The limited amounts of starting plant material resulted in relatively small amounts of crude alkaloid extract and even smaller amounts of purified compounds. For this analysis, reference compounds were necessary for co-tlc and gc comparisons of the alkaloids. These were obtained from different sources. Cytisine was obtained from the seeds using the procedure outlined by Keller (104). N-methylcytisine, sparteine, oxosparteine, lupanine and lupinine were supplied by Dr. Tom Mabry which were obtained commercially from Light and Co., Ltd., England. Dr. William Keller supplied a sample of 11 allylcytisine as well as gc retention data concerning Δ^5 , 6-dehydrolupanine,

rhombifoline, B-isosparteine and anagyrine. The gc-ms of these standard compounds was performed using a Finnigan GC attached to a Finnigan Mass Spectrometer.

Instrumentation

The following instruments were utilized for this analysis: uv, Perkin Elmer Double Beam Spectrophotometer; ir, Beckman IR 5A and 33 Double Beam Spectrophotometer; nmr, Varian A-60 60 megacycle instrument with and without FT cross correlation; mp, Thomas Hoover Uni-melt Capillary Melting Point Apparatus and a Fisher John melting stage.

Synthesis of Cytisine Derivatives

Synthesis of N-methylcytisine from cytisine.--For compound 4, 100 mg of cytisine was dissolved in 5 ml anhydrous methanol in a 25 ml round bottom flask and stirred with a magnetic stirrer. The flask was maintained at 0° by partial immersion in a beaker with ice. To this solution was added 0.2 ml formaldehyde solution and allowed to react for 0.5 hours. Afterwards, 0.5 g of sodium borohydride was added in 3 equal batches over a period of 30 minutes. The reaction was allowed to come to room temperature, then 10 ml of water was added to react the remaining sodium borohydride. The reaction mixture was partitioned with chloroform. The organic solvent was dried with magnesium sulfate, filtered and evaporated to dryness. The resultant yellow syrup was further purified by preparative tlc procedures using solvent system A.

Synthesis of N-ethyl-cytisine from cytisine.--For compound 6, 100 mg of cytisine was dissolved in 5 ml anhydrous methanol in a 25 ml round bottom flask and stirred with a magnetic stirrer. The flask was maintained at 0° by partial immersion in a beaker with ice. ~~0.2 ml~~ added 0.2 ml acetaldehyde solution and allowed to react for 0.5 hours. Afterwards, 0.5 g of sodium borohydride was added in 3 equal batches over a period of 30 minutes. The reaction was allowed to come to room temperature, then 10 ml of water was added to react the remaining sodium borohydride. The reaction mixture was partitioned with chloroform. The organic solvent was dried with magnesium sulfate, filtered and evaporated to dryness. Four compounds in the reaction mixture resulted, none of which had a Rf or tlc similar to compound 6. A gc-ms (Figure 28) of the reaction mixture revealed the anticipated product to be present in the mixture but did not correspond to N-formylcytisine by gc. No further attempts to purify the individual components were pursued.

Synthesis of N-formylcytisine from cytisine.--One-half ml formic acid was added to 100 mg of cytisine in a 15 ml round bottom flask and reacted under reflux for 6 hours. The reaction mixture was cooled and taken to dryness on a rotary evaporation apparatus. The material was dissolved in chloroform and purified by preparative tlc plates using solvent system A. The resultant clear oil resisted all attempts to crystallization but showed a single spot on tlc and gc.

Synthesis of N-acetyl cytisine.--Cytisine from the seeds of *S. secundiflora* was reacted under reflux with acetic anhydride.

Three ml of acetic anhydride was added to 100 mg of cytosine and refluxed for 8 hours. The reaction mixture was allowed to cool and blown dry with air filtered through cotton. The material was purified tlc plates to yield a clear syrup which was determined to be chromatographically pure by tlc, gc and mass.

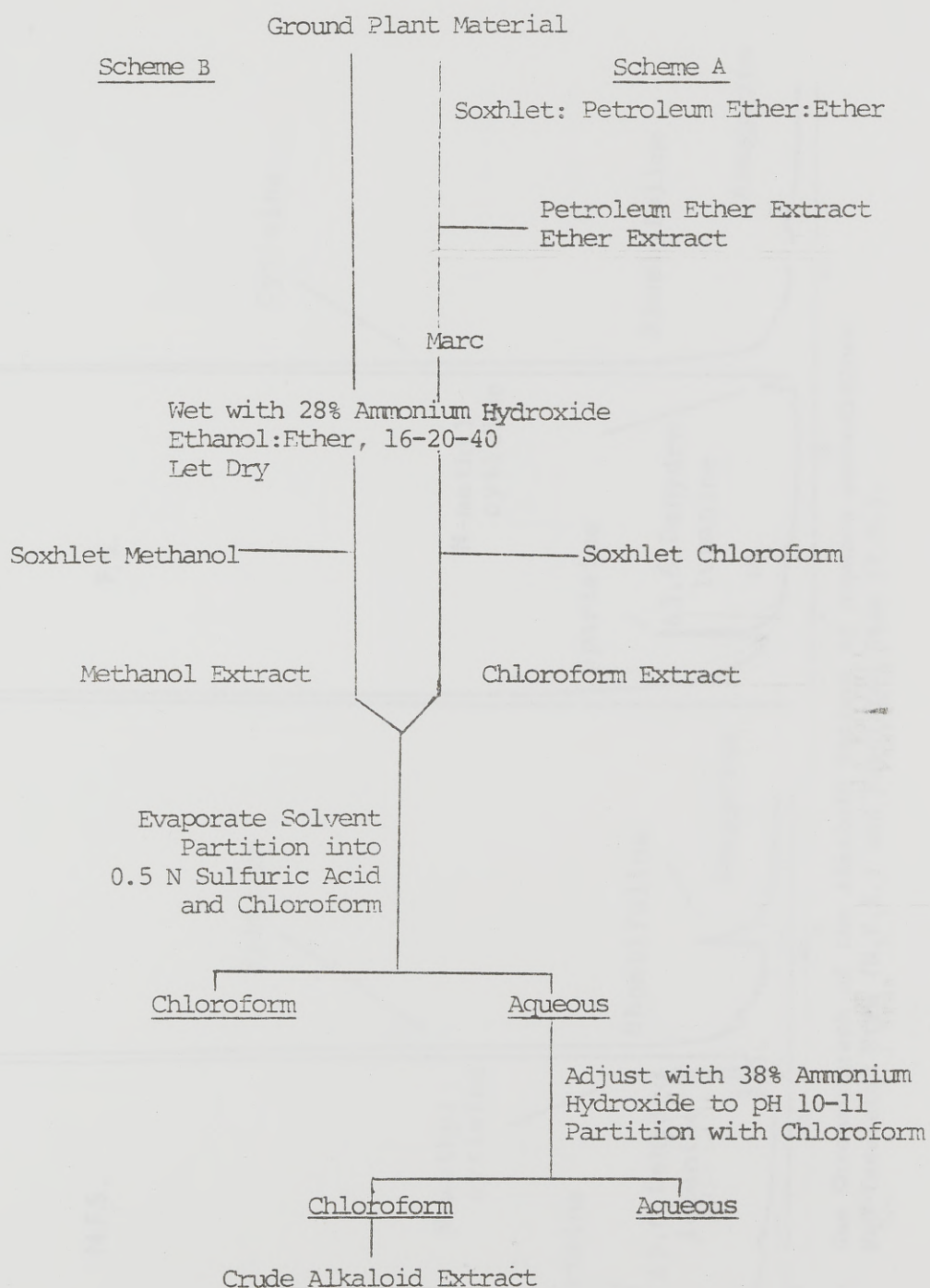


Figure 4. Extraction Schemes for Alkaloids.

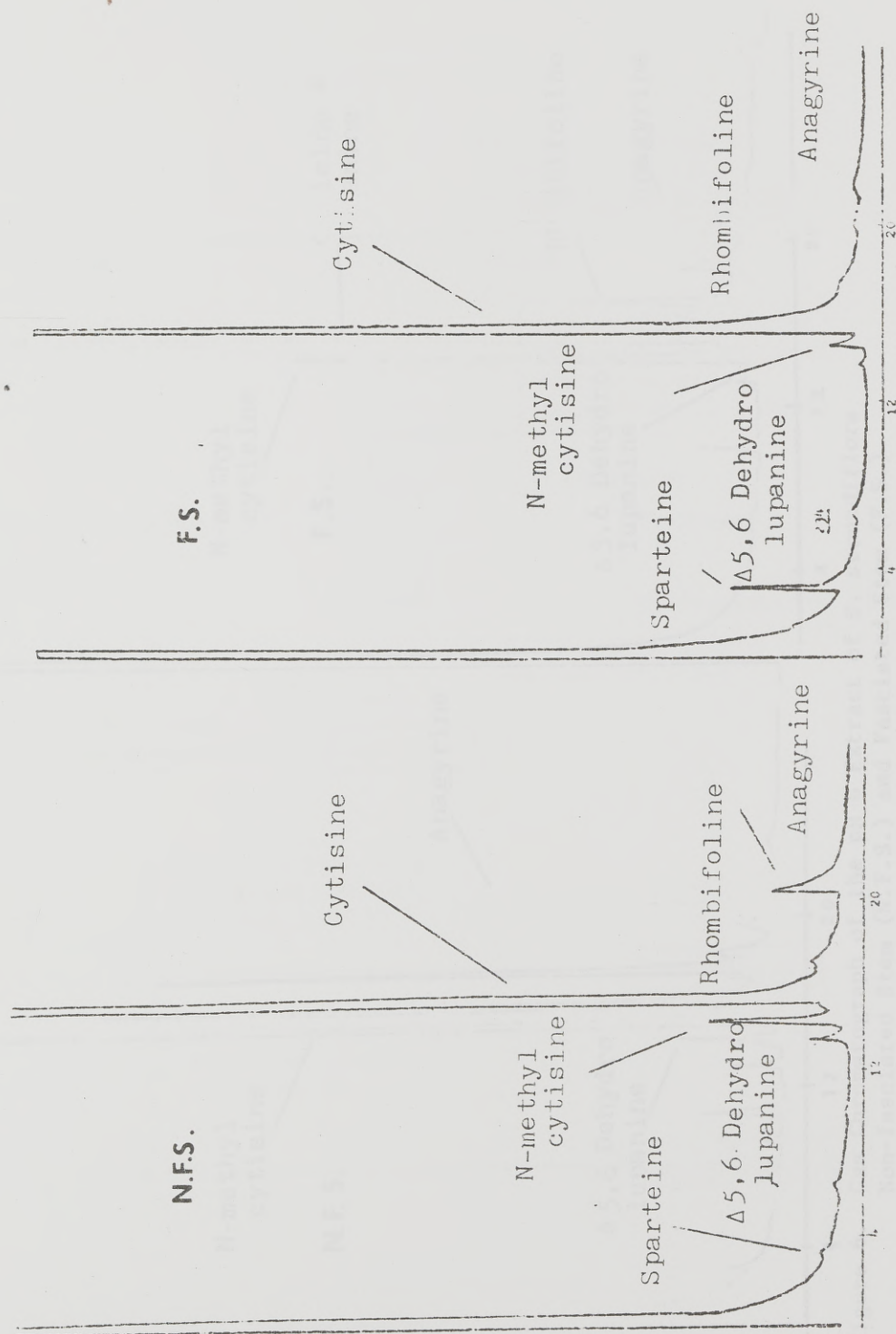


Figure 5. Gas Chromatograph of the Alkaloid Extract of *Sophora secundiflora* Non-fasciated Stem (N.F.S.) and Fasciated Stem (F.S.).

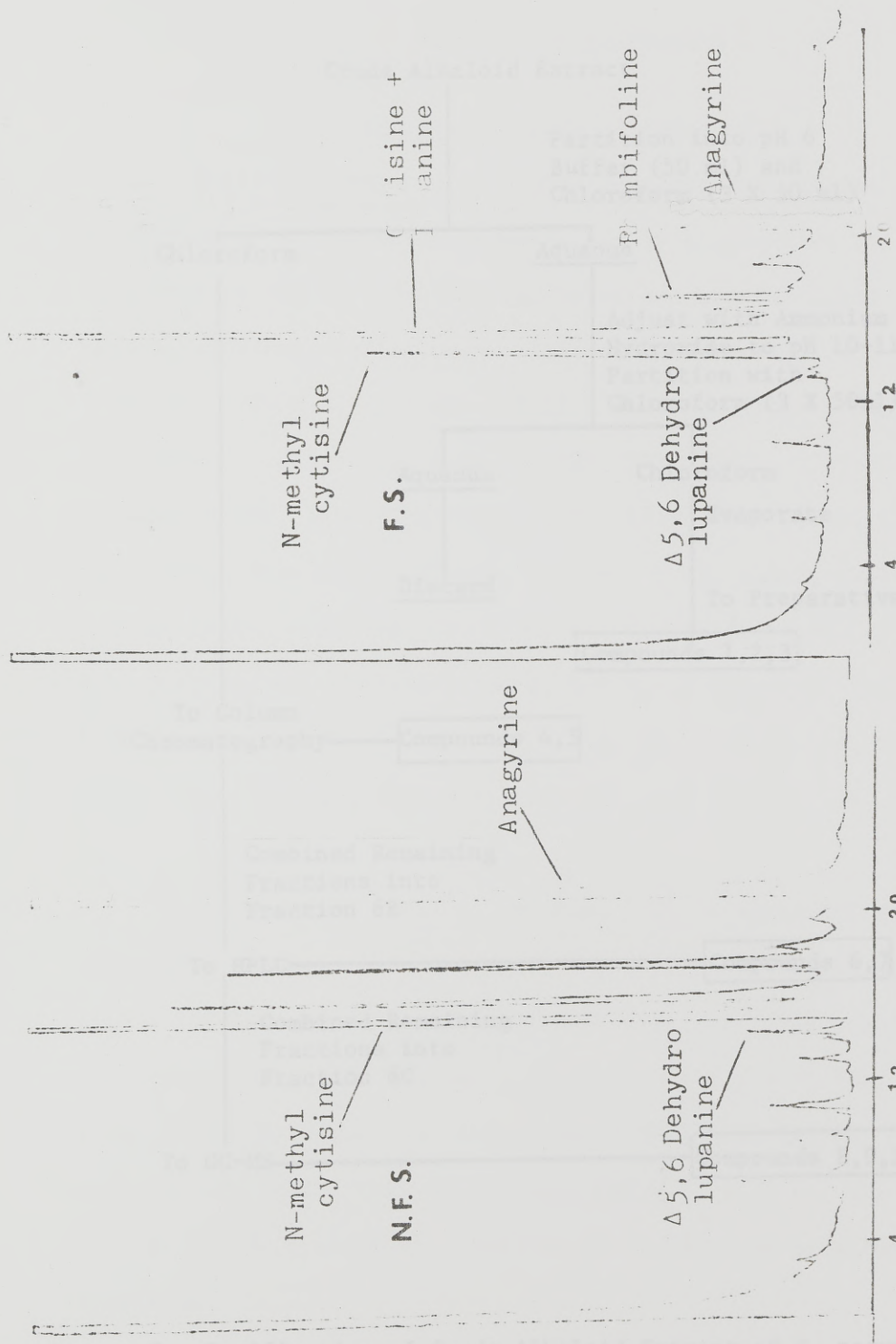


Figure 6. Gas Chromatograph of the pH 6 Extract of *S. secundiflora* Non-fasciated Stem (N.F.S.) and Fasciated Stem (F.S.).

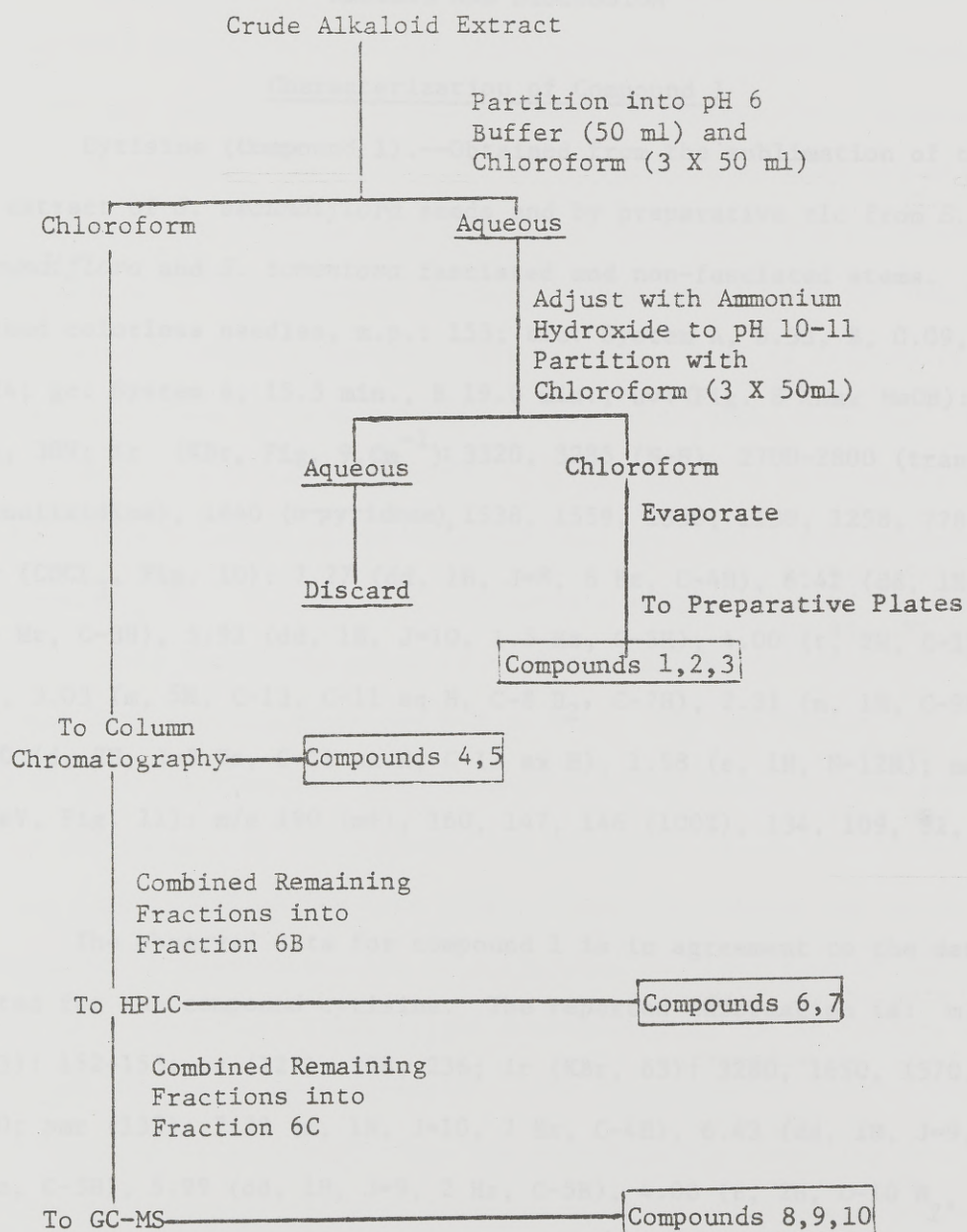


Figure 7. Purification of Crude Alkaloid Extract of *S. secundiflora*

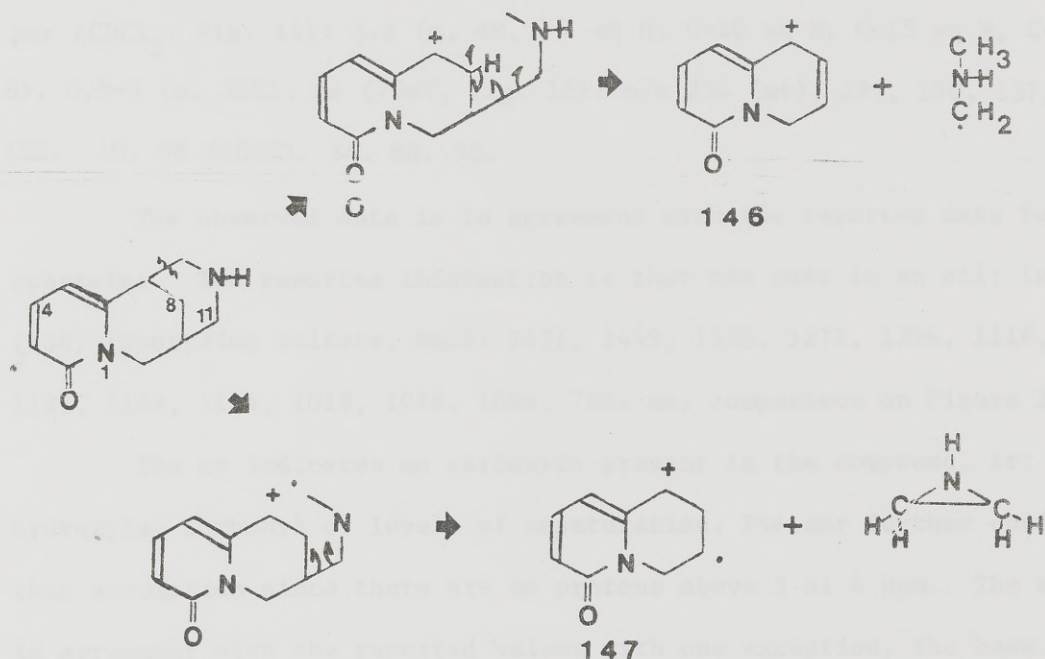
RESULTS AND DISCUSSION

Characterization of Compound 1

Cytisine (Compound 1).--Obtained from the sublimation of the pH 11 extract of *S. secundiflora* seeds and by preparative tlc from *S. secundiflora* and *S. tomentosa* fasciated and non-fasciated stems. Obtained colorless needles, m.p.: 153; tlc: System A, 0.33, B, 0.09, C, 0.24; gc: System A, 15.5 min., B 19.0 min.; uv: (Fig. 8 λ_{Max} MeOH): 234, 309; ir (KBr, Fig. 9, cm^{-1}): 3320, 3285 (N-H), 2700-2800 (trans-quinolizidine), 1640 (α -pyridone), 1538, 1559, 1330, 1130, 1258, 778, 723; pmr (CDCl_3 , Fig. 10): 7.27 (dd, 1H, $J=8, 6$ Hz, C-4H), 6.42 (dd, 1H, $J=6, 1.5$ Hz, C-3H), 5.92 (dd, 1H, $J=10, 1.5$ Hz, C-5H), 4.00 (t, 2H, C-10 H_2), 3.03 (m, 5H, C-13, C-11 eq H, C-8 H_2 , C-7H), 2.31 (m, 1H, C-9H), 1.90 (d, 2H, $J=3$ Hz, C-11 ax H, C-13 ax H), 1.58 (s, 1H, N-12H); ms (70eV, Fig. 11): m/e 190 (m^+), 160, 147, 146 (100%), 134, 109, 82, 77, 58.

The observed data for compound 1 is in agreement to the data reported for the compound cytisine. The reported information is: m.p. (113): 152-153; uv (127): 308, 236; ir (KBr, 63): 3280, 1650, 1570, 1550; pmr (132): 7.29 (m, 1H, $J=10, 7$ Hz, C-4H), 6.43 (dd, 1H, $J=9, 2$ Hz, C-3H), 5.99 (dd, 1H, $J=9, 2$ Hz, C-5H), 4.00 (t, 2H, C-10 H_2), 2.96 (m, 5H, C-11H, C-13 eq H, C-7H, C-8 H_2), 2.32 (m, 1H, C-9H), 1.95 (t, 2H, C-11H, C-13 ax H), 1.68 (s, 1H, N-12H); ms is presented in Figure 11.

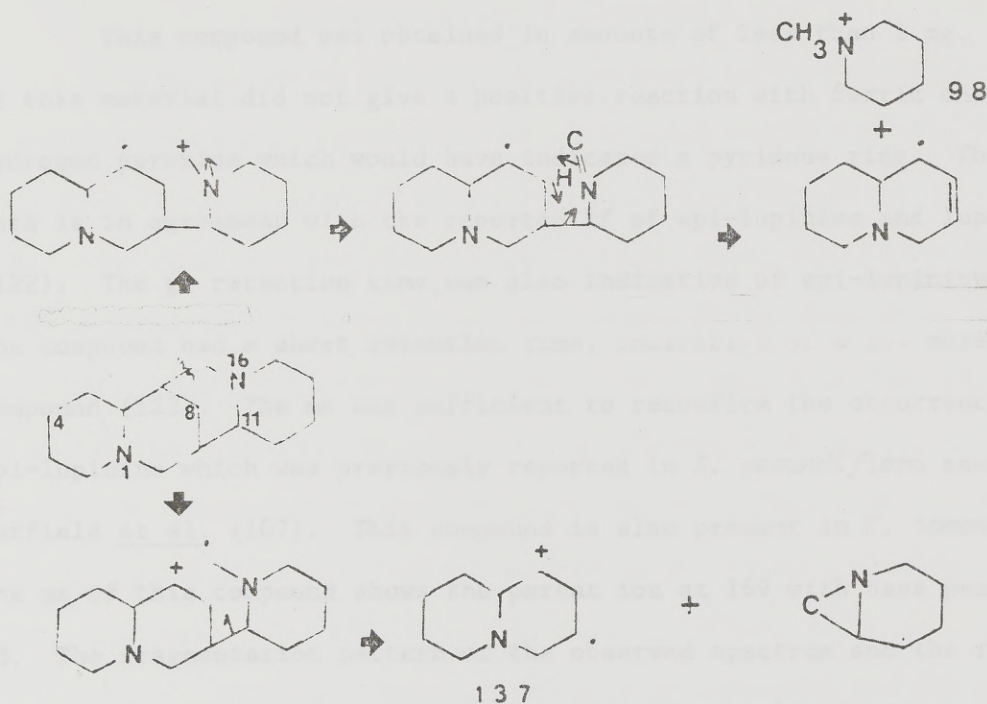
The presence of the pyridone ring was confirmed by several means. The compound on a tlc plate appeared orange in color when sprayed with ferric chloride and light blue when sprayed afterwards with hydrogen peroxide. This color change is typical for compounds with the α pyridone ring (105). The ir absorption at 1640 and uv at 309 indicates the presence of a conjugated carbonyl. The relationships of the proton in this aromatic system is indicated by the pmr which demonstrates 3 aromatic protons. The proton at 7.27 is coupled to two dissimilar protons to form a double doublet with ortho coupling. The protons at 6.46 and 5.99 show ortho coupling split by meta coupling of 1.5 Hz. This relation can be obtained only when three protons are on an aromatic ring. The pmr also indicates a singlet at 1.58 for the N-H at C-12. This functional group is confirmed by absorption in the ir at 3320 and 3285 attributed to the N-H at N-12. Cho and Martin (123) indicated that the base peak and the molecular ion were the same peak of 190 in cystisine. In our analysis the base peak is 146 and the molecular ion peak is 45% of the base peak. Those differences may be due to differences in instrumentation or difference in conditions of operation of the ms. Other than these slight discrepancies, the ms spectra of both compounds is in good agreement. The fragmentation pattern indicates loss of 44 mass units from the main molecule resulting from a MacLafferty rearrangement with hydrogen transfer after formation of the enamine. The M-43 can be formed without the hydrogen transfer (129).



gc: System A, 2.9 min., B, 9.5 min.; uv (Fig. 12 λ Max MeOH): no absorption above 220 nm; ir (neat, Fig. 13 cm^{-1}): 1469, 1451, 1350, 1271, pmr (CDCl_3 , Fig. 14): 3.5 (m, 4H, C-2 eq H, C-10 eq H, C-15 eq H, C-17 eq H), 0.9-3 (m, 22H); ms (70eV, Fig. 15): m/e 234 (m⁺), 193, 150, 137, 136, 122, 110, 98 (100%), 84, 68, 55.

The observed data is in agreement with the reported data for sparteine. The reported information is that the base is an oil; ir (134) (Sparteine Sulfate, NaCl) 1471, 1449, 1355, 1271, 1294, 1116, 1129, 1149, 1184, 1018, 1076, 1084, 786; ms, comparison on Figure 15.

The uv indicates no carbonyls present in the compound, ir: no hydroxyls, carbonyl or levels of unsaturation. The nmr further confirms this assumption since there are no protons above 5 or 4 ppm. The ms is in agreement with the reported values with one exception, the base peak is 137 in the reported spectra and 98 in the observed spectrum. This difference may be due to instrumental differences or the fact that the free base was used in this analysis and the salt was used in the reported spectrum. To check this hypothesis, the free base from commercially obtained sparteine sulfate was used. In this spectrum of the free base, the base peak was 98 as in our natural product spectral analysis. The major fragments were at m/e 98 and m/e 137. These fragments are obtained from the molecule in the following manner:

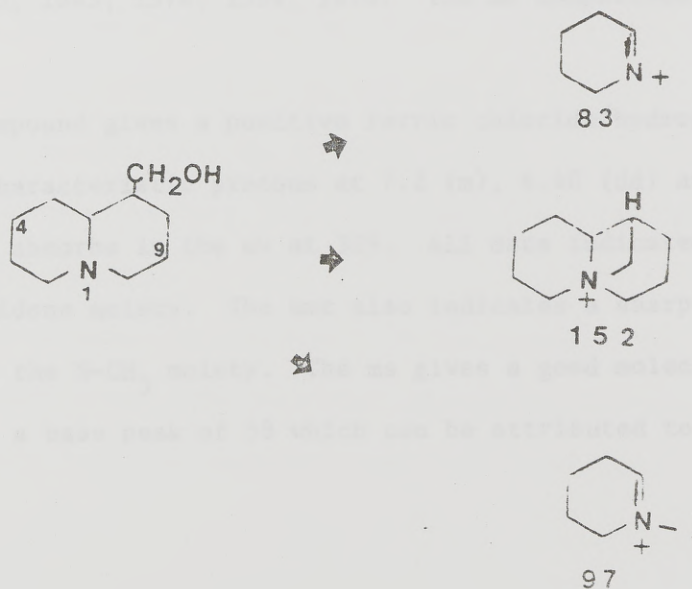


In addition to the agreement between the observed and recorded data the compound was co-chromatographed with a standard of sparteine by gc, gc-ms and tlc in 3 solvent systems and gave identical retention times with the standard. An isomer of sparteine, β -isosparteine, has identical spectral data to sparteine but differs according to Keller (personal communication) with respect to gc retention time in a temperature program on OV-17. Data from such an analysis indicates that this compound is sparteine.

Characterization of Compound 3

Epi-lupinine (Compound 3).--Obtained from *S. secundiflora* and *S. tomentosa* as an oil: tlc: System A, 0.08, B, 0.67, C, 0.42; gc: System A, 1.0 min., B, 6.7 min. ms (70eV, Fig. 16): m/e 169 (m⁺), 168, 152, 138, 111, 110, 97, 96, 83 (100%), 55.

This compound was obtained in amounts of less than 1 mg. Tlc of this material did not give a positive reaction with ferric chloride/hydrogen peroxide which would have indicated a pyridone ring. The tlc data is in agreement with the reported Rf of epi-lupinine and lupinine (122). The gc retention time was also indicative of epi-lupinine since the compound had a short retention time, indicative of a low molecular compound (123). The ms was sufficient to reconfirm the occurrence of epi-lupinine which was previously reported in *S. secundiflora* seed by Hatfield et al. (107). This compound is also present in *S. tomentosa*. The ms of this compound shows the parent ion at 169 with base peak of 83. The fragmentation pattern of the observed spectrum and the reported spectrum is compared in Figure 16. The complex fragmentation of epi-lupinine has the following fragment:

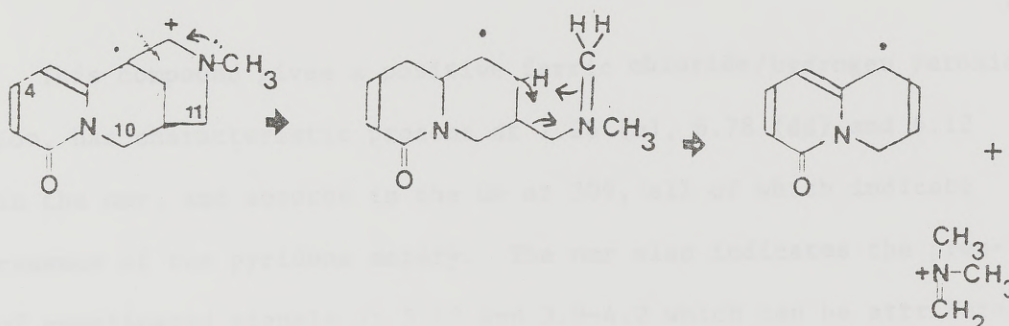


Characterization of Compound 4

N-methylcytisine (Compound 4).--Obtained as clear needles from *S. secundiflora* and *S. tomentosa*. mp: 136; tlc: System A, 0.68, B, 0.41, C, 0.77; gc: System A, 14.2 min., B, 17.6 min.; uv (Fig. 17 λ Max MeOH): 309, 234; ir (KBr, Fig. 18, cm^{-1}): 2938, 2900, 2830, 2700-2800 (trans quinolizidine), 1645, 1568, 1541, 1254, 1133, 1045, 790, 739; pmr (CDCl_3 , Fig. 19): 7.36 (t, 1H, $J=8, 6$ Hz, C-4H), 6.48 (dd, 1H, $J=6, 1.5$ Hz, C-3H), 6.01 (dd, 1H, $J=10, 1.5$ Hz, C-5H), 4.00 (m, 2H, C-10 H_2), 2.90 (m, 2H, C-13 eq H, C-11 eq H), 2.45 (m, 1H, C-7H), 2.18 (s, 3H, N-12 CH_3), 1.5-2.0 (m, 4H, C-11 ax H, C-13 ax H, C-8 H_2); ms (Fig. 20, 70 eV): 204 (m^+), 189, 160, 146, 132, 117, 58 (100%), 42.

The observed data for compound 4 is in agreement for the data reported for the compound N-methylcytisine. The reported information for N-methylcytisine is mp (112): 136; uv (68): 309, 234; ir (83): 2937, 2833, 2779, 1660, 1645, 1574, 1559, 1470. The ms comparisons are on Figure 20.

This compound gives a positive ferric chloride/hydrogen peroxide reaction, has characteristic protons at 7.2 (m), 6.40 (dd) and 6.02 (dd) in the nmr, and absorbs in the uv at 309. All data indicates the presence of the pyridone moiety. The nmr also indicates a sharp three proton singlet for the N- CH_3 moiety. The ms gives a good molecular ion peak at 204 and a base peak of 58 which can be attributed to the fragments:



58

Synthetic N-methylcytisine was identical to the natural product. The natural products and the synthetic compound were co-chromatographed with a standard in the tlc, gc and gc-ms and gave identical results.

Characterization of Compound 5

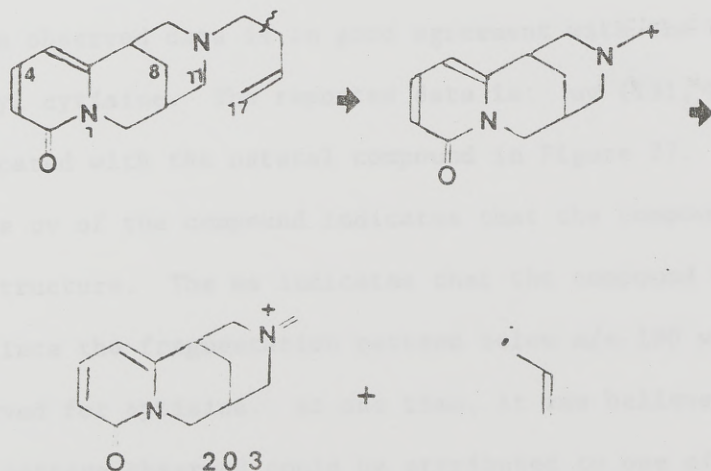
Rhombifoline (Compound 5).--Obtained as a yellow oil from *S. secundiflora*. tlc: System A, 0.81, B, 0.82; gc: System A, 18.3 min., B, 20.6 min.; uv (Fig. 21, λ Max, MeOH): 309, 234; ir (KBr, chloroform, Fig. 22 cm^{-1}): spectra in chloroform shows absorption at 1650; pmr (CDCl_3 , Fig. 23, partial): 7.27 (m, 1H, C-4H), 6.72 (d, 1H, $J=11$ Hz, C-5 H), 6.42 (m, 1H, C-16H), 6.24 (d, 1H, $J=9$ Hz, C-5H), 5.18 (m, 1H, C-17 Hb), 4.0-4.8 (m, 4H, C-14 H₂, C-15 H₂), 0.7-4 (rest of protons); ms (70eV, Fig. 24) m/e 244 (m⁺), 215, 163, 149, 134, 122, 98, 97, 84, 68, 58 (100%), 55, 41.

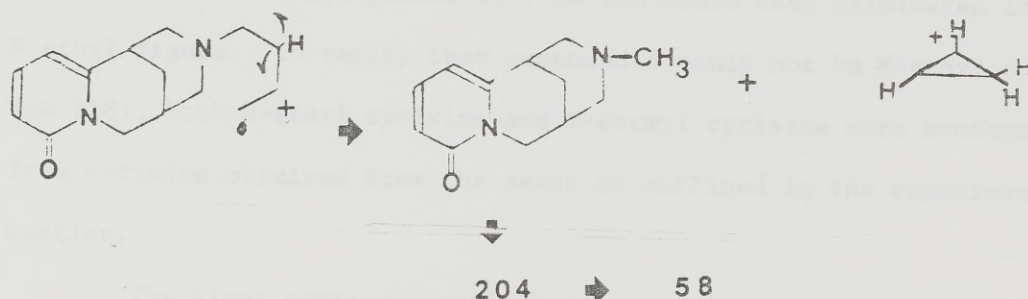
The observed data for compound 5 is in agreement for the data reported for rhombifoline. The reported information for rhombifoline is: ir (CaF, 135): 1651 for carbonyl; nmr: (not reported): ms: comparison on Figure 25.

This compound gives a positive ferric chloride/hydrogen peroxide reaction, has characteristic protons at 7.26 (m), 6.78 (dd) and 6.12 (dd) in the nmr, and absorbs in the uv at 309, all of which indicate the presence of the pyridone moiety. The nmr also indicates the presence of complicated signals at 5.42 and 3.9-4.2 which can be attributed to the butenyl attached to the N-12. The 15-H proton is interpreted to be deshielded by the lone pair of protons on the nitrogen and therefore situated at 6.4.

The ir spectrum of the compound was achieved in chloroform and the spectrum indicates mostly the spectrum of chloroform. The band at 1650 is due to the compound.

The ms is in sound agreement with the reported spectrum. The spectrum indicates a mass of m/e 244 with a base peak of 58. The main fragmentation process is believed to originate as a result of the formation of N-methylcytisine which is charged to form the peak at 204 and which fragments to the typical N-methylcytisine pattern with 58 as the base peak:





Characterization of Compound 6

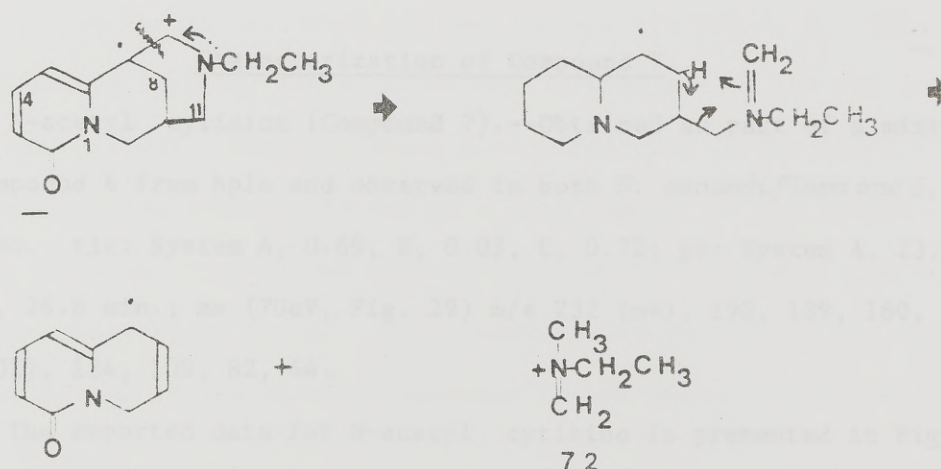
N-formyl cytisine (Compound 6).--Obtained as an oil from *S. secundiflora* and detected in *S. tomentosa*. tlc: System A, 0.64, B, 0.02, C, 0.69; gc: System A, 24.5 min., B, 26.9 min.; uv (Fig. 25, λ Max, (MeOH): 234, 310; pmr (CDCl₃, Fig. 26): 7.84 (d, 1H, J=20, C-14H), 7.37 (t, 1H, J=8, 6 Hz, C-4H), 6.51 (dd, 1H, J=6, 1.5 Hz, C-3H), 6.03 (m, 1H, C-5H), 4.2-4.8 (m, 2H, C-11, C-13 eq H), 4.00 (m, 2H, C-10 H₂), 0.7-3.8 (rest of protons); ms (70eV, Fig. 27): m/e 218 (m⁺), 190, 160, 159, 147, 146 (100%), 132, 118, 55.

The observed data is in good agreement with the data reported for N-formyl cytisine. The reported data is: uv (131, MeOH): 309, 234 and ms compared with the natural compound in Figure 27.

The uv of the compound indicates that the compound possesses a pyridone structure. The ms indicates that the compound was related to cytisine since the fragmentation pattern below m/e 190 was identical to that observed for cytisine. At one time, it was believed that the fragmentation pattern observed could be attributed to one of two compounds,

N-ethyl cytosine or N-formyl cytosine. The nmr peak showing a high upfield shifted formyl proton at 7.84 PPM would have eliminated the N ethyl figure. To verify that compound 6 could not be N-ethyl cytosine (mw 218), both N-ethyl cytosine and N-formyl cytosine were synthesized from cytosine obtained from the seeds as outlined in the experimental section.

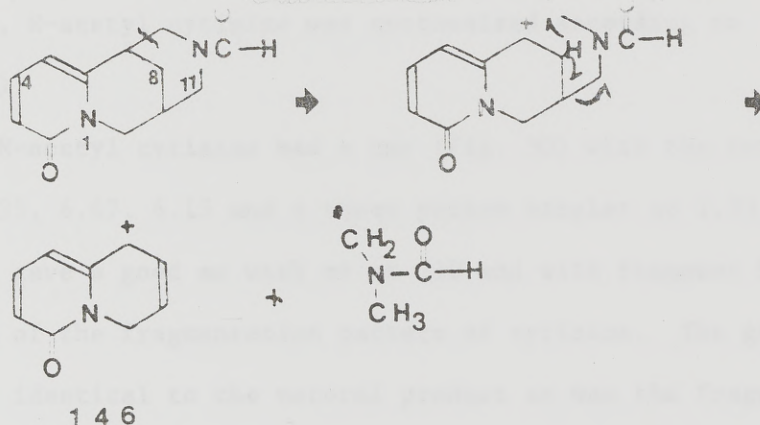
The first compound synthesized to assist in the identification of compound 6 was N ethyl cytosine. The ms spectrum (Figure 28) was similar to N-methylcytosine plus 14 mass units with m^+ at 218 and the base peak of 72. This fragmentation could be achieved in the following manner:



The second compound synthesized to determine the structure of compound 6 was N-formyl cytosine. Both the synthetic material and the natural product gave a uv 309 indicating a pyridone structure. The gc

retention of both compounds was identical as was the gc-ms and nmr. The tlc on 3 solvent systems was the same. Both gave a positive ferric chloride test. Compound 6 therefore is N-formyl cytosine.

The ms of the natural and synthetic N-formyl cytosine indicate the following fragments:



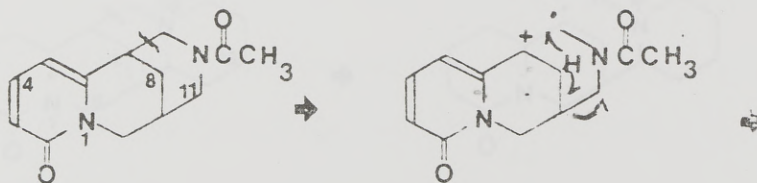
Characterization of Compound 7

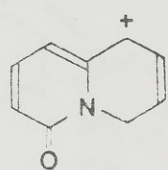
N-acetyl cytosine (Compound 7).--Obtained as part of a mixture with compound 6 from hplc and observed in both *S. secundiflora* and *S. tomentosa*. tlc: System A, 0.69, B, 0.03, C, 0.72; gc: System A, 23.6 min., B, 26.8 min.; ms (70eV, Fig. 29) m/e 232 (m+), 190, 189, 160, 147, 146 (100%), 134, 109, 82, 44.

The reported data for N-acetyl cytosine is presented in Figure 29 and consists of the ms of the natural product and the data presented in the literature for N-acetyl cytosine. The fragmentation of the natural compound in the ms was similar to the ms spectrum of cytosine below m/e 190 and was structurally related to cytosine. The molecular ion was 42 mass units higher than cytosine and since the peaks between

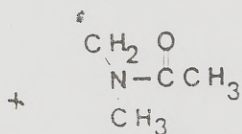
190 and 232 were of low intensity, N-alkyl cytosine was ruled out. N-alkyl cytosines have the base peak generated by a fragment which includes the N-12 as part of the peak. The natural product was believed to be related to N-formyl cytosine and to differ from it by 14 mass units. This compound would be N-acetyl cytosine. To confirm this initial hypothesis, N-acetyl cytosine was synthesized according to Ohmiya *et al.* (114).

Synthetic N-acetyl cytosine had a nmr (Fig. 30) with the pyridone protons at 7.35, 6.47, 6.15 and a three proton singlet at 2.03 PPM. The material gave a good ms with m^+ at 232 and with fragment below 190 characteristic of the fragmentation pattern of cytosine. The gc retention time was identical to the natural product as was the fragmentation pattern on the gc-ms. With tlc and solvent systems A, B, and C synthetic N-acetyl cytosine had an identical R_f with one of the two components in the mixture. Both compounds gave a positive ferric chloride/hydrogen peroxide reaction. Addition of a small amount of the synthetic N-acetyl cytosine to spike the mixture enhanced the height of one of the two peaks observed on the gc. The fragmentation pattern of the synthetic compound and of the natural compounds in the mixture both indicated the pattern:





1 4 6



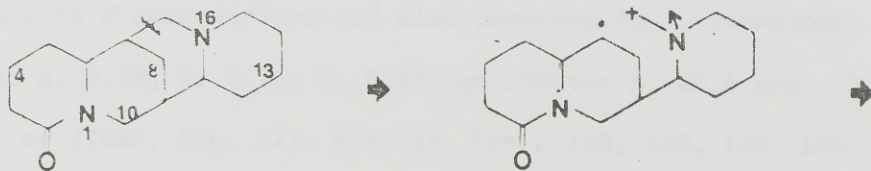
Identification of Compounds 8, 9, 10

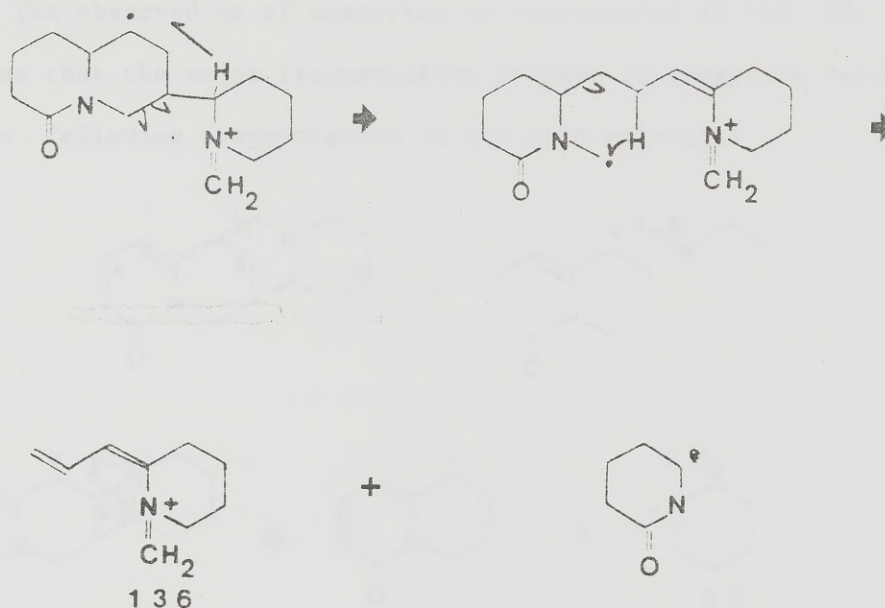
The next three compounds, compound 8, 9 and 10 were obtained as a mixture from *S. secundiflora*. The amount of the mixture was 1.8 mg. Secondary methods of identification were employed. It was believed that these alkaloids had previously been reported in *S. secundiflora*. The identification of these three compounds would, therefore be a reconfirmation of the occurrence of these compounds in *S. secundiflora*.

Compound 8

Lupanine (Compound 8).--Obtained as a three component mixture. tlc: System A, 0.20, B, 0.66, C, 0.57; gc: System A, 17.2 min., B, 19.8 min.; ms (70eV, Fig. 31): m/e 248 (m⁺), 219, 149, 136 (100%), 124, 110, 98, 97, 84.

The observed ms of lupanine is represented in Figure 31. The ms indicates that the major fragmentation process in lupanine results from the following fragmentation:



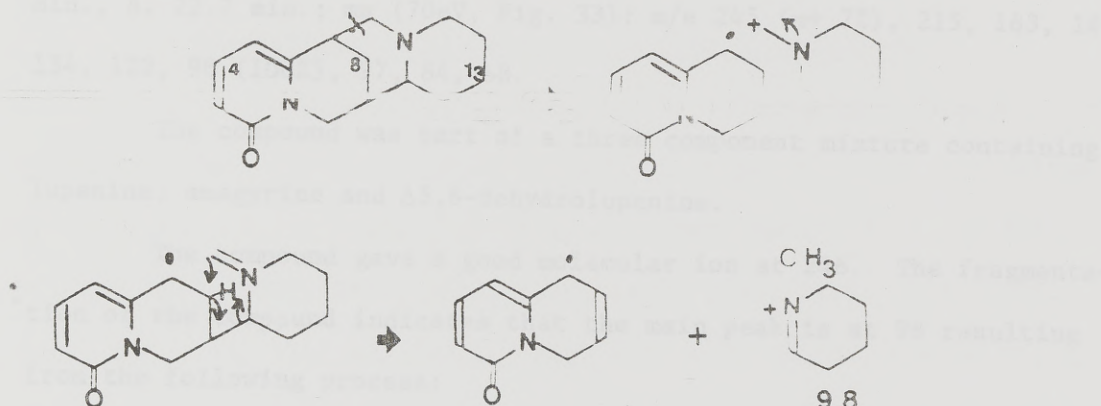


The compound gave a negative ferric chloride/hydrogen peroxide test indicating the absence of a pyridone structure. A gc of the three component mixture with an authentic sample of lupanine indicated that the authentic sample had retention times of 17.2 min. and 19.8 min. in gc system A and B, respectively. The gc-ms of the authentic sample and one of the components of the mixture was identical. This information reconfirmed the presence of lupanine in *S. secundiflora* as indicated initially by Keller et al. (107). Compound 8 is therefore lupanine.

Compound 9

Anagryne (Compound 9).---Obtained as part of a 3 component mixture in *S. secundiflora* and also observed in *S. tomentosa*. tlc: System A, 0.58, B, 0.76, C, 0.55; gc: System A, 20.6 min., B, 25.1 min.; ms (70eV, Fig. 32): m/e 244 (m^+), 188, 160, 146, 136, 117, 98 (100%), 55, 41.

The observed ms of anagyrine is represented in Fig. 32. The ms indicates that the major fragmentation process in anagyrine resulted from the following fragmentation of the main molecule:



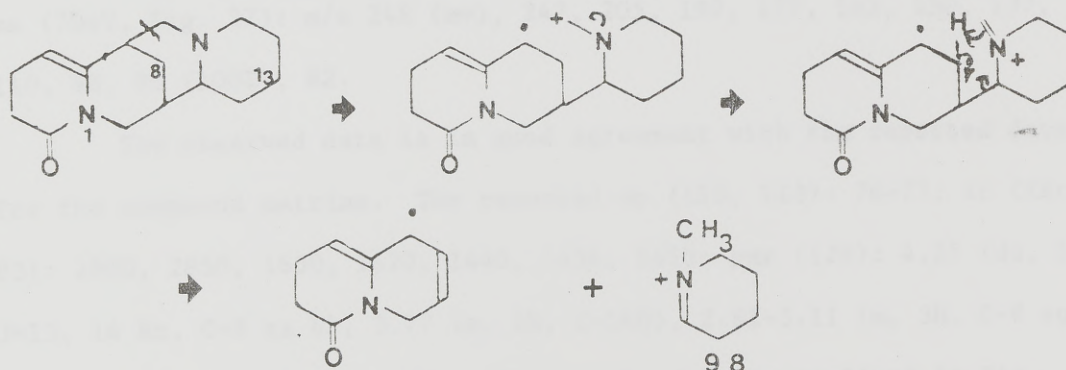
The compound gave a positive ferric chloride/hydrogen peroxide test indicating the presence of a pyridone structure. No authentic sample for anagyrine was available but a 2 mg sample of thermopsine, the C-11 isomer of anagyrine was available. Cho and Martin (123) indicated that anagyrine and thermopsine could be separated on a QF-1 column using a temperature program similar to gc system A. Injection of thermopsine into gc system A resulted in a retention time of this compound of 23.0 min. The three component mixture of crude compounds did not have a compound with the retention time of thermopsine even though it gave a similar ms fragmentation pattern to one of the compounds. Compound 9 is therefore anagyrine.

Compound 10

$\Delta 5,6$ -Dehydrolupanine (Compound 10).--Obtained as part of a 3 component mixture. tlc: system A, 0.73, C, 0.76; gc: System A, 13.4 min., B, 22.7 min.; ms (70eV, Fig. 33): m/e 245 (m^+ 7%), 215, 163, 149, 134, 122, 98 (100%), 97, 84, 68.

The compound was part of a three component mixture containing lupanine, anagrine and $\Delta 5,6$ -dehydrolupanine.

The compound gave a good molecular ion at 246. The fragmentation of the compound indicates that the main peak is at 98 resulting from the following process:



This fragmentation is in good agreement with the fragmentation reported for $\Delta 5,6$ -dehydrolupanine

This compound gave a negative ferric chloride/hydrogen peroxide test indicating the absence of a pyridone structure. The compound had a retention time of 13.4 min. and 22.7 min. with gc system A and B. Cho and Martin (123) observed that on a QF-1 column similar to gc system A, $\Delta 5,6$ -dehydrolupanine had a relative retention time in front of

N-methylcytisine and cytisine. On an OV-17 system, $\Delta 5,6$ -dehydrolupanine comes after N-methylcytisine and cytisine according to Keller (personal communication). Compound 10 behaves in a similar manner on the gc.

Identification of Compound 11 from *S. tomentosa*

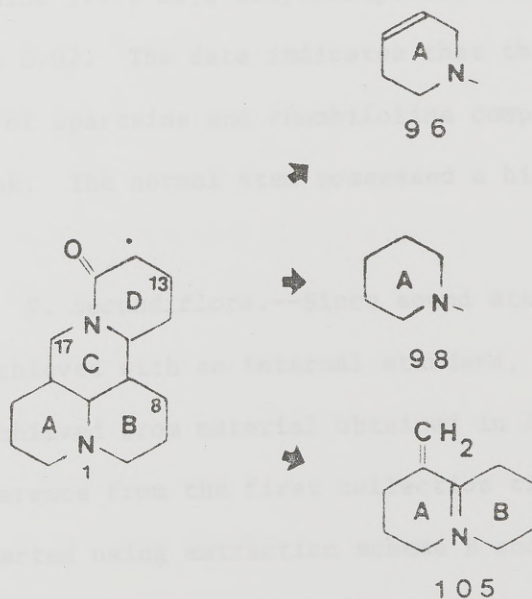
Compound 11 (compound 11).--Obtained as white needles from *S. tomentosa*. mp: 76; tlc: System A, 0.75, B, 0.64, C, 0.83; gc: System A, 18.4 min., B, 22.2 min.; uv (Fig. 34, λ Max, MeOH): 23.2; ir (KBr, Fig. 35 cm^{-1}): 2920, 2860, 1624, 1435, 1415, 1353, 1228, 1135, 1095, 1069, 860, 783, 730; pmr (CDCl_3 , Fig. 36): 4.25 (m, 1H, C-8 ax H), 3.75 (m, 1H, C-14H), 2.8-3.2 (m, 3H, C-8 eq H, C-3H, C-5 H), 1.7 (m, 19H); ms (70eV, Fig. 37): m/e 248 (m+), 247, 205, 192, 177, 162, 150, 137, 110, 98, 96 (100%), 82.

The observed data is in good agreement with the reported data for the compound matrine. The reported mp (112, 113): 76-77; ir (KBr, 83): 2900, 2850, 1630, 1620, 1440, 1434, 1413; pmr (126): 4.25 (dd, 1H, $J=13, 14$ Hz, C-8 ax H), 3.77 (m, 1H, C-14H), 2.81-3.11 (m, 3H, C-8 eq H, C-3H, C-5H), 1.74 (m, 19H). The reported ms is outlined in Figure 37.

This compound did not give a positive ferric chloride/hydrogen peroxide test indicating the absence of a pyridone moiety in the compound. The ir spectra indicated absorption at 2850 indicating a trans quinolizidine compound and at 1620 indicating a lactam carbonyl. The pmr indicates no aromatic or olefinic protons since no signals above 4.5 were observed. The ms gave a molecular ion of 248 and indicated

that the main fragmentation occurred to generate fragments characteristic to the matrine series of quinolizidine alkaloids.

One of the distinguishing features of the matrine series of compounds in the ms is the strong peaks with m/e 98 and 96. In addition, the matrine series also has the parent or the $m-1$ ion peak as the base peak. In the observed spectra of matrine, the base peak was 96. The difference between the observed and the reported is most likely due to differences in instrumentation. The fragmentation of matrine is complicated and the chief fragments reported by Iskandarov and Yunusov (137) appear to arise from the A and B rings of the molecule:



Semi-quantitative Study

Study 1. *S. secundiflora*.--The initial gc comparisons of the fasciated and non-fasciated stems of *S. secundiflora* were performed in 1978 and indicated that there were some quantitative differences of the minor alkaloids. For this study, a 3% QF 1 column was used. Exact quantitative examinations could not be achieved however but relative interpretation could be made on 4 of the major peaks. Figure 5 shows the gc spectra of 1.8 ul of fasciated stem crude alkaloid extract and 1.8 ul of normal stem extract from the same amount of plant material extracted according to scheme A. In the non-fasciated spectra, the peak ratio of individual compounds peak height to that of N-methylcytisine was sparteine 0.03, Δ 5,6-dehydrolupanine 0.26, rhombifoline 0.06 and anagryne 0.48. In the fasciated spectra, the corresponding ratios were sparteine 3.17, Δ 5,6-dehydrolupanine 0.20, rhombifoline 0.20 and anagryne 0.02. The data indicates that the fasciated stem had higher levels of sparteine and rhombifoline compared to the N-methylcytisine peak. The normal stem possessed a higher ratio of anagryne.

Study 2. *S. secundiflora*.--Since sound statistical data could be better achieved with an internal standard, a second quantitative study was achieved from material obtained in 1979 at about 1 year 1 month difference from the first collection time. The plant material was extracted using extraction scheme B and this extract was analyzed using gc system B. The internal standard used was a 2 mg/ml solution of oxosparteine. The spectra for the fasciated and non-

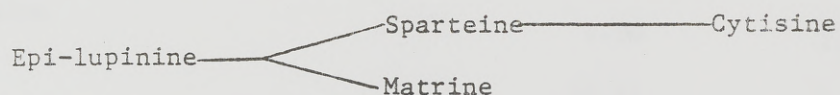
fasciated stem are presented in Figure 38. Table 2 indicates the relationship between the different peak ratios relative to the internal standard.

In all instances but two, the non-fasciated stem had a higher ratio of alkaloids relative to the internal standard. The amounts of sparteine and $\Delta 5,6$ -dehydrolupanine were relatively equal in both stem types while the amount of N-methylcytisine, cytisine and N-formyl cytisine were approximately 50% higher in the non-fasciated stems. The relative amount of sparteine will be discussed further. The area/area ratio of sparteine/N-methylcytisine was 5.3 for the non-fasciated stem and 19.5 for the fasciated stem thus reconfirming the relationship observed in the first study.

An observation not made in the first study was the relationship between cytisine and sparteine and cytisine and the internal standard. The area/area ratio of sparteine/cytisine was 0.27 for the non-fasciated stem and 0.39 for the fasciated stem. This observation would indicate that the fasciated stem produced higher levels of sparteine than the non-fasciated stem relative to the major constituent cytisine. Since Nowachi and Waller (120) have indicated that the tetracyclic sparteine is the biogenetic precursor to the tricyclic cytisine, this higher ratio may be indicative of biosynthetic blockage occurring in the fasciated stem. A second observation is that the area/area ratio of cytisine/internal standard is 25% less in the fasciated stem. This decrease in the amount of major constituent was unexpected especially since the fasciated stems produced a greater amount of the alkaloid extract as noted on page 43.

Study 2. *S. tomentosa*.--The examination of *S. tomentosa* resulted in Figure 39 and Table 3. In *S. tomentosa* the non-fasciated stem had higher ratios of N-methylcytisine, matrine and compound 12 (structurally related to matrine). The fasciated stem had higher ratios of epi-lupinine, cytisine, anagyrine and N-formyl cytisine.

Non-fasciated and fasciated stems of *S. tomentosa* were examined for alkaloids. Epi-lupinine like precursor would first be formed followed by either matrine types or sparteine-cytisine types of alkaloids:



In the non-fasciated stems, matrine and related alkaloids predominated quantitatively. In the fasciated stem there was a decrease in the matrine/internal standard ratio and a relative increase in the sparteine-cytisine group of alkaloids as well as the precursor epi-lupinine. These differences may be due to a quantitative shift from the matrine type of alkaloids to the sparteine-cytisine group.

TABLE 2. Semi-quantitative Comparison of Non-fasciated Stem (N.F.S.) and Fasciated Stem (F.S.) of *S. secundiflora*. Area of Compound Relative to the Internal Standard Oxosparteine.

<u>COMPOUND</u>	<u>N.F.S. (A)</u>	<u>F.S. (B)</u>	<u>A/B</u>
Epi-lupinine	—	—	—
Sparteine	28.50	27.87	1.02
N-methylcytisine	5.36	1.43	2.74
Oxosparteine	1.00	1.00	1.00
Cytisine	102.79	70.51	1.46
Lupanine	0.87	0.50	1.74
Δ 5,6-Dehydrolupanine	2.42	2.25	1.07
Anagyrine	7.91	4.85	1.63
N-formyl cytisine	1.67	1.02	1.64
N-acetyl cytisine	—	—	—

TABLE 3. Semi-quantitative Comparison of Non-fasciated Stem (N.F.S.) and Fasciated Stem (F.S.) of *S. tomentosa*. Area of Compound Relative to the Internal Standard Oxosparteine.

<u>COMPOUND</u>	<u>N.F.S.(A)</u>	<u>F.S.(B)</u>	<u>A/B</u>
Epi-lupinine	0.041	0.209	0.198
N-methylcytisine	0.043	0.024	1.758
Oxosparteine	1.00	1.00	1.00
Cytisine	0.238	0.313	0.762
Matrine	1.654	0.556	2.922
Compound 12	0.031	0.015	2.066
Anagyrine	0.040	0.066	0.612
N-formyl cytisine	0.106	0.555	0.190

SUMMARY

S. secundiflora

The compounds isolated from the seed of *S. secundiflora* by (104) Izadost et al. (105), Hatfield (106) and Keller and Hatfield (107) are epi-lupinine, sparteine, β -isosparteine, cytisine, N-methylcytisine, 11-allyl cytisine, lupanine, Δ 5,6-dehydrolupanine, rhombifoline, anagryne and thermopsine. In our analysis of the normal and fasciated stems of *S. secundiflora* all but three of these compounds were encountered: 11-allyl cytisine, β -isosparteine and thermopsine. Two quinolizidine alkaloids were detected in the stems which had not been reported previously in this plant, N-formyl cytisine and N-acetyl cytisine.

The differences between the seed and the stem alkaloids may be explained in several ways. The differences may be of a quantitative nature and the alkaloids missing in the stems could be detected by extraction and fractionation of larger amounts of plant material. Alternatively, the stems may produce a slightly different composition of alkaloids than the seeds. A dramatic example of this phenomenon is evident in the alkaloids of the poppy where the morphine alkaloids are not produced in the seeds. A third possibility is that the two previously unreported compounds are artifacts. Both N-formyl cytisine and N-acetyl cytisine have been reported in *S. franchetiana* by Ohmiya et al. (72) and *S. tomentosa* by Ohmiya et al. (114), respectively. Murakoshi et al. (115) have isolated the enzyme acetyl-coenzyme A:

cytisine N-acetyltransferase from *S. tomentosa* which catalyzed the conversion of cytisine to N-acetyl cytisine. Indeed, one of the reasons we examined *S. tomentosa* was to obtain spectral data on N-acetyl cytisine. It is, therefore, possible but unlikely that N-formyl cytisine and N-acetyl cytisine are available.

The semi-quantitative study on *S. secundiflora* normal and fasciated stems indicated a quantitative difference of alkaloids between the two stem types. This difference is possibly due to the diseased conditions of the fasciated stem. Since the cause of the fasciation is unknown, it is difficult to speculate why these quantitative differences do occur.

S. tomentosa

Ohimya et al. (114) reported the isolation of cytisine, N-methylcytisine, matrine, matrine N-oxide, baptifoline, N-acetyl cytisine and anagyrine from the seeds of *S. tomentosa*.

In our analysis matrine, cytisine, N-acetyl cytisine and N-methylcytisine were detected by a combination of gc-ms and other techniques. Also detected by this method were epi-lupinine and N-formyl cytisine, two previously unreported alkaloids from this species of *Sophora*. In our analysis of *S. tomentosa* isolation of specific components from the fasciated and non-fasciated stems was hampered by the limited amount of plant material. The comparison of the fasciated and non-fasciated stems in *S. tomentosa* indicated that the differences between these two stems types were difficult to inter-

pret due to the complexity of the mixtures. A general interpretation for fasciations in *Sophora* is that fasciation may decrease the amount of the major constituent and increase the relative accumulation of simpler compounds and/or biogenetically branched compounds.



Figure 2. Infrared and Ultraviolet Spectra of Compound 1 (Cytidine).

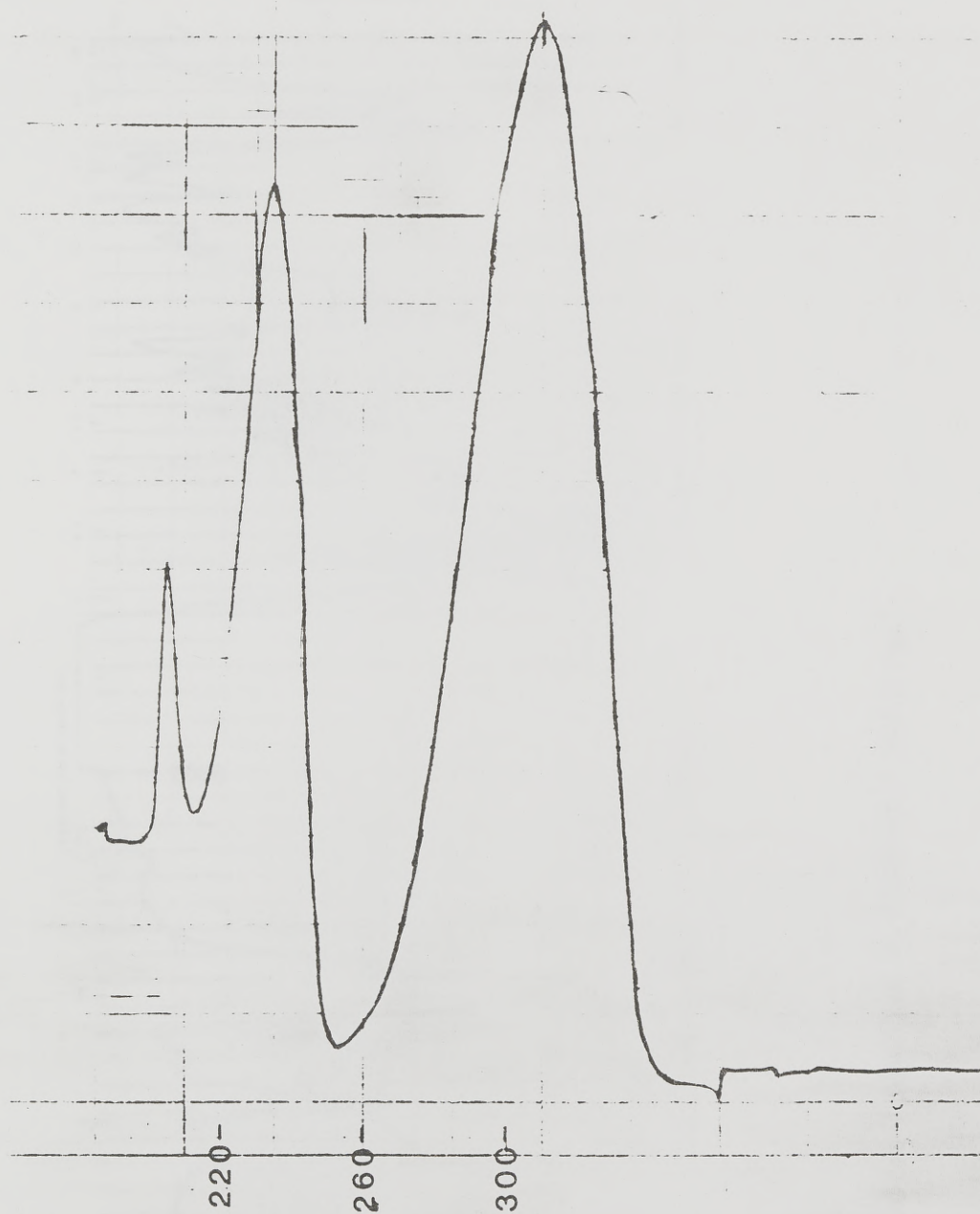
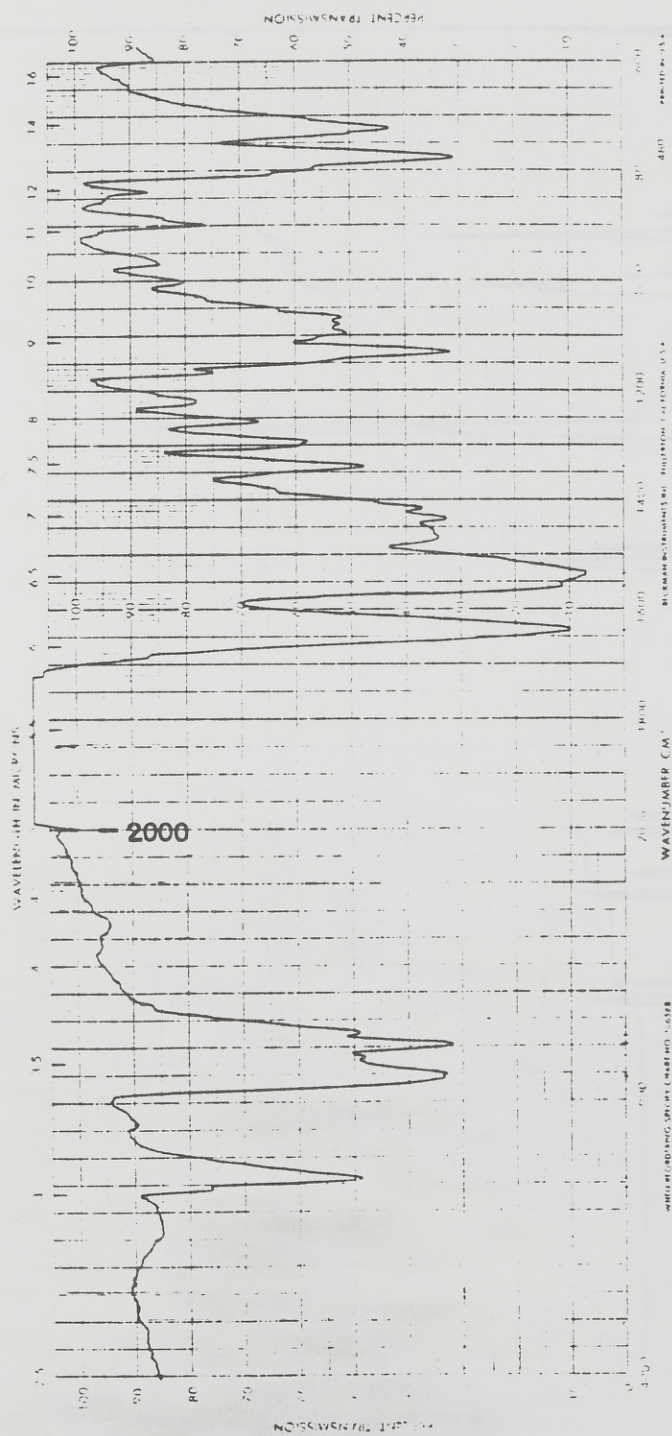


Figure 8. Visible and Ultra-violet Spectrum of Compound 1 (Cytisine).



9. Infrared spectrum of Compound 1 (Cytisine).

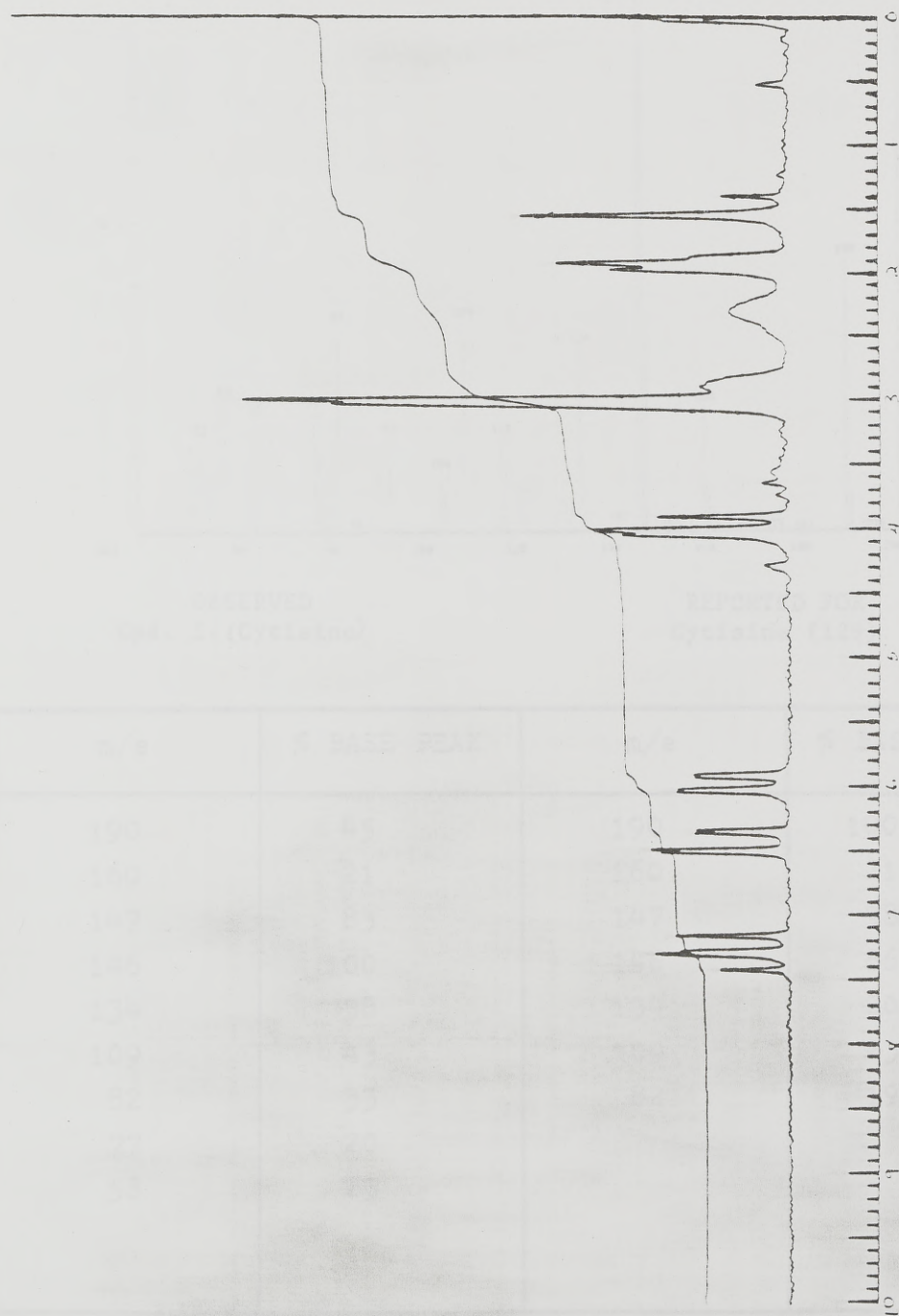
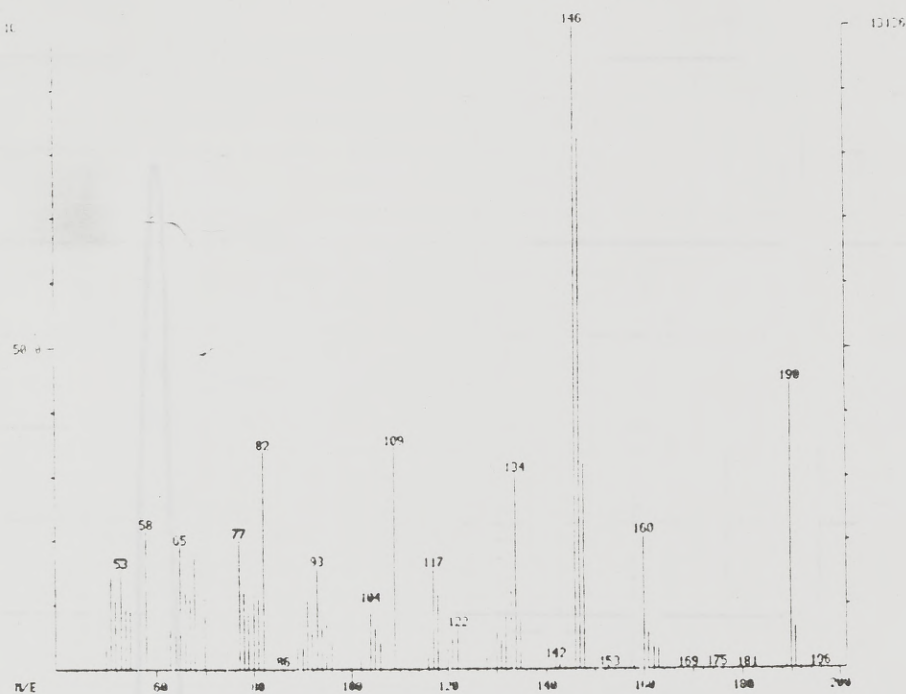


Figure 10. Nuclear Magnetic Resonance Spectrum of Compound 1 (Cytisine).



OBSERVED
Cpd. 1. (Cytisine)

REPORTED FOR
Cytisine (129)

m/e	% BASE PEAK	m/e	% BASE PEAK
190	45	190	100
160	21	160	21
147	83	147	98
146	100	146	86
134	38	134	20
109	43	109	23
82	33	82	19
77	20		
58	23		

Figure 11. Mass Spectrum of Compound 1 (Cytisine).

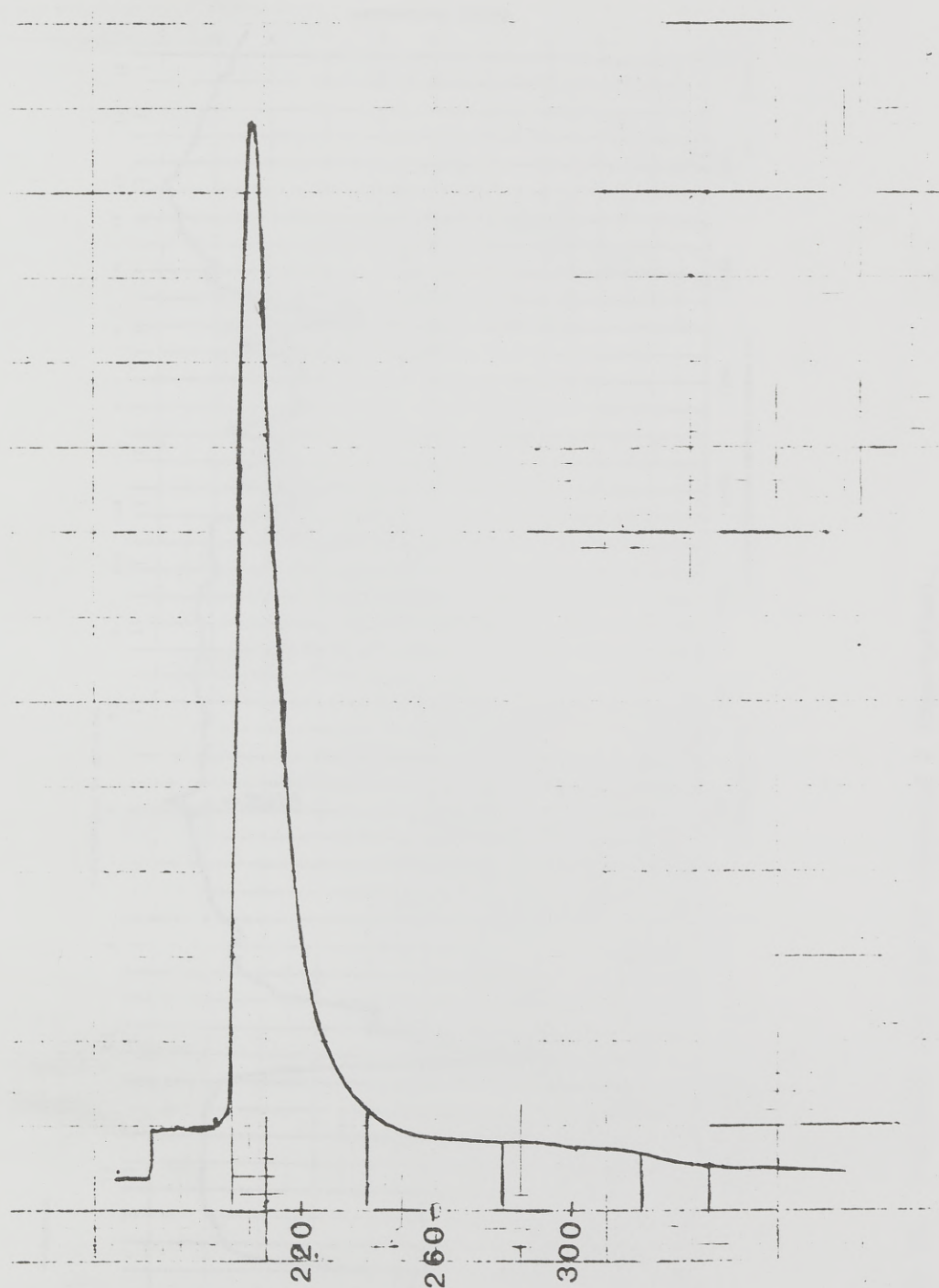


Figure 12. Visible and Ultra-violet Spectrum of Compound 2 (Sparteine).

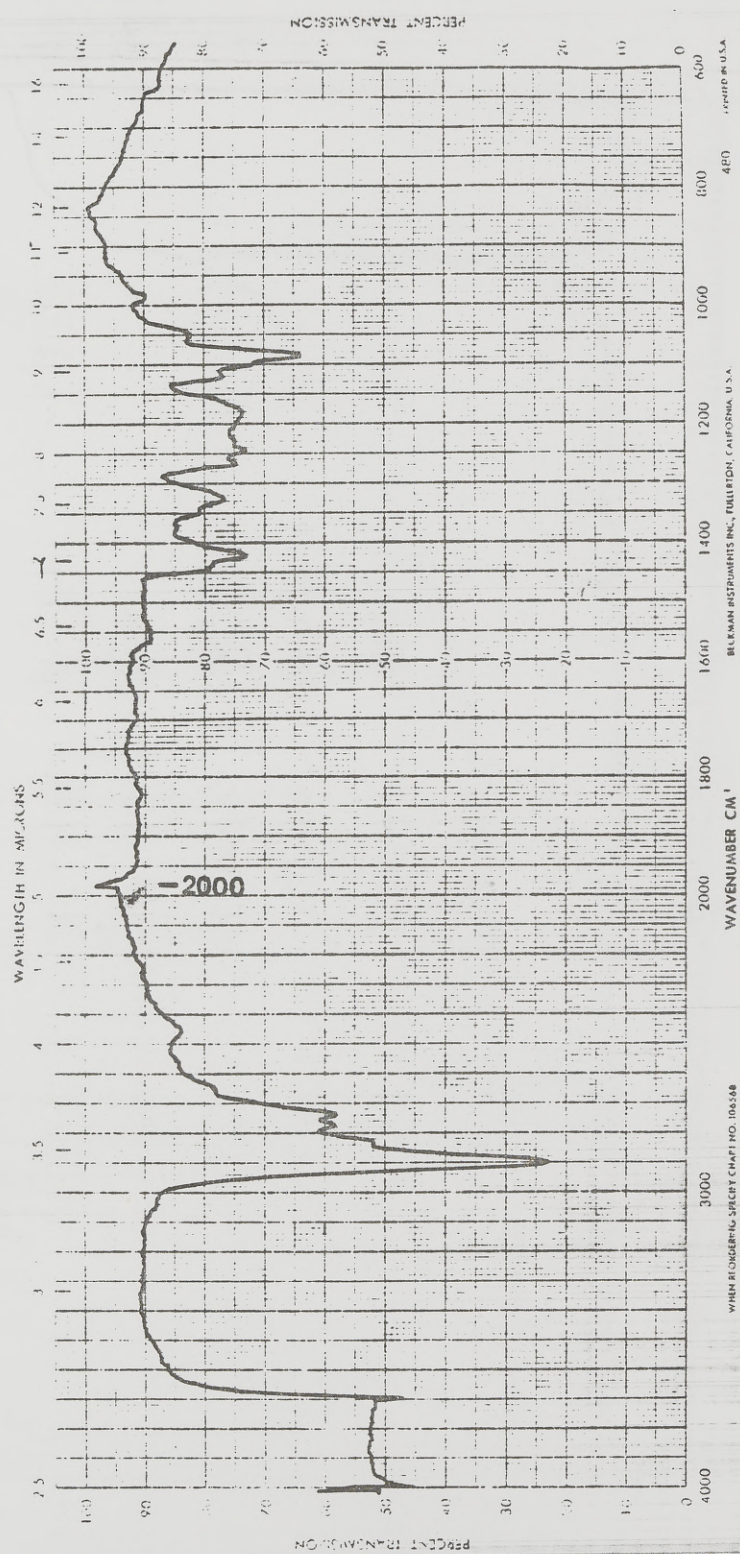


Figure 13. Infrared spectrum of Compound 2 (Sparteine).

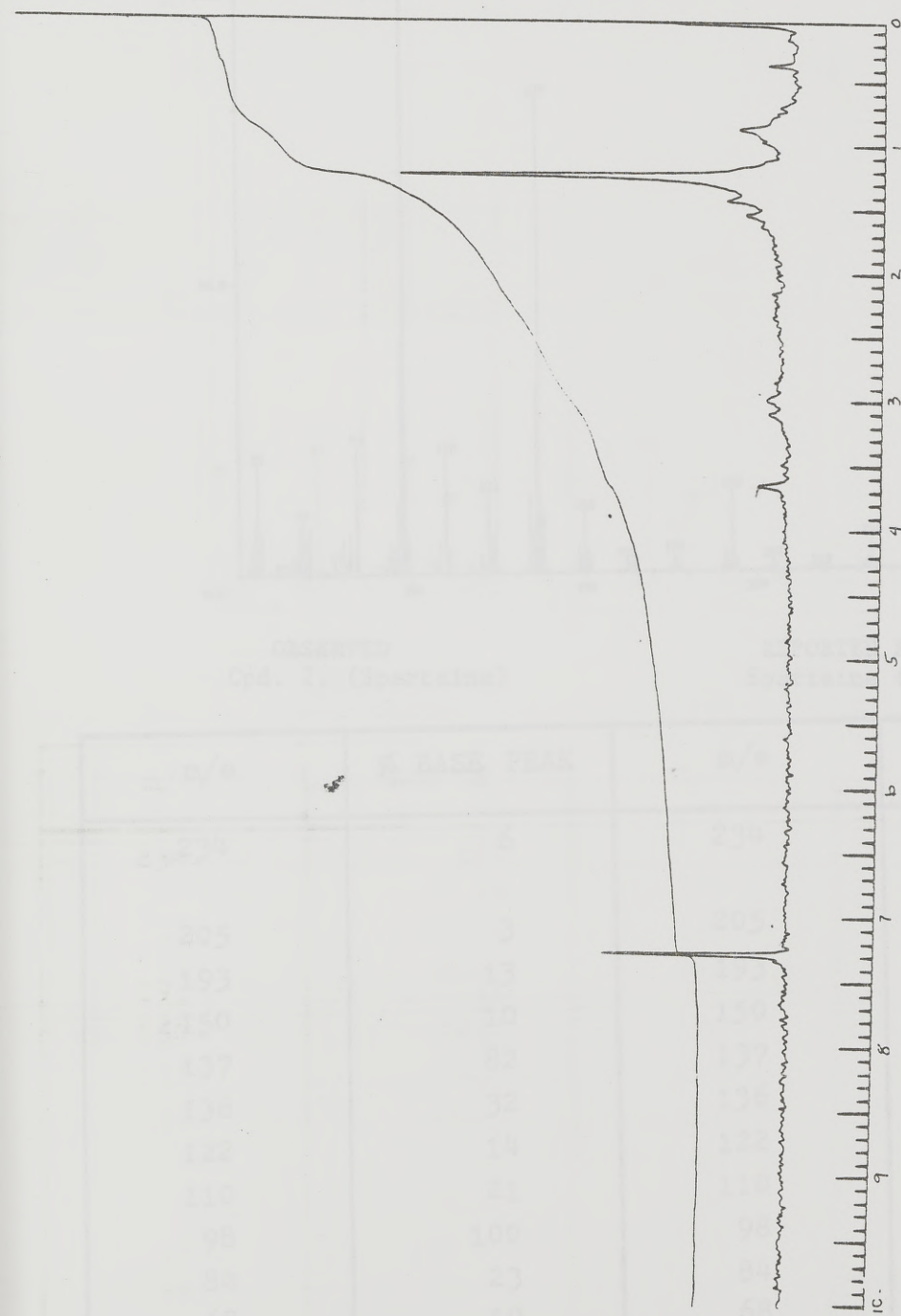
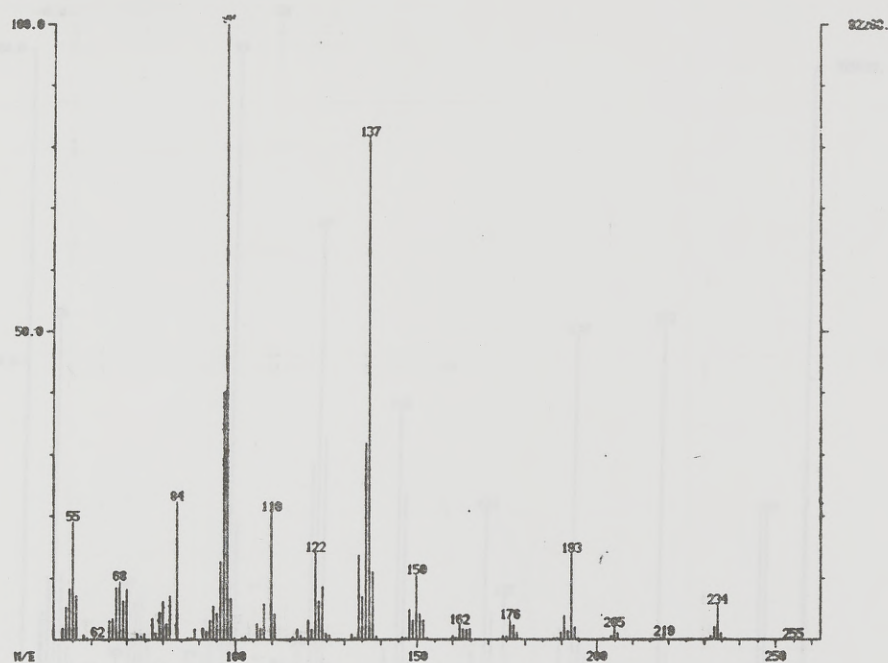


Figure 14. Nuclear Magnetic Resonance Spectrum of Compound 2 (Sparteine).

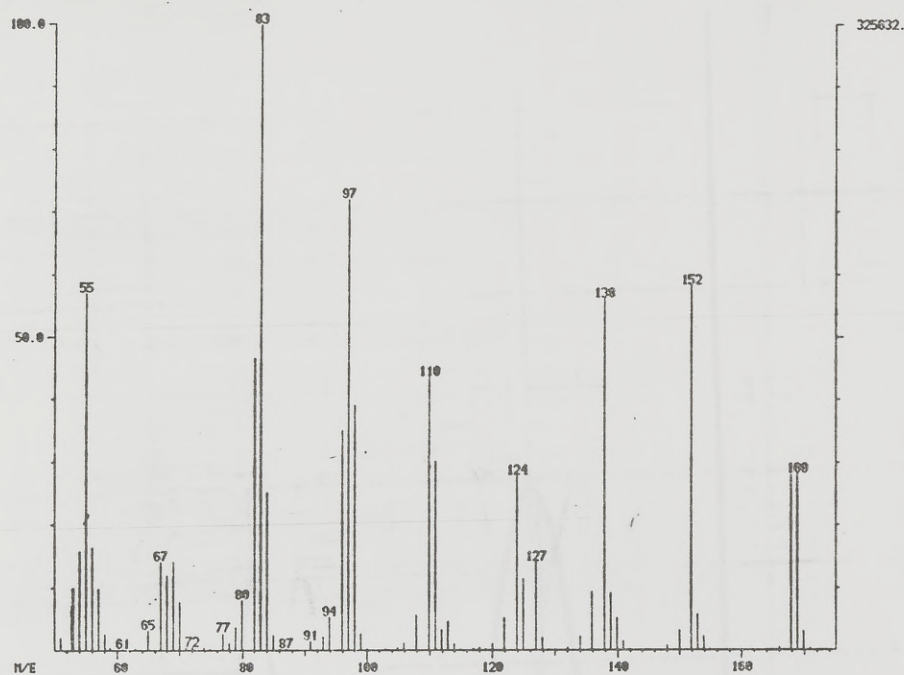


OBSERVED
Cpd. 2. (Sparteine)

REPORTED FOR
Sparteine (129)

m/e	% BASE PEAK	m/e	% BASE PEAK
234	6	234	36
205	3	205	0
193	13	193	32
150	10	150	10
137	82	137	100
136	32	136	28
122	14	122	6
110	21	110	16
98	100	98	92
84	23	84	16
68	10	68	10

Figure 15. Mass Spectrum of Compound 2 (Sparteine).



OBSERVED
Cpd. 3. (Epi-lupinine)

REPORTED FOR
Epi-lupinine (129)

m/e	% BASE PEAK	m/e	% BASE PEAK
169	28	169	74
168	28	168	43
152	58	152	78
138	56	138	74
111	30	111	43
110	43	110	52
97	72	97	69
96	35	96	56
83	100	83	100
55	57	55	59

Figure 16. Mass Spectrum of Compound 3 (Epi-lupinine).

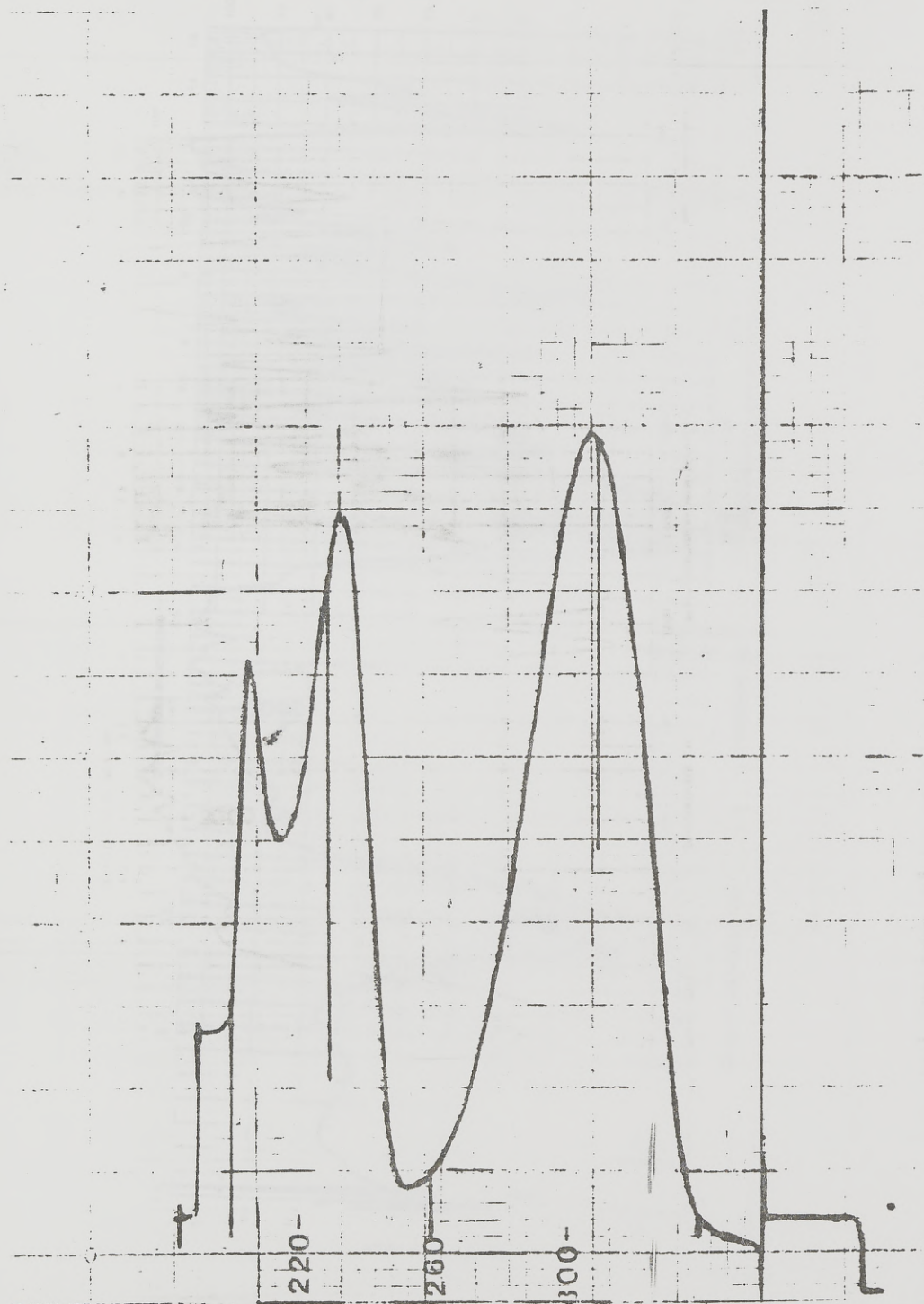


Figure 17. Visible and Ultra-violet Spectrum of Compound 4 (N-methylcytisine).

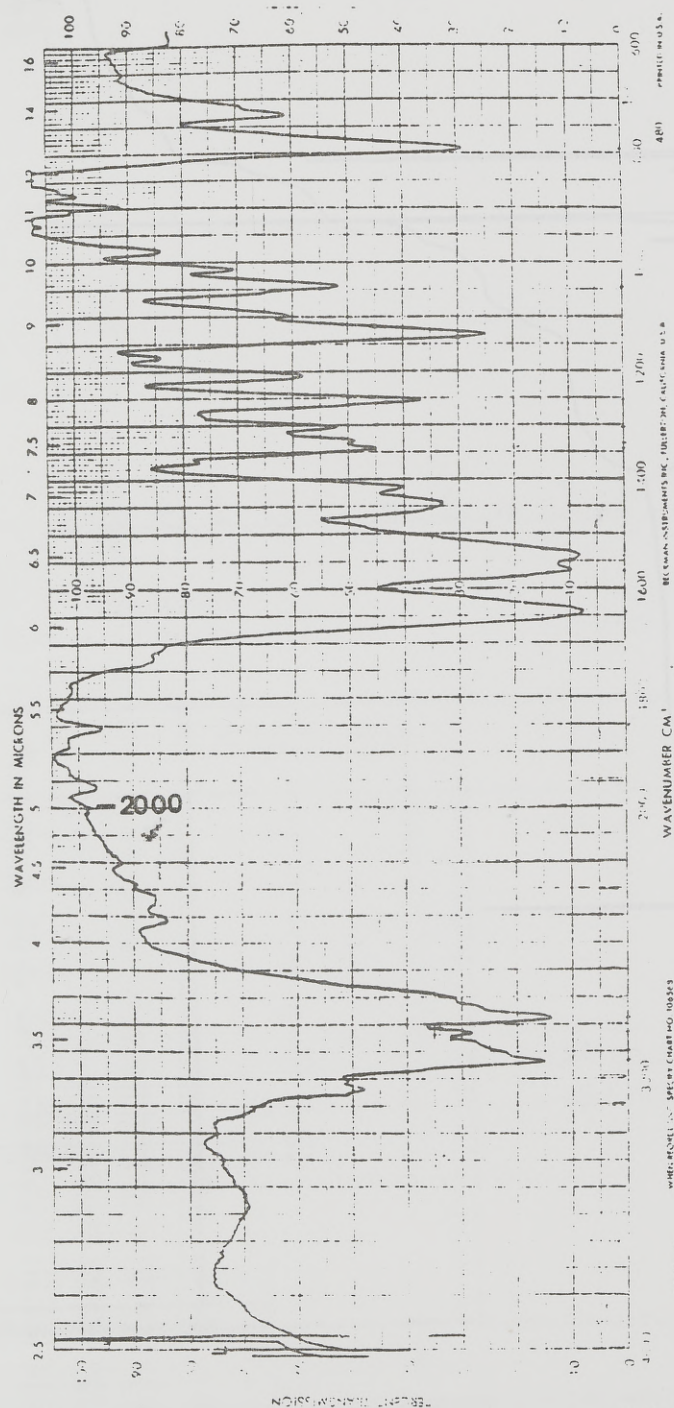


Figure 18. Infrared Spectrum of Compound 4 (N-methylcytisine).

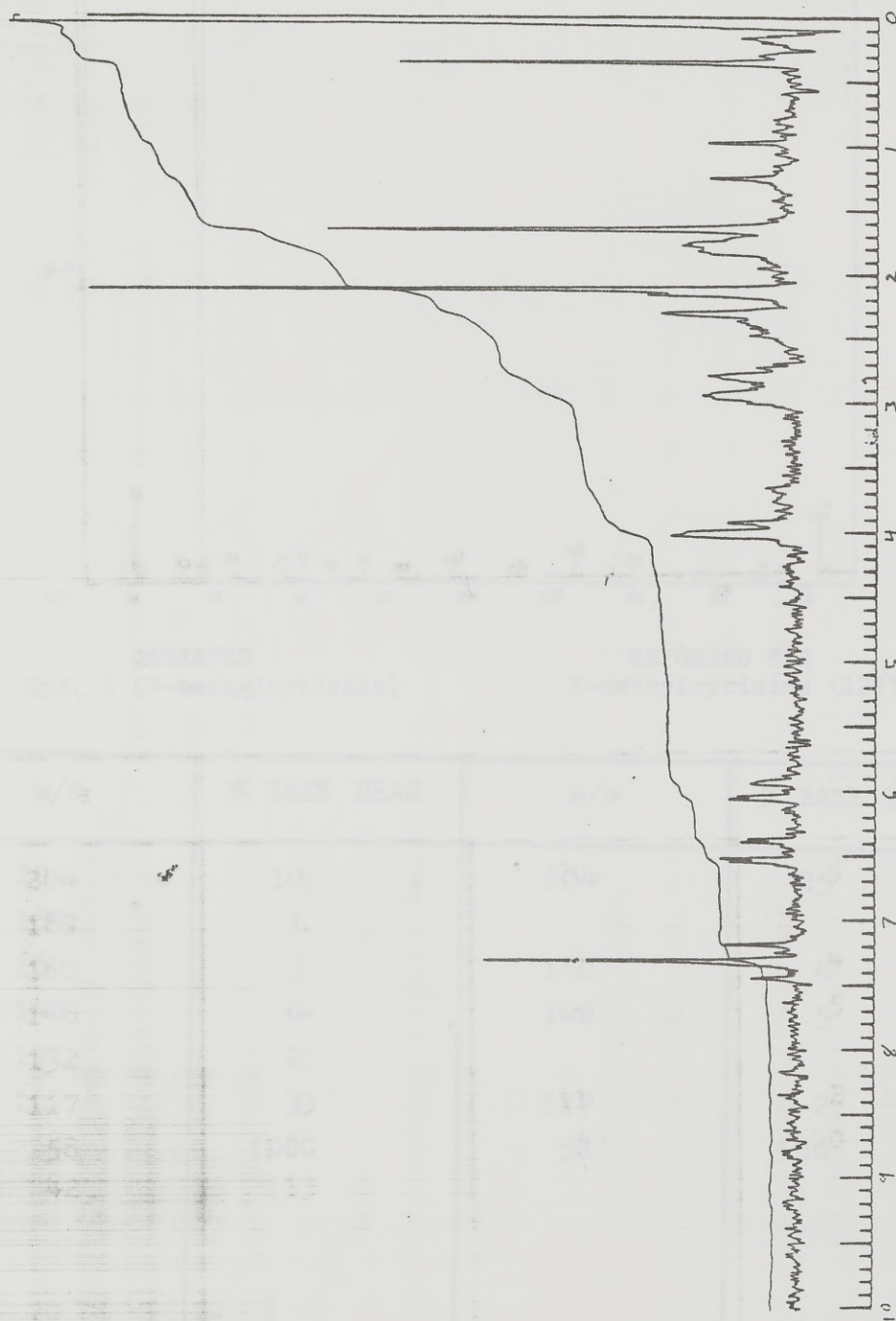
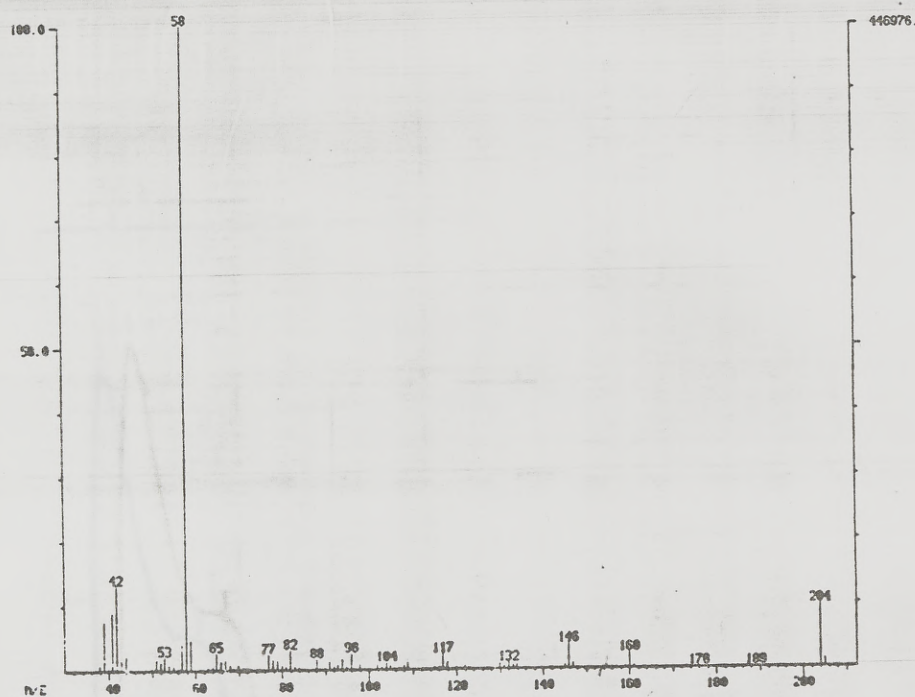


Figure 19. Nuclear Magnetic Resonance Spectrum of Compound 4 (N-methylcytisine).



OBSERVED
Cpd. 4 (N-methylcytisine)

REPORTED FOR
N-methylcytisine (129)

m/e	% BASE PEAK	m/e	% BASE PEAK
204	10	204	25
189	1		
160	3	160	4
146	4	146	5
132	2		
117	3	117	2
58	100	58	100
42	13		

Figure 20. Mass Spectrum of Compound 4 (N-methylcytisine).

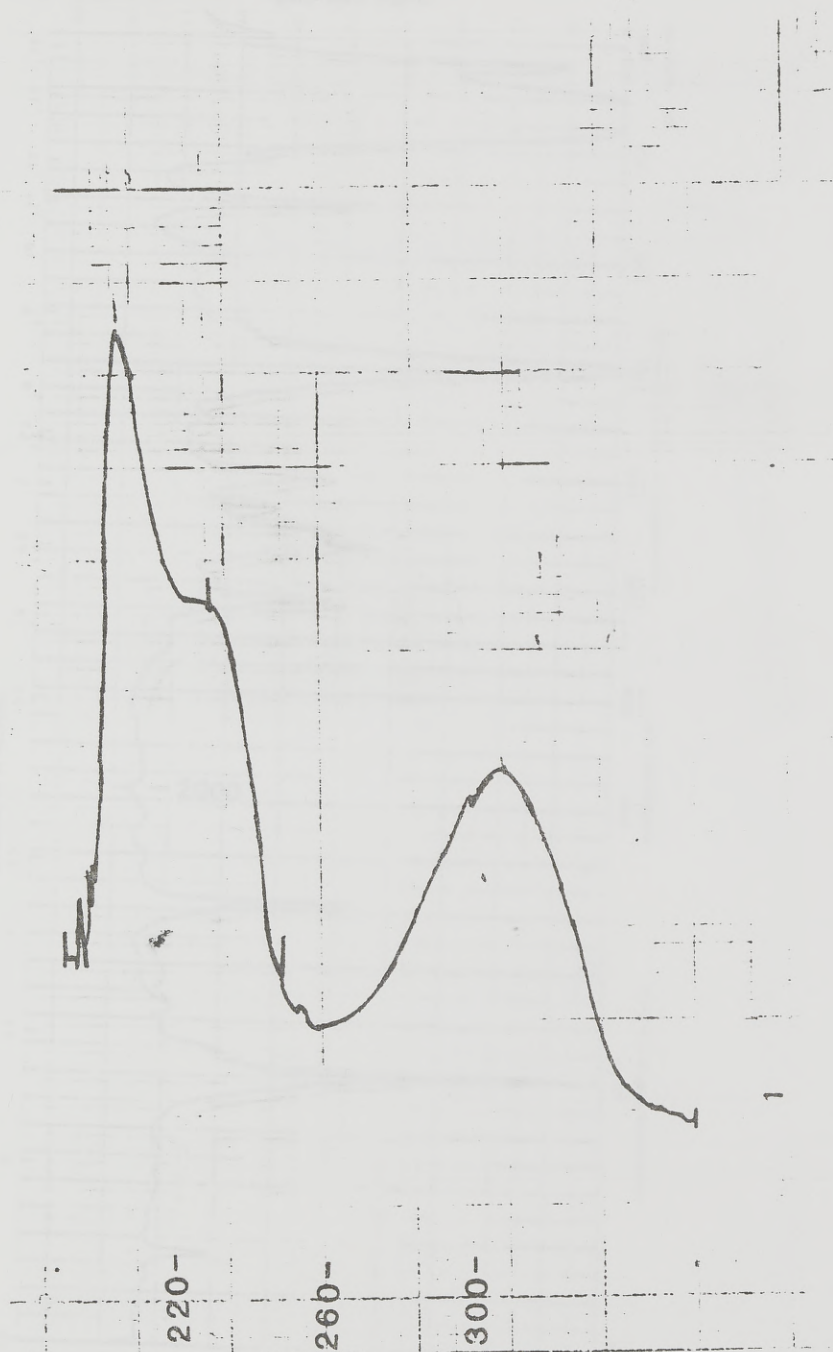


Figure 21. Visible and Ultra-Violet Spectrum of Compound 5 (Rhombifoline).

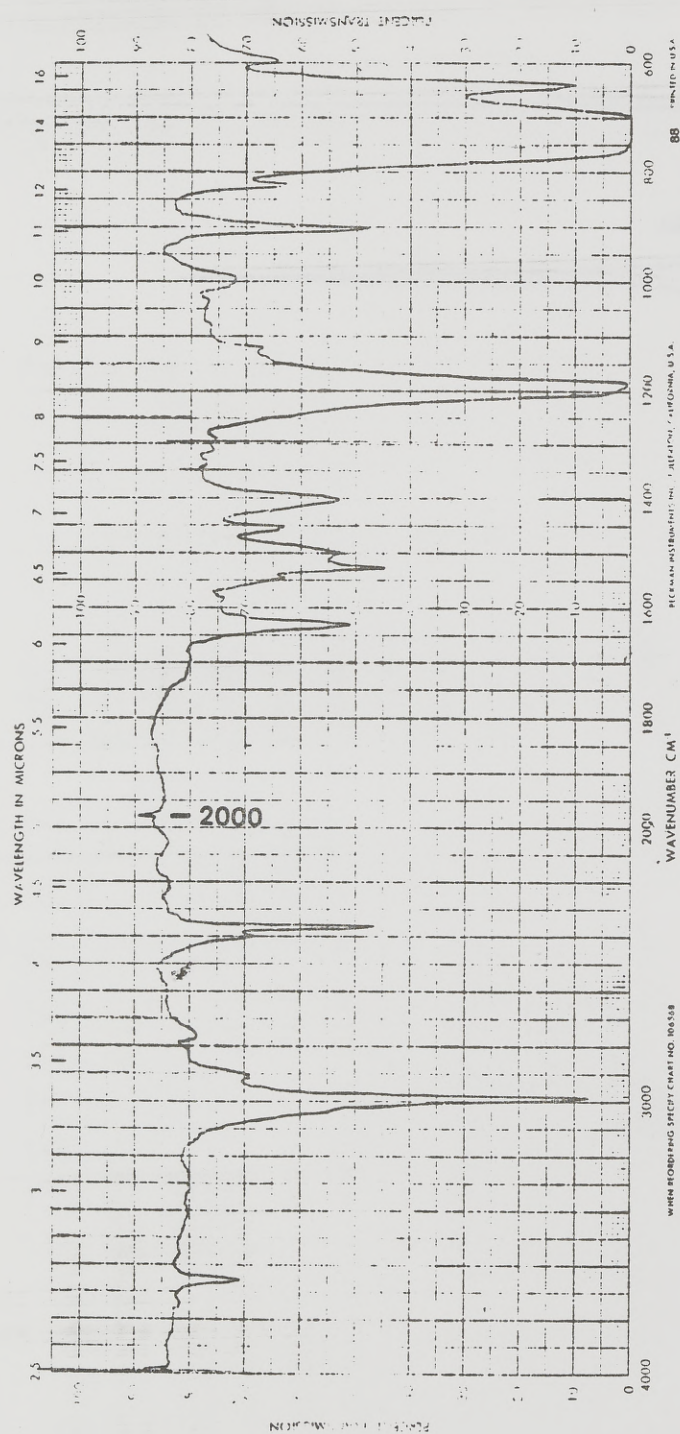


Figure 22. Infrared Spectrum of Compound 5 (Rhombifoline).

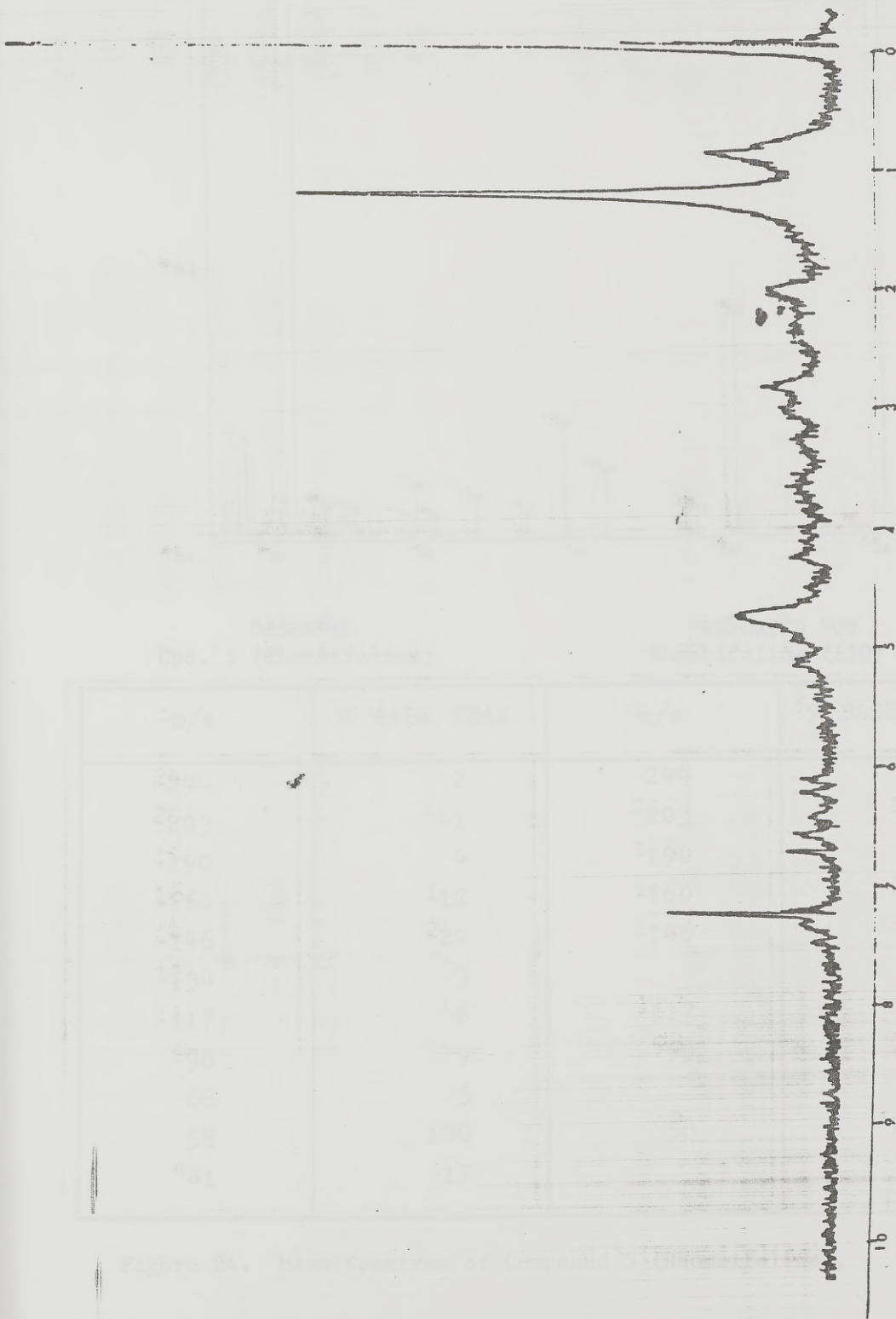
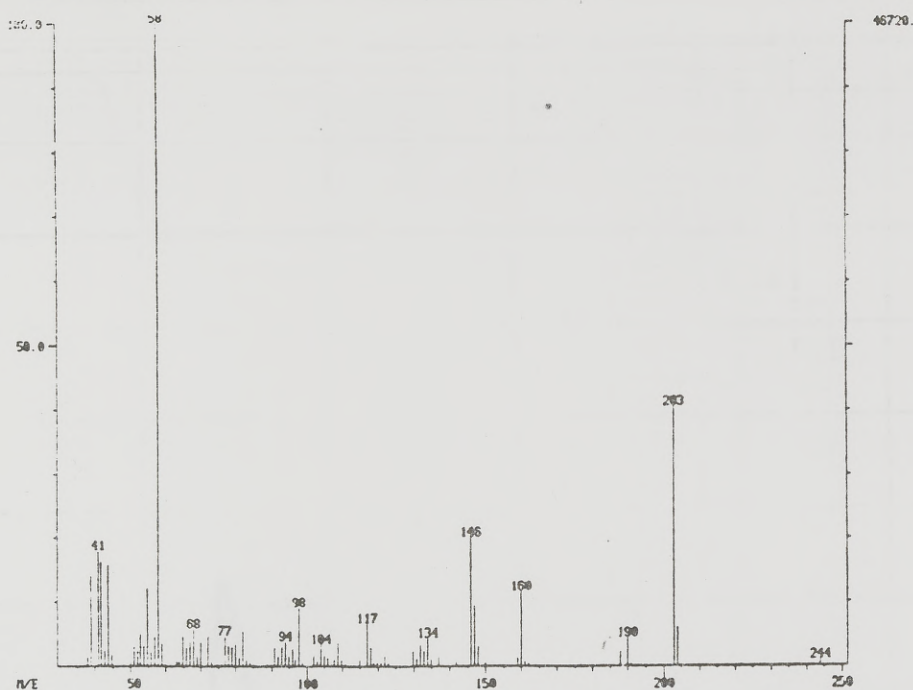


Figure 23. Nuclear Magnetic Resonance Spectrum of Compound 5 (Rhombifoline).



OBSERVED
Cpd. 5 (Rhombifoline)

REPORTED FOR
Rhombifoline (130)

m/e	% BASE PEAK	m/e	% BASE PEAK
244	2	244	5
203	41	203	10
190	4	190	4
160	12	160	10
146	20	146	15
134	3		
117	6	117	3
98	9	98	31
68	5		
58	100	58	100
41	17		

Figure 24. Mass Spectrum of Compound 5 (Rhombifoline).

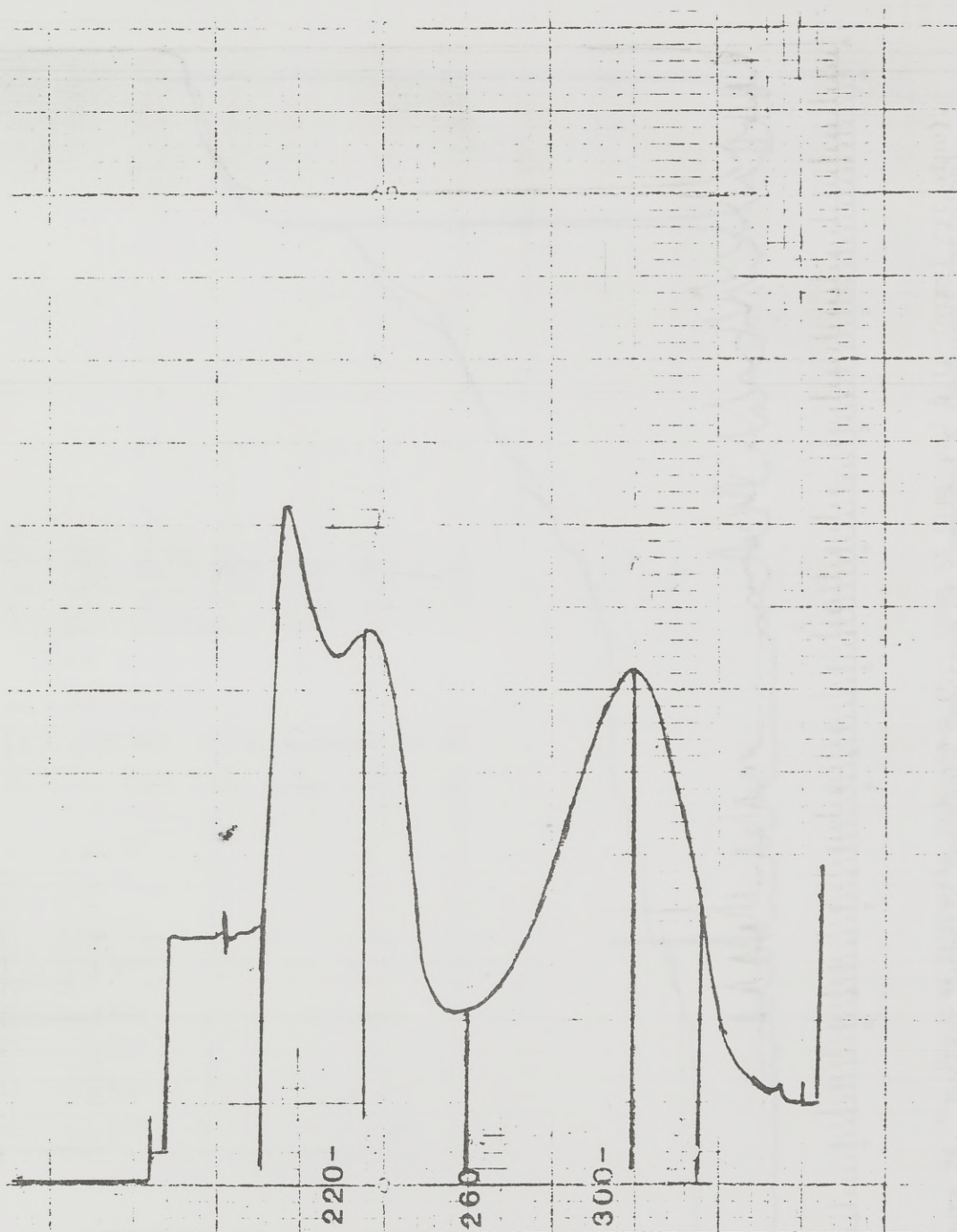


Figure 25. Visible and Ultra-violet Spectrum of Compound 6 (N-formyl cytosine).

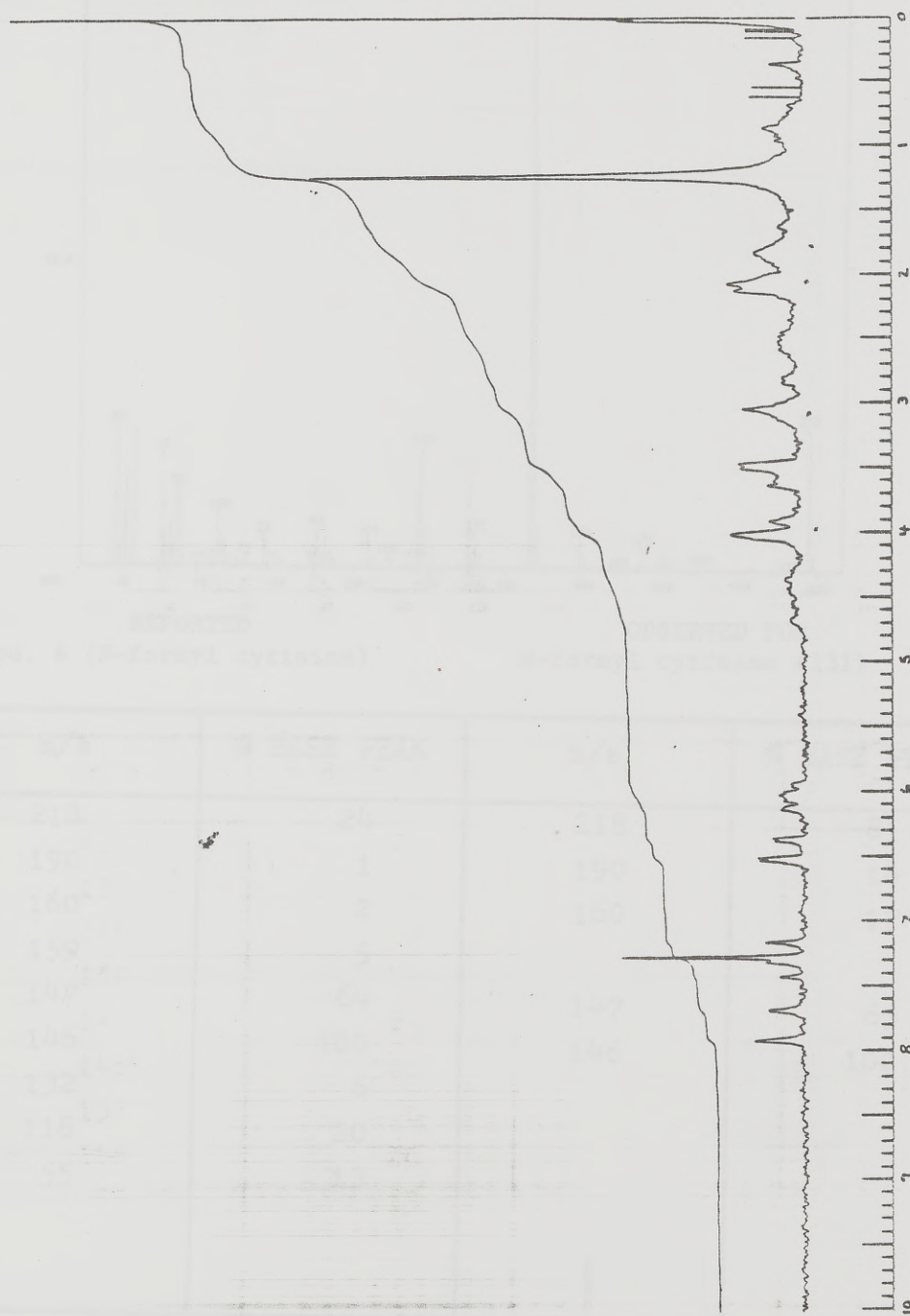
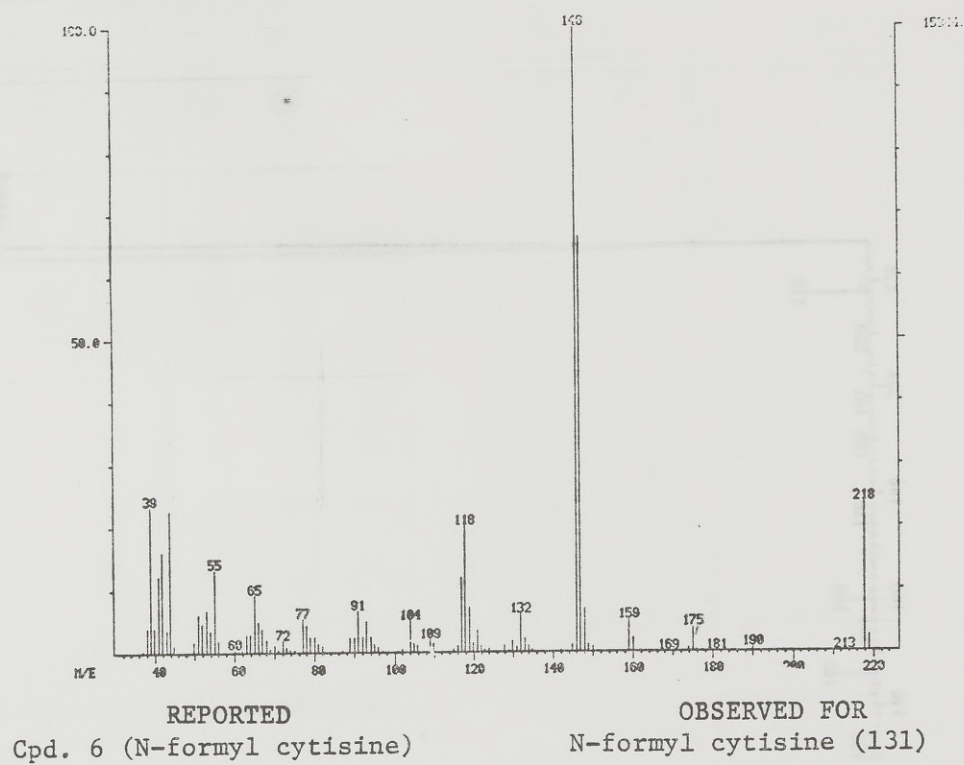


Figure 26. Nuclear Magnetic Resonance Spectrum of Compound 6 (N-formyl cytosine).



m/e	% BASE PEAK	m/e	% BASE PEAK
218	24	218	81
190	1	190	14
160	2	160	17
159	5		
147	64	147	63
146	100	146	100
132	6		
118	20		
55	13		

Figure 27. Mass Spectrum of Compound 6 (N-formyl cytosine).

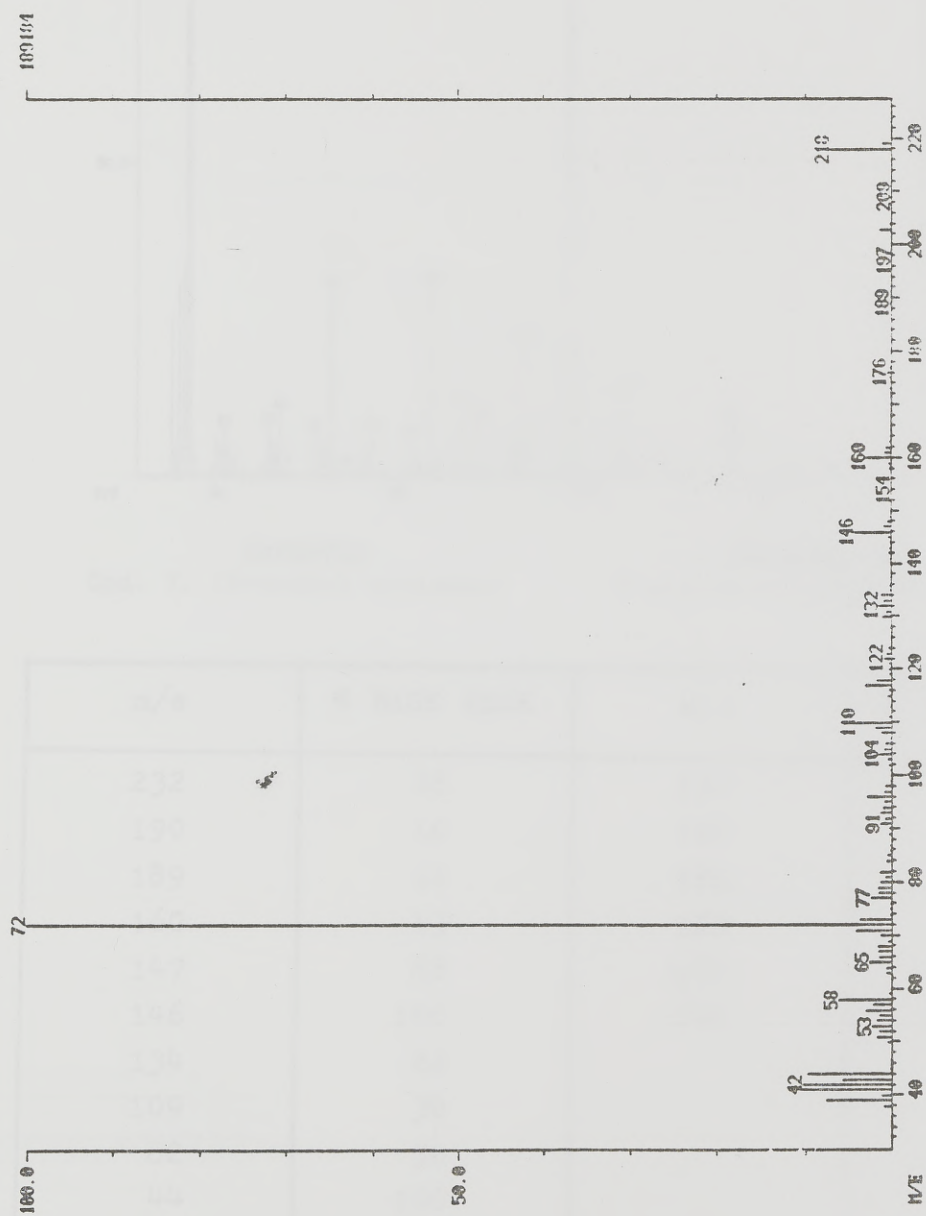
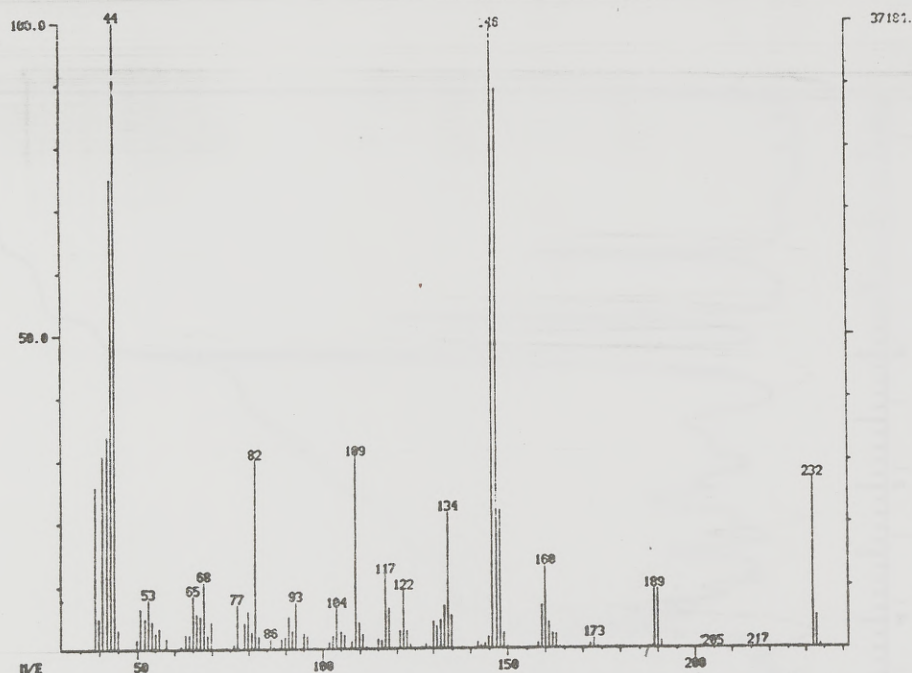


Figure 28. Mass Spectrum of Synthetic N-ethyl cytosine.



OBSERVED
Cpd. 7. (N-acetyl cytosine)

REPORTED FOR
N-acetyl cytosine (131)

m/e	% BASE PEAK	m/e	% BASE PEAK
232	28	232	61
190	10	190	18
189	10	189	16
160	13	160	21
147	88	147	91
146	100	146	100
134	22		
109	30		
82	30		
44	100		

Figure 29. Mass Spectrum of Compound 7 (N-acetyl cytosine).

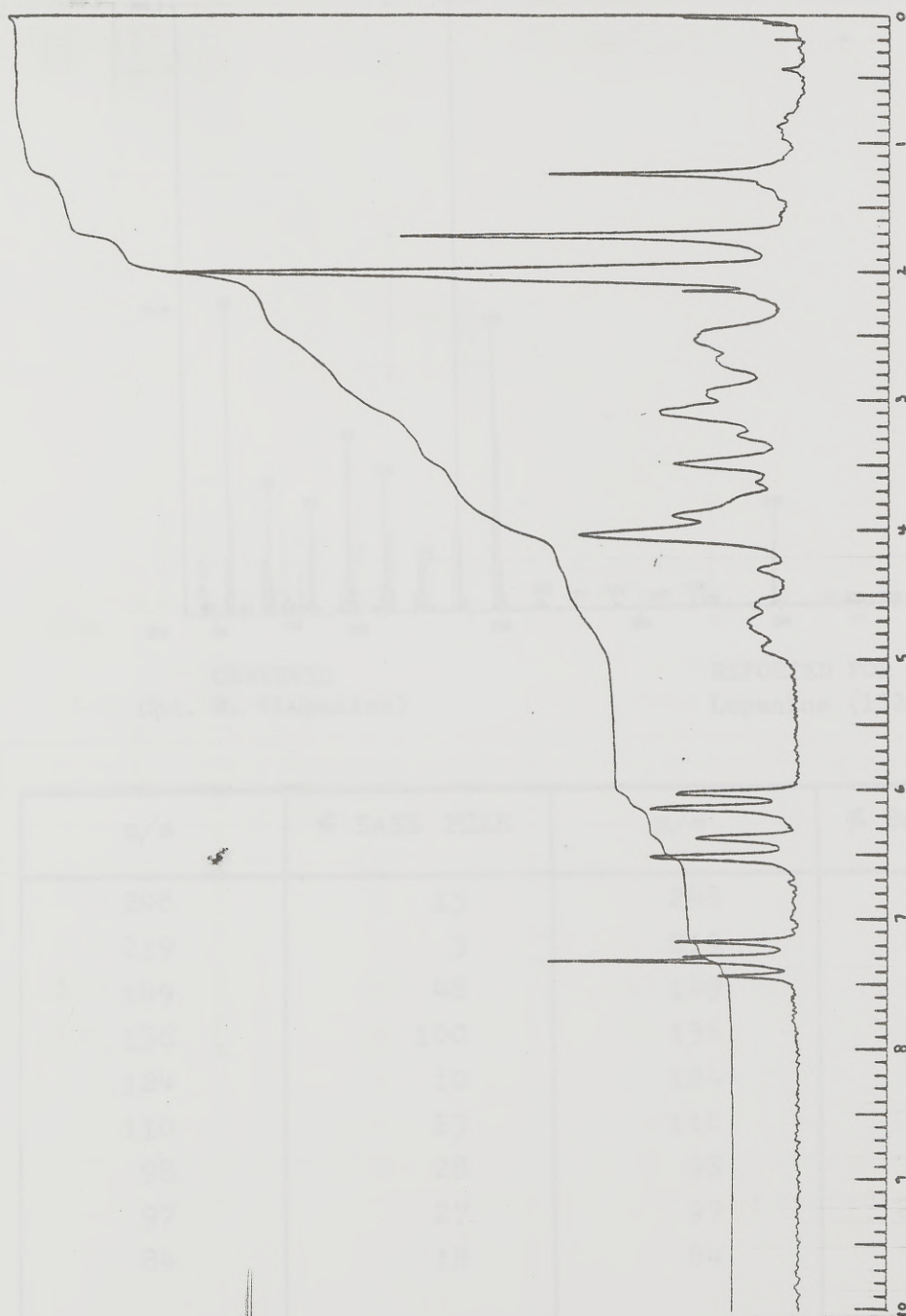
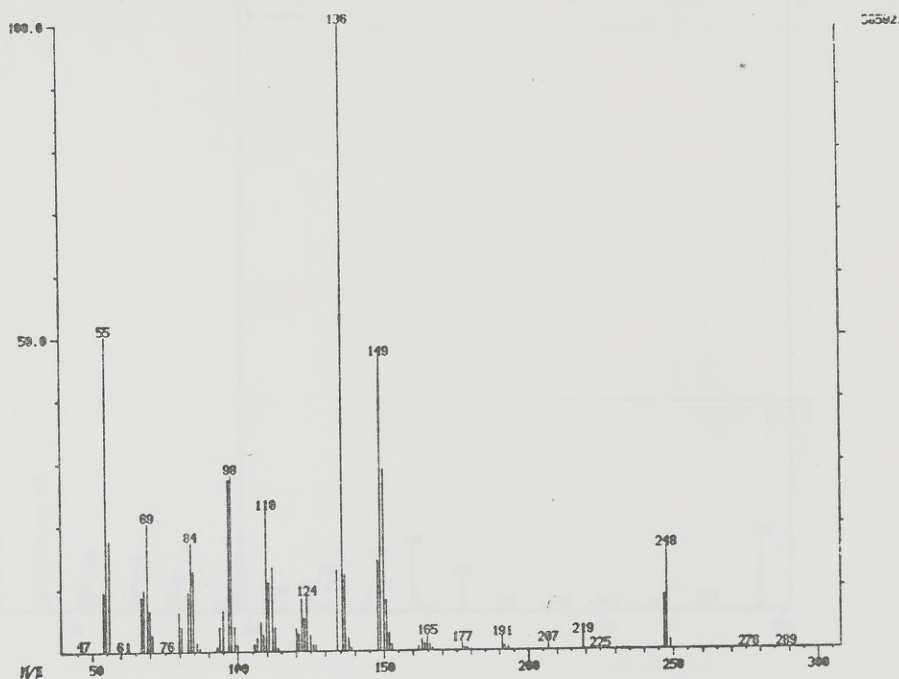


Figure 30. Nuclear Magnetic Resonance Spectrum of Synthetic N-acetyl cytosine.



OBSERVED
Cpd. 8. (Lupanine)

REPORTED FOR
Lupanine (132)

m/e	% BASE PEAK	m/e	% BASE PEAK
248	15	248	67
219	3	219	4
149	48	149	58
136	100	136	100
124	10	124	40
110	23	110	21
98	28	98	21
97	27	97	25
84	18	84	19

Figure 31. Mass Spectrum of Compound 8 (Lupanine).

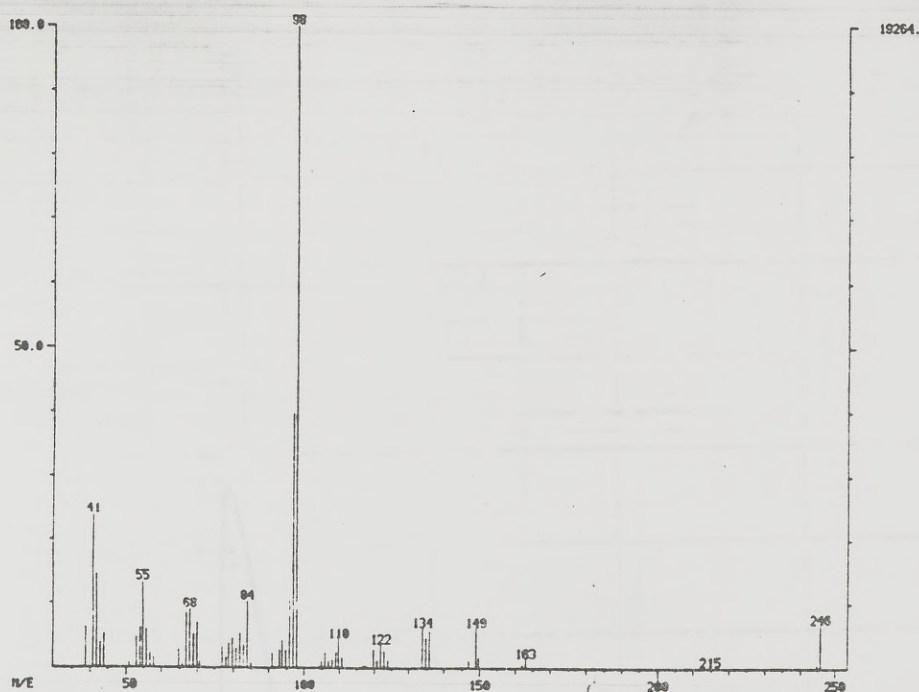


OBSERVED
Cpd. 9 (Anagyrine)

REPORTED FOR
Anagyrine HBr (129)

m/e	% BASE PEAK	m/e	% BASE PEAK
244	13	244	51
188	1	190	5
160	5	160	10
146	10	146	16
136	8	136	6
117	4	117	4
98	100	98	100
55	8		

Figure 32. Mass Spectrum of Compound 9 (Anagyrine).



OBSERVED
Cpd. 10. (Δ 5,6 Dehydrolupanine

REPORTED FOR
 Δ 5,6-Dehydrolupanine (128)

m/e	% BASE PEAK	m/e	% BASE PEAK
246	7	246	33
215	1	215	2
163	2	163	4
149	6	149	2
134	6	134	6
122	3	122	6
98	100	98	100
97	40	97	43
84	10	84	8
68	8	68	8

Figure 33. Mass Spectrum of Compound 10 (Δ 5,6-Dehydrolupanine).

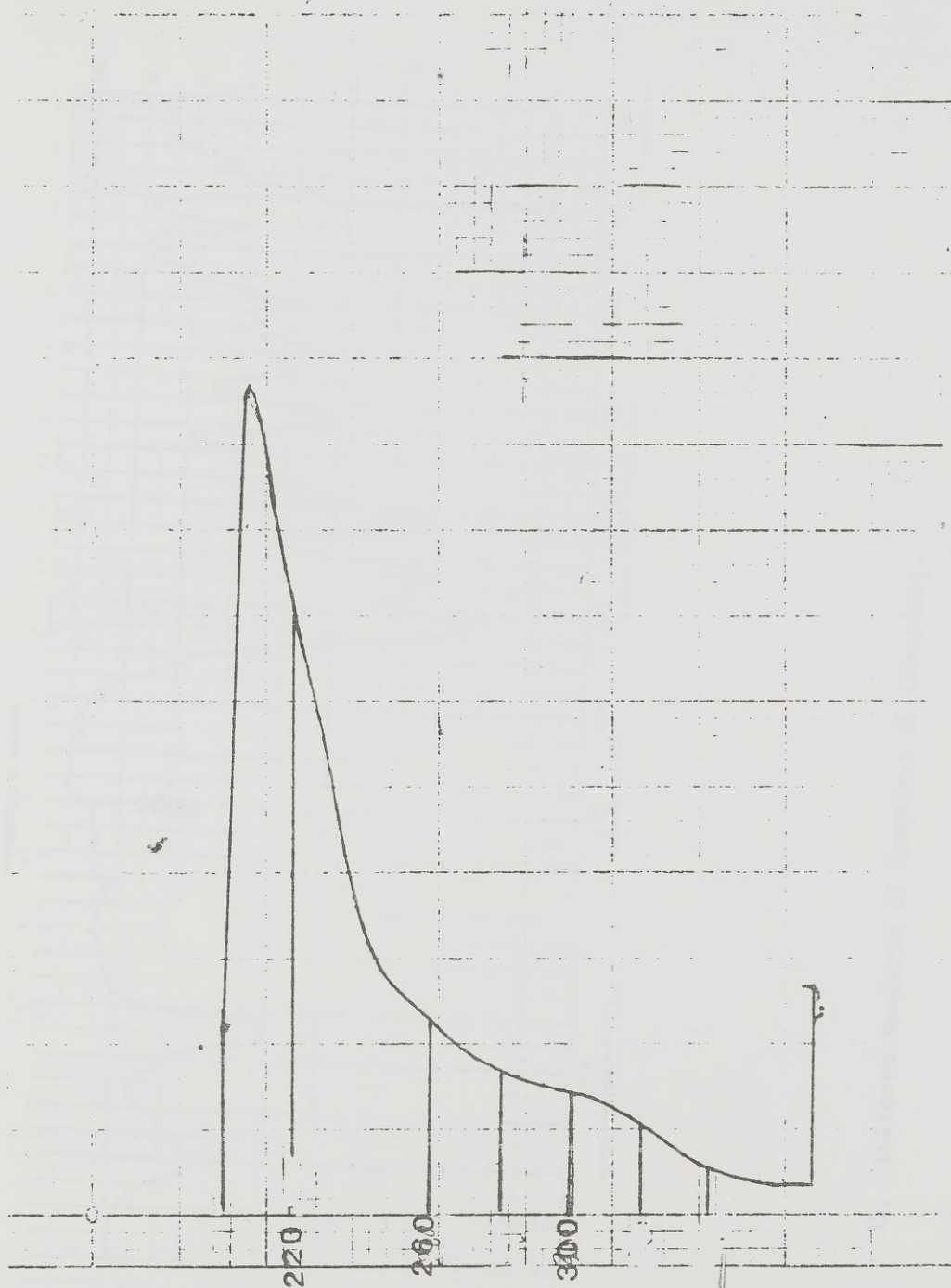
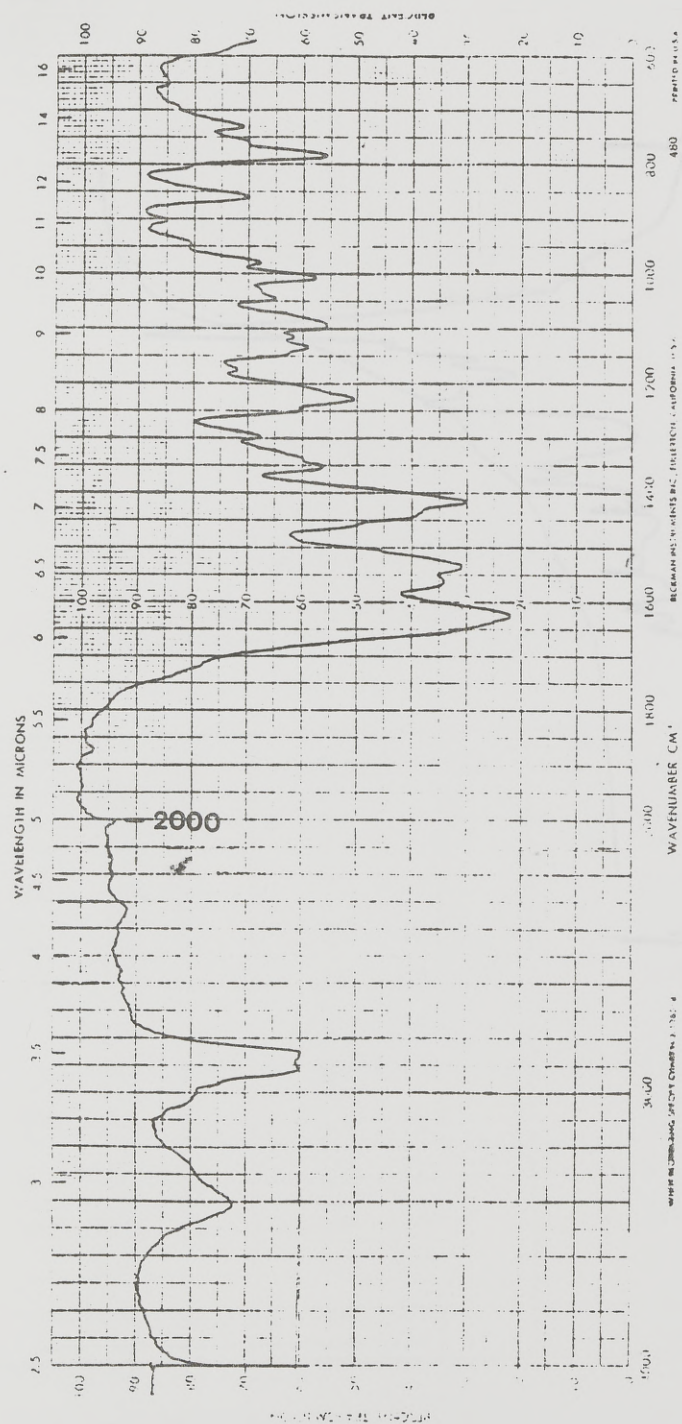


Figure 34. Visible and Ultra-violet Spectrum of Compound 11 (Matrine).



35. Infrared Spectrum of Compound 11 (Matrine).

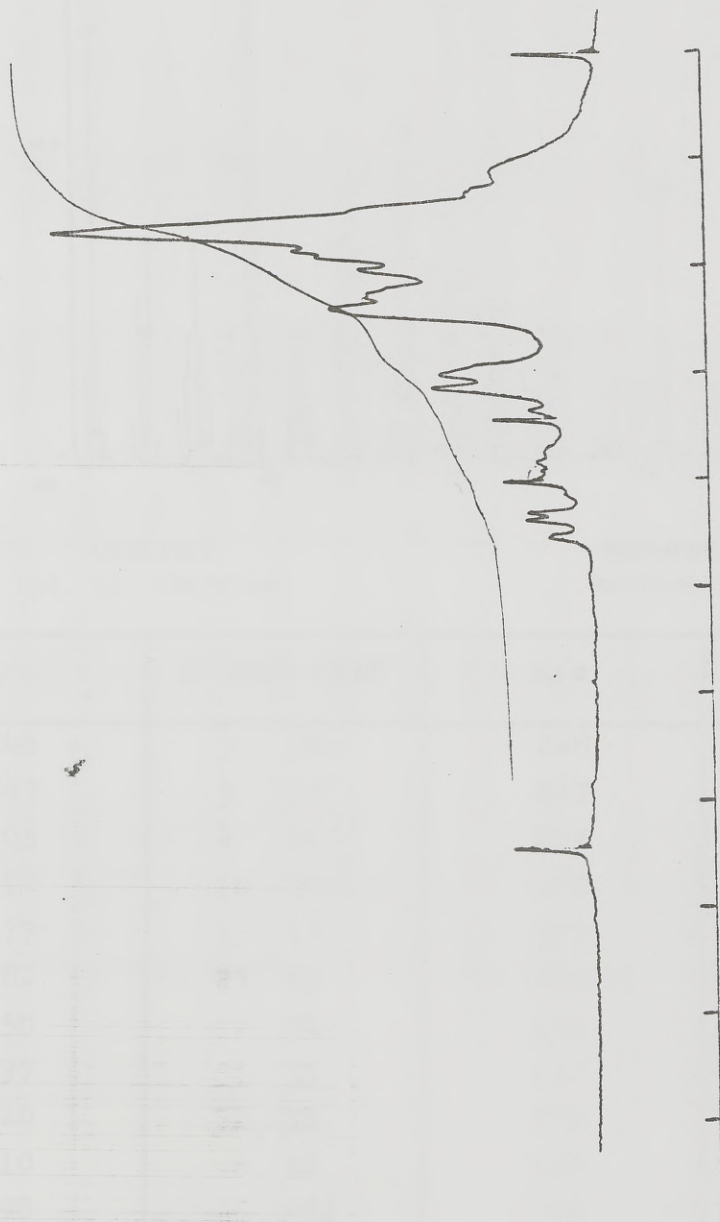
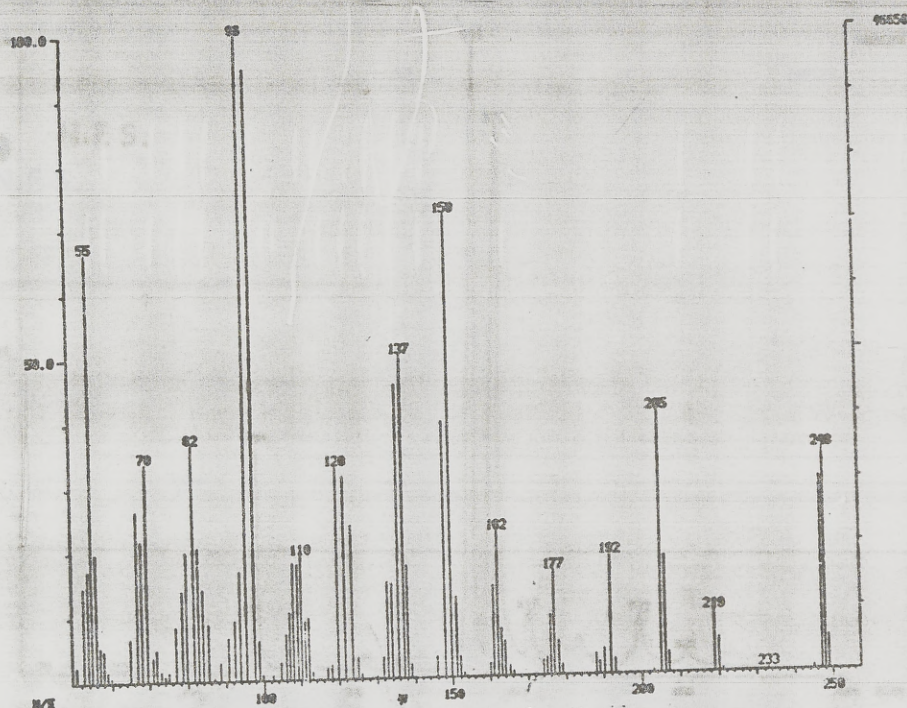


Figure 36. Nuclear Magnetic Resonance Spectrum of Compound 11 (Matrine).



OBSERVED
Cpd. 11. (Matrine)

REPORTED FOR
Matrine (134)

m/e	% BASE PEAK	m/e	% BASE PEAK
248	34	248	100
247	30	247	63
205	40	205	45
192	18	192	40
177	15	177	29
162	23	162	20
150	72	150	55
137	51	137	47
120	32	120	32
110	18	110	19
98	96	98	43
96	100	96	95

Figure 37. Mass Spectrum of Compound 11 (Matrine).

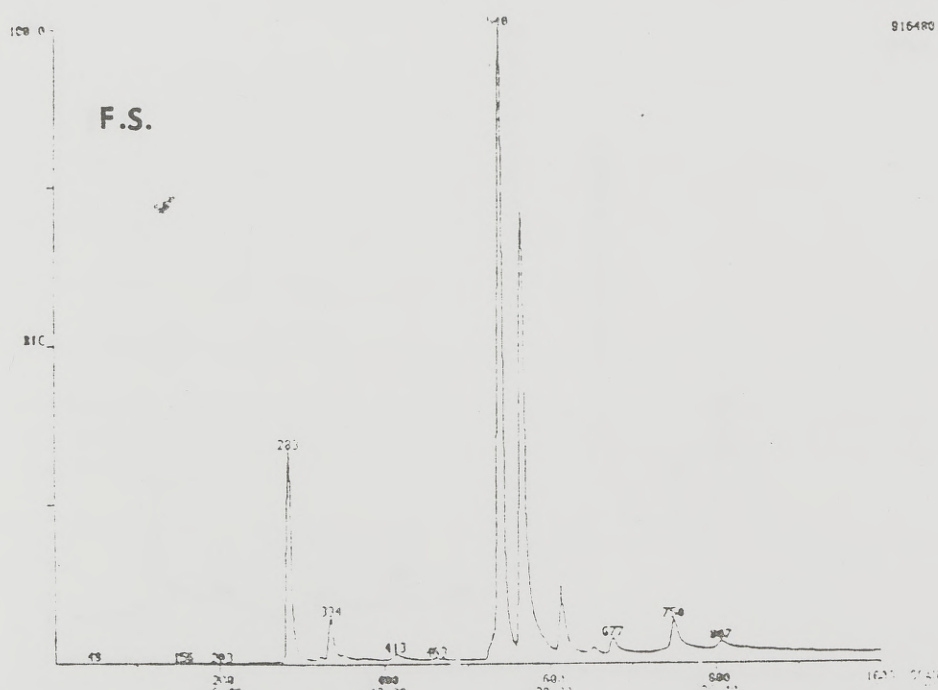
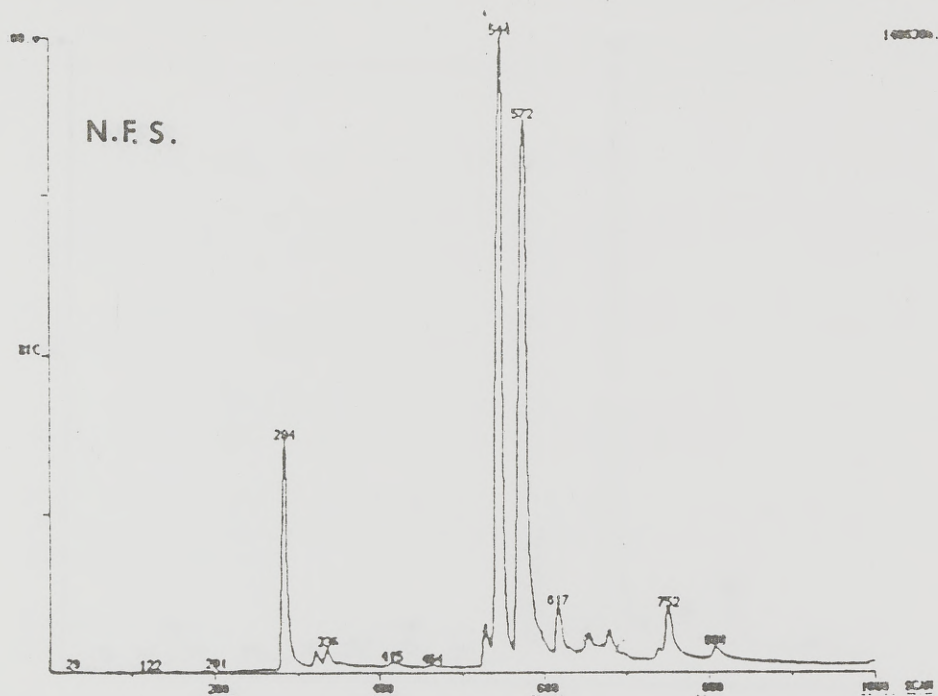


Figure 38. Semi-quantitative GC Comparison of Non-fasciated Stem (N.F.S.) and Fasciated Stem (F.S.) of *S. secundiflora* with Oxosparteine as Internal Standard 544 and 540.

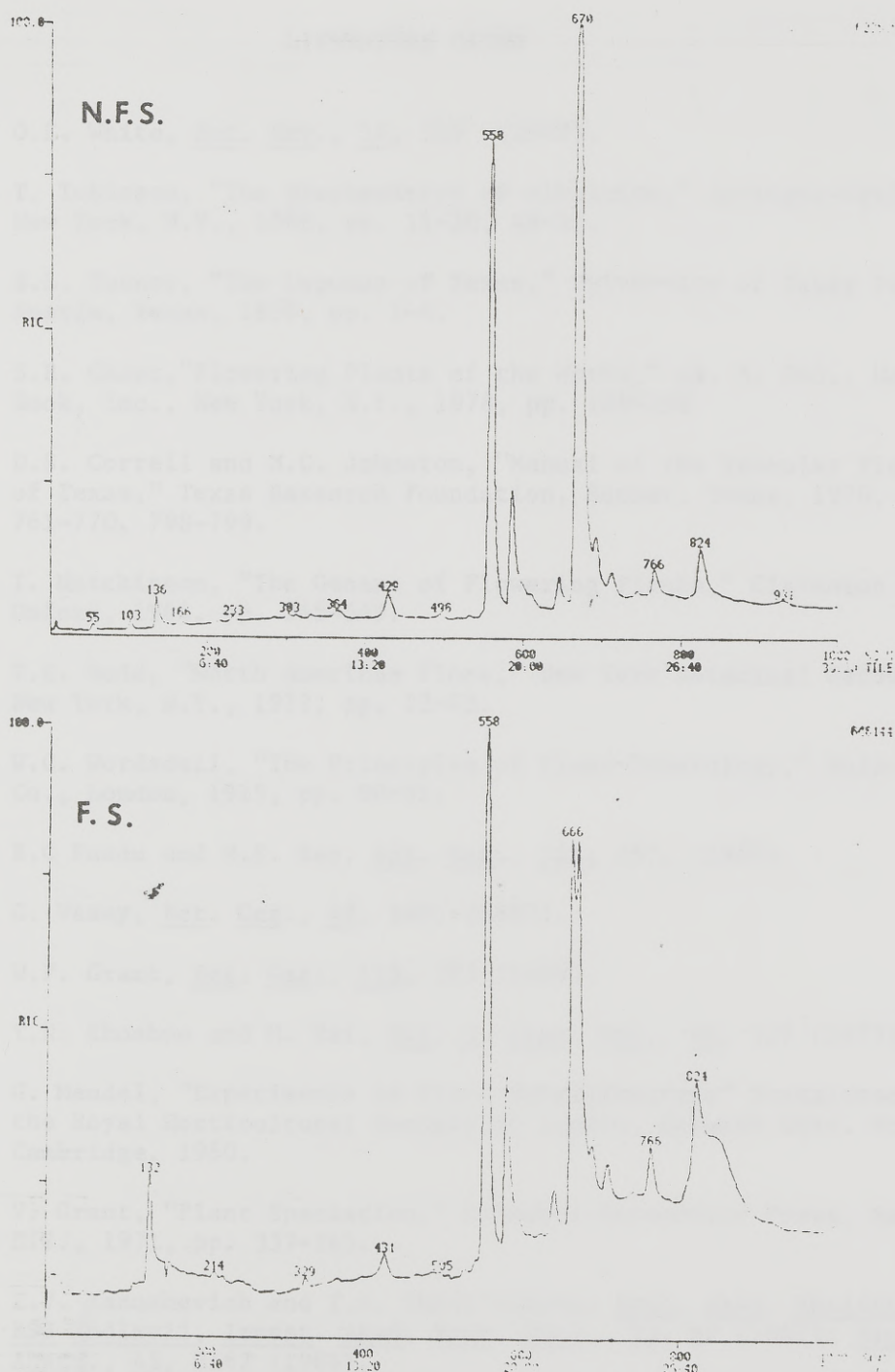


Figure 39. Semi-quantitative GC Comparison of Non-fasciated Stem (N.F.S.) and Fasciated Stem (F.S.) of *S. tomentosa* with Oxosparteine as Internal Standard.

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