AN ABSTRACT OF THE THESIS OF

Jeannette Talbot Hovermale for the degree of Doctor of Philosophy in Chemistry presented on January 22, 1998. Title: Metabolism of Pyrrolizidine Alkaloids by Ruminal Microbes.

Abstract approved: Redacted for Privacy

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The plant *Senecio jacobaea* (tansy ragwort) produces several macrocyclic pyrrolizidine alkaloids which cause irreversible liver cirrhosis. All of the pyrrolizidine alkaloids in *Senecio jacobaea* are macrocyclic diesters of the necine base retronecine, with the two most predominant being seneciphylline and jacobine. Unlike horses and cattle, sheep and goats are generally resistant to chronic pyrrolizidine alkaloid toxicosis due to metabolism of the toxic pyrrolizidine alkaloids by ruminal bacteria.

In this study, metabolism of jacobine and seneciphylline by ruminal bacteria was investigated, focusing on two possible metabolic pathways. One pathway involved hydrolysis of jacobine and seneciphylline and the subsequent production of the necine base retronecine. For use in these studies, retronecine was isolated and labeled with deuterium. A method was developed for the determination of retronecine to 0.02 µg/mL. Significant hydrolysis of jacobine and seneciphylline was not observed in either ovine whole rumen fluid or with a mixed culture of anaerobic microbes derived from ovine rumen fluid which metabolizes jacobine and

seneciphylline. Direct metabolism of retronecine independent of the macrocyclic pyrrolizidine alkaloids was also not observed.

The second metabolic pathway studied involved the production of the necine base modified by the conversion of the 1,2-double bond to an external methylene group. Previously this conversion has been observed during metabolism of pyrrolizidine alkaloids by the organism *Peptostreptococcus heliotrinreducens*. In this study, *P. heliotrinreducens* was used to convert the pyrrolizidine alkaloids heliotrine and lasiocarpine to the known 1-methylene-pyrrolizidines. The mixed culture of ovine anaerobic microbes also metabolized heliotrine and lasiocarpine rapidly to identical methylene products. This mixed culture metabolized jacobine and seneciphylline rapidly with production of very low levels of the corresponding 1-methylene compounds. In contrast, metabolism of jacobine or seneciphylline by *P. heliotrinreducens* was not observed. The mixed culture has demonstrated the ability to metabolize a greater variety of pyrrolizidine alkaloids than *P. heliotrinreducens*.

Although the metabolites of jacobine and seneciphylline were not conclusively identified, it was determined that hydrolysis of jacobine and seneciphylline is not occurring. The second pathway studied appears to be more probable, with the production of 1-methylene compounds as intermediates, although not as end-products.

Metabolism of Pyrrolizidine Alkaloids by Ruminal Microbes

by

Jeannette Talbot Hovermale

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CONTRIBUTION OF AUTHORS

Dr. Morrie Craig was involved in the design, analysis, and writing of each manuscript. Dr. Fraser Fleming assisted in the experimental design and manuscript writing for the synthesis portion of this work. The isolation and synthesis were performed in the laboratory of Dr. James White who also assisted in the experimental design for the synthesis portion of this work.

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METABOLISM OF PYRROLIZIDINE ALKALOIDS BY RUMINAL MICROBES

Chapter 1

INTRODUCTION

BACKGROUND

Throughout the world, pyrrolizidine alkaloids are produced by nearly 8000 species of plants (Culvenor, 1980). Approximately 300 unique pyrrolizidine alkaloids have been isolated and identified. Many pyrrolizidine alkaloids are hepatotoxic to both livestock and humans, with the principle pathology being irreversible liver cirrhosis with pronounced fibrosis and biliary hyperplasia. Large scale pyrrolizidine alkaloid poisoning in humans has usually arisen from the contamination of grains with pyrrolizidine alkaloid containing plants. Three major instances have occurred in South Africa, the Central Asian Republics of the USSR, and Jamaica. In South Africa in the 1920s, wheat was contaminated mainly with *Senecio burchellii* and *S. ilicifolius* which caused bread poisoning. (Bull et al., 1968; Cheeke and Shull, 1985; Kingsbury, 1964) This bread poisoning was found to be similar to veno-occlusive disease found in humans in the West Indies and elsewhere (Kingsbury, 1964). In India and Afghanistan in the early 1970s grain contaminated with *Crotalaria* seeds also caused veno-occlusive disease (Cheeke and Shull, 1985; Culvenor, 1978; Tandon et al., 1978). In central Asia (1931-1945) cereal grains were contaminated with *Heliotropium lasiocarpum* (Bull et al., 1968).

As recently as 1992, flour contaminated with *Heliotropium* spp. in Tadjikistan caused 3,906 cases of toxic liver injury (Chauvin et al., 1993). Human poisoning also can occur when herbal teas and medicines are contaminated with pyrrolizidine alkaloid-containing plants. In Africa and the West Indies bush tea often is made with plants of the *Crotalaria* and *Senecio* spp. (Cheeke and Shull, 1985), and poisoning in the United States resulting from tea made from *Senecio longilobus* has been reported (Stillman et al., 1977).

The majority of the plants implicated in livestock poisonings have belonged to one of three genera: 1) *Senecio* 2) *Crotalaria* or 3) *Heliotropium* (Cheeke and Shull, 1985; Hooper, 1978; Kingsbury, 1964). The species most often causing outbreaks of pyrrolizidine alkaloid poisoning will be discussed here. All of the diseases discussed affecting livestock are characterized by liver lesions and often death by hepatic liver cirrhosis, and have after the fact been identified as cases of pyrrolizidine alkaloid poisonings. Lung lesions are also involved in some cases, most often when plants of the genera *Crotalaria* are involved.

The genus *Senecio* consists of more than 1200 species of plants (Kingsbury, 1964; Sharrow et al., 1988), several of which are linked with pyrrolizidine alkaloid poisoning. *Senecio jacobaea* has been the cause of stomach staggers in Wales (early 1800s), Pictou disease (horses and cattle) in Nova Scotia (<1860) and Winton disease (horses and cattle) in New Zealand (Bull et al., 1968; Cheeke and Shull, 1985; Hosking and Brandt, 1936; Kingsbury, 1964; Long, 1917). *S. latifolius* has been identified as the cause of Molteno horse disease and cattle sickness (also called dunziekte) in South Africa (Bull et al., 1968; Cheeke and Shull, 1985; Hosking

and Brandt, 1936; Kingsbury, 1964; Long, 1917). In Norway, sirasyke disease, a liver disease in calves, has been linked with *S. aquacticus* (Bull et al., 1968; Hosking and Brandt, 1936; Kingsbury, 1964). In Czechoslovakia and South Bohemia, zdar disease in horses has been linked with *S. erracticus* (Bull et al., 1968; Kingsbury, 1964). In the West Indies, veno-occlusive disease in animals has been linked with *Senecio* spp. (Kingsbury, 1964). In Germany, Schweinsberger disease in horses, which is a cirrhosis of the liver, has been linked with *S. vernalis* (Bull et al., 1968). *S. riddelli* has caused walking disease in horses in the United States, (Bull et al., 1968; Cheeke and Shull, 1985) and *S. burchellii* has proven fatal to an ox (Long, 1917). Also in the United States, both *S. longilobus* and *S. vulgaris* have both caused severe cattle losses (Cheeke and Shull, 1985; Johnson and Molyneux, 1984). On several occasions cattle losses in Australia have been attributed to *S. lautus*, especially after feeding experiments induced sickness and death in calves and pathological changes consistent with pyrrolizidine alkaloid poisoning (Noble et al., 1994; Seawright et al., 1991b; Walker and Kirkland, 1981). Recently, *S. raphanifolius* has been implicated in cases of pyrrolizidine alkaloid poisoning in yaks (Winter et al., 1992, 1993, 1994) and *S. selloi* has been implicated in cases of pyrrolizidine alkaloid poisoning in Argentinian cattle (Odriozola et al., 1994).

The genera *Crotalaria* also contains several species of plants which are linked with pyrrolizidine alkaloid poisoning by various diseases affecting the liver. Missouri bottom disease, or walking disease in horses has been attributed to *C. sagittalis* in the United States, and *C. dura* in South Africa where it is called jaagsiekte disease (Bull et al., 1968; Cheeke and Shull, 1985; Culvenor, 1980;

Kingsbury, 1964). *C. spectabilis* has caused poisonings of swine and cattle in the United States (Cheeke and Shull, 1985), and has also been linked with losses of horses and fowl (Kingsbury, 1964). In western Australia, *C. retusa* has been linked with Kimberley horse disease, or 'walkabout' (Bull et al., 1968; Cheeke and Shull, 1985), as well as major losses of fowl (Kingsbury, 1964).

In Australia, two pyrrolizidine alkaloid containing plants belonging to the genera *Heliotropium* are known to cause severe losses of livestock. *H. europaeum* has caused poisoning of sheep, cattle (Bull et al., 1968; Cheeke and Shull, 1985; Kingsbury, 1964), and swine (Jones et al., 1981). Rather than the typical liver cirrhosis type symptoms, both *H. europaeum* and *H. lasiocarpum* have been known to cause a sudden hematogenous jaundice in sheep, which is characterized by an abnormally high copper content in the liver (Bull et al., 1968; Culvenor, 1980; Kingsbury, 1964).

Other pyrrolizidine alkaloid containing plants which are known to cause problems are those in the genera *Amsinckia* and *Trichodesma*. *A. intermedia* has caused poisoning of horses, cattle and swine, both experimentally and in the field (Kingsbury, 1964). In the United States it has caused walking disease in horses and hard liver disease in both cattle and swine (Bull et al., 1968; Cheeke and Shull, 1985; Kingsbury, 1964). *T. incanum* has been linked with suiljuk disease (characterized by lesions in the liver and the lungs) of horses and cattle in central Asia (Bull et al., 1968; Culvenor, 1980).

Veno-occlusive disease, whether described in animals or humans, is considered to be the result of poisoning from the pyrrolizidine alkaloids contained

in *Crotalaria* and *Senecio* spp. specifically (Kingsbury, 1964). Veno-occlusive disease in characterized by fibrosis and occlusion of hepatic veins resulting from endothelial damage and blood clotting (Johnson et al., 1985). Due to the formation of lesions in the liver, the liver loses its ability to produce urea. Death is often a result of the increased blood ammonia concentration and ammonia intoxication (Kingsbury, 1964).

Variations in species susceptibility to pyrrolizidine alkaloid poisoning have also been observed. Hooper (1978) has rated several species sensitivity to pyrrolizidine alkaloid poisoning, based on the approximate ratio of the amount of plant material required to induce poisoning. The ratios are, for sheep and goats, 200; mice, 150; rats, 50; cattle and horses, 14; chickens, 5; and swine, 1. Feeding trials by Cheeke have yielded very similar ratios for these animals, and have also measured the lethal dose for several other rodents and young poultry (Cheeke, 1984; Cheeke and Pierson-Goeger, 1983). Gerbils were found to be the most resistant, consuming 36 times their body weight of plant material. Hamsters are about the same as sheep and goats, and guinea pigs and rabbits are slightly more sensitive, yet still fairly resistant. Chicks and turkey poults were both extremely sensitive to pyrrolizidine alkaloid poisoning (Cheeke and Pierson-Goeger, 1983). Goeger et al., (1982) demonstrated that goats are more resistant than cattle to pyrrolizidine alkaloid poisoning, with the lethal dose being >100% of their initial body weight. Swick et al. (1982b) demonstrated that although guinea pigs can consume large quantities of *S. jacobaea*, they are not completely resistant to pyrrolizidine alkaloid poisoning from this plant.

A similar variation of species resistance to specific pyrrolizidine alkaloids is also observed. Hopkirk and Cunningham (1936) were able to demonstrate the resistance of guinea pigs to the pyrrolizidine alkaloid jacobine which had been isolated from *S. jacobaea*. In the same trials, rats were very susceptible to the alkaloid, developing liver lesions similar in type to those observed in cattle which have ingested *S. jacobaea*. It has also been demonstrated that monocrotaline is not toxic to guinea pigs (Chesney and Allen, 1973; Swick et al., 1982a) but that rats are susceptible to poisoning by monocrotaline (Chesney and Allen, 1973).

Variations in resistance due to method of pyrrolizidine alkaloid introduction have also been observed. Pierson et al. (1977) has shown that rabbits are resistant to pyrrolizidine alkaloid poisoning when consuming a diet of *S. jacobaea* (112% of body weight over 263 days), but that they are susceptible to pyrrolizidine alkaloid poisoning when injected intraperitoneally with purified pyrrolizidine alkaloid isolated from *S. Jacobaea*. Sheep, which are generally considered resistant to pyrrolizidine alkaloid poisoning under chronic conditions, have been observed to ingest 200% of their body weight of toxic plant over a one year period without symptoms (Craig et al., 1986). Sheep are susceptible to acute toxicity caused by pyrrolizidine alkaloid poisoning, and have died after a single intraperitoneal injection of alkaloids (Hooper, 1978).

As previously mentioned, sheep tend to be tolerant to ingestion of pyrrolizidine alkaloid containing plants, but are not completely immune to the toxic effects. In feeding trials with both sheep and cattle using *S. longilobus*, sheep generally required twice as much plant material to display the same symptoms as

cattle (Dollahite, 1972; Kingsbury, 1964). Although sheep are generally considered to be resistant to the chronic hepatotoxic effects of pyrrolizidine alkaloid poisoning, sheep can die after long periods of exposure to pyrrolizidine alkaloids. In some cases these deaths have been attributed to copper toxicity because after exposure to pyrrolizidine alkaloids, copper tends to accumulate in the liver (Bull et al., 1968; Cheeke and Shull, 1985; Culvenor, 1980). In experimental feeding trials, both rats and broiler chicks have also been observed to have high concentrations of copper in the liver after exposure to pyrrolizidine alkaloids (Huan et al., 1992; Swick et al., 1982a, 1982c). In feeding trials in which sheep were fed diets containing a combination of *H. Europaeum* and copper, it was concluded that the combination of the two substances enhanced the toxicity of each one individually and caused an excessive accumulation of copper in the liver (Howell et al., 1991). A recent study using rats being fed a combination of the pyrrolizidine alkaloid retrorsine and copper concluded that toxicity was enhanced when compared to rats getting either retrorsine or copper alone (Morris et al., 1994).

Pyrrolizidine alkaloids can cause either acute or chronic toxicity. Acute pyrrolizidine alkaloid toxicity is the result of a large intake of pyrrolizidine alkaloids, and seldom occurs under natural conditions (Kingsbury, 1964). Symptoms of acute toxicity are a massive generalized hemorrhage and acute liver toxicity (Sharrow et al., 1988). Under natural conditions chronic toxicity is more commonly observed. Chronic toxicity is characterized by the gradual loss of liver function due to a cirrhosis-like condition which is brought on by small daily doses over the course of several weeks or months (Kingsbury, 1964; Sharrow et al., 1988). Chronic

symptoms in an animal are often not observed until just a few days or weeks before it dies (Molyneux et al., 1988; Sharrow et al., 1988). Symptoms of cattle ingesting chronic, lethal amounts of *Senecio* spp. plants include disinterest in food and surroundings, signs of discomfort, gradual weight loss or failure to gain weight, a dull rough coat, diarrhea or constipation, behavioral changes (stubbornness), and intestinal edema develops (Sharrow et al., 1988). In the terminal stages, the animal may pace incessantly and appear blind (Sharrow et al., 1988).

The total dose necessary to cause acute toxicity is generally less than that obtained under conditions of chronic toxicity (Hooper, 1978; Kingsbury, 1964). Sheep are capable of metabolizing pyrrolizidine alkaloids within their rumen (Craig et al., 1992; Dick et al., 1963; Lanigan, 1970; Lanigan and Smith, 1970; Wachenheim et al., 1992a), and under chronic conditions, the small daily dose obtained may be completely metabolized, whereas a large intake of pyrrolizidine alkaloid may overwhelm ruminal microbes ability to function (Craig et al., 1992). This may explain why the chronic dose in sheep is approximately 100 times larger than the acute dose (Hooper, 1978). Under chronic conditions in horses and cattle, fatalities generally occur after ingestion of 10% of the animals body weight of toxic plant, although there is some evidence to indicate that toxicity is dependent more on the rate at which the dose is received, and not necessarily on the cumulative dose (Johnson et al., 1985).

PYRROLIZIDINE ALKALOID STRUCTURES

The chemical structures of pyrrolizidine alkaloids are complicated and very diverse. Pyrrolizidine alkaloids are all alkylamine esters: a nitrogen containing necine base attached through ester linkages to mono- or dicarboxylic necic acids. Examples of both are shown in Figures 1.1 and 1.2. Over 350 pyrrolizidine alkaloids have been structurally elucidated, and the number is continually growing (Logie et al., 1994; Segall et al., 1991).

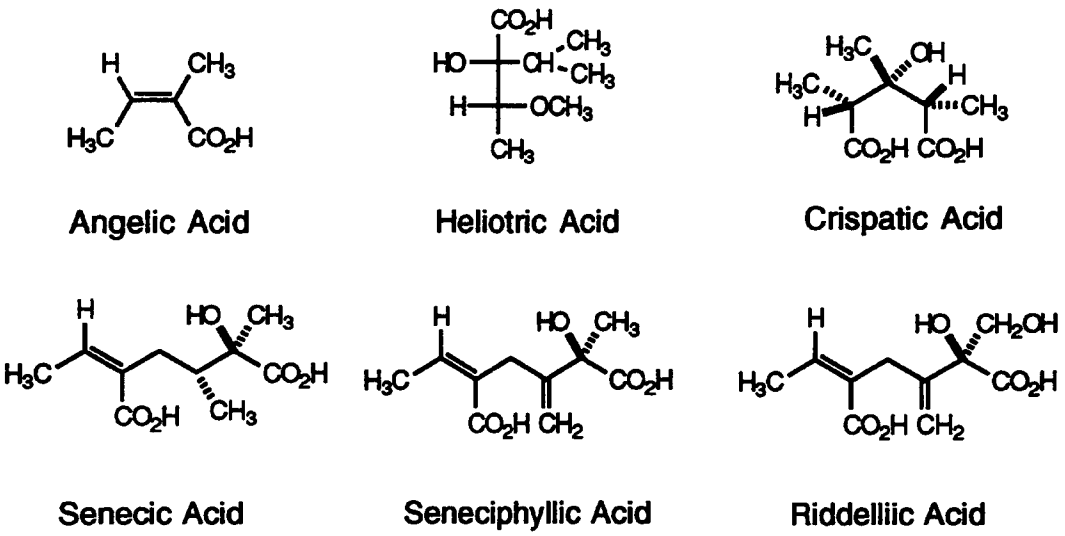


Figure 1.1 Examples of necic acids.

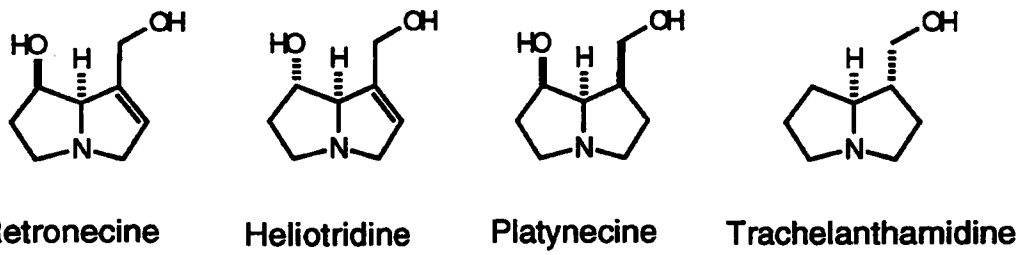
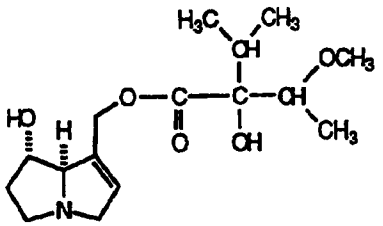


Figure 1.2 Examples of necine bases.

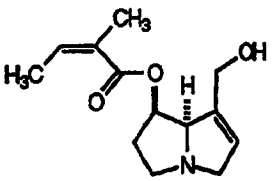
The necic acid is typically C7-C10 in length, oxygenated and highly branched. The necine base can have varying degrees of unsaturation and stereochemistry. Although numerous necine bases have been identified, nearly half of all pyrrolizidine alkaloids which have been structurally elucidated contain the necine base retronecine. The pyrrolizidine alkaloids resulting from the combination of the necic acids and bases are either single-branched monoesters, noncyclic diesters or cyclic diesters, the latter of which are often referred to as macrocyclic pyrrolizidine alkaloids (Cheeke and Shull, 1985). Examples of each are shown in Figure 1.3.

In plants, pyrrolizidine alkaloids are present in two chemical forms, the free base and the N-oxide (Bull et al., 1968; Mattocks, 1986; Molyneux et al., 1991). Some studies have indicated that the N-oxides are not as toxic as the free base of pyrrolizidine alkaloids, but other evidence suggests that the mode of pyrrolizidine alkaloid introduction can have a dramatic influence on the toxicity (Mattocks, 1971). In studies where N-oxides are given intraperitoneally they are about 1/5 as toxic as the parent alkaloids (Bull et al., 1958; Downing and Peterson, 1968), but when given orally they display nearly identical toxicity (Barnes et al., 1964; Mattocks, 1971; Molyneux et al., 1991; Schoental and Magee, 1959). It has been proposed that the nearly equivalent toxicity of the two forms could be due to the conversion of the N-oxide to the free base within the rumen (Molyneux et al., 1991) or the intestine (Mattocks, 1971). This conversion has been observed for heliotrine within the rumen (Lanigan, 1970).

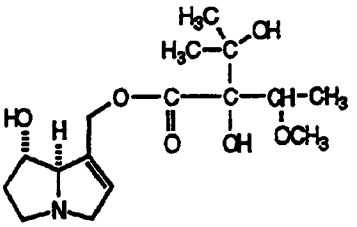
Monoesters



Heliotrine

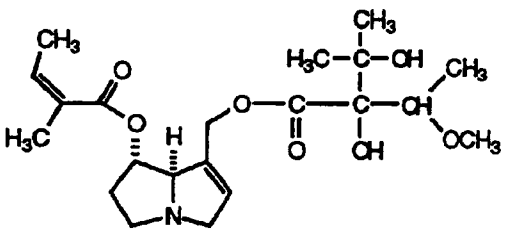


7-Angelyl Retronecine

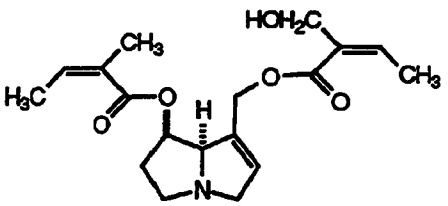


Europine

Noncyclic Diesters

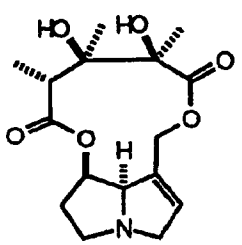


Lasiocarpine

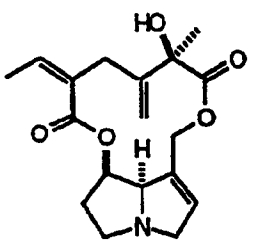


Triangularine

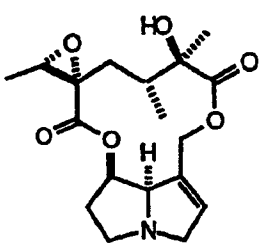
Macrocyclic



Monocrotaline



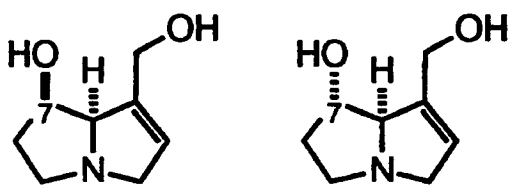
Seneciphylline



Jacobine

Figure 1.3 Examples of different types of pyrrolizidine alkaloids.

Not all pyrrolizidine alkaloids are toxic, and the degree of toxicity can also be associated with a pyrrolizidine alkaloids structural characteristics. Toxic pyrrolizidine alkaloids all contain a 1,2 double bond in the necine base and a branch in the ester group (Cheeke and Shull, 1985; Culvenor et al., 1976; Kingsbury, 1964; Schoental, 1968). Cyclic diesters are more toxic than non-cyclic diesters which are more toxic than monoesters (Cheeke and Shull, 1985; Culvenor et al., 1976; Schoental, 1968). Some evidence exists to suggest that pyrrolizidine alkaloids containing the necine base heliotridine are generally more toxic than those containing the necine base retronecine (Cheeke and Shull, 1985; Culvenor et al., 1976; Schoental, 1968). Heliotridine and retronecine are structural isomers of each other with heliotridine having an α-OH at C7 and retronecine having a β-OH at C7, as shown in Figure 1.4 (Bull et al., 1968; Mattocks, 1986). This may by true only for natural pyrrolizidine alkaloid compounds which are resistant to hydrolysis (Mattocks, 1981), although there is evidence that the structure of the acid is more important than the configuration of the base moiety (Mattocks and Bird, 1983), with necic acids containing α,β-unsaturation being more toxic than saturated necic acids (Hincks et al., 1991). It has been proposed that between similar necic acids, those with acetoxy- or methoxy- groups are more toxic than those with hydroxyl groups (Schoental, 1968).



Retronecine Heliotridine

Figure 1.4 Structures of retronecine and heliotridine.

LIVER METABOLISM

After ingestion, pyrrolizidine alkaloids are absorbed into the bloodstream and transported to the liver. Once in the liver there are several possible reactions that can take place, as demonstrated in Figure 1.5 (Cheeke and Shull, 1985; Culvenor, 1980; Segall et al., 1991; Swick, 1984). The pyrrolizidine alkaloids could be hydrolyzed to the corresponding necic acid and base, which are then excreted in the urine (Eastman and Segall, 1982; Mattocks, 1981). The pyrrolizidine alkaloids could also be converted to their corresponding N-oxides which, being highly water soluble, are also excreted in the urine (Mattocks and White, 1971). Both of these pathways are considered to be non-toxic pathways.

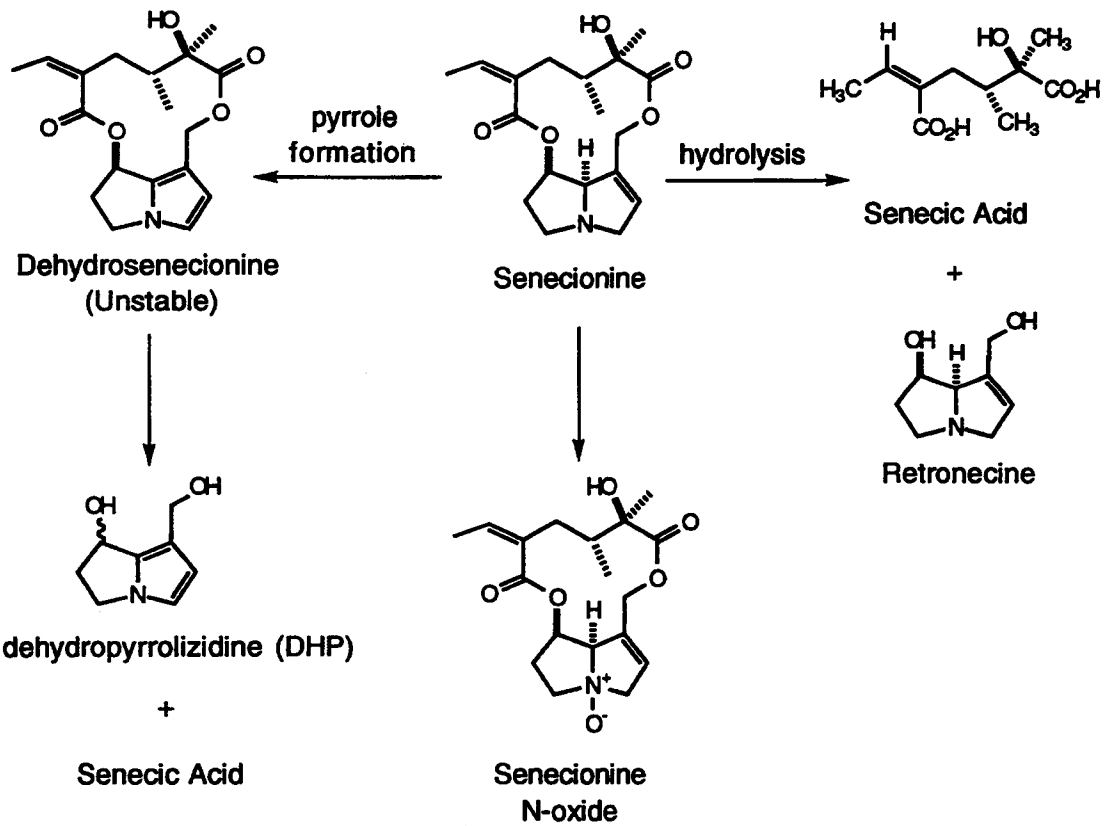


Figure 1.5 Major pathways of pyrrolizidine alkaloid metabolism in the liver.

The third possible pathway is the bioactivation of the pyrrolizidine alkaloids. This bioactivation of pyrrolizidine alkaloids occurs when they are oxidized by mixed function oxidases to potentially toxic pyrroles which are more reactive than the original alkaloid, and act as alkylating agents (Mattocks and White, 1971). The presence of the 1,2-double bond greatly facilitates this conversion to pyrroles, and the esters attached at C9 or C7 cause the pyrrolic metabolites to be very strong alkylating agents (Culvenor et al., 1976). Once formed, these extremely electrophillic alkylating agents either react almost immediately with nucleophiles in the cell (Mattocks, 1969, 1978; Swick, 1984), or hydrolyze to the more stable dehydroaminoalcohols (pyrroles of necine bases) which can react further or be eliminated from the body (Mattocks, 1969; Peterson et al., 1972).

Initial methods used by numerous researchers to detect the pyrroles of necine bases were not very sensitive, and they could not distinguish between the pyrrole formed from retronecine or the pyrrole formed form heliotridine. Early reports of the pyrrole formation assumed that if the parent pyrrolizidine alkaloid contained retronecine, then the pyrrole of retronecine was the product, and likewise for pyrrolizidine alkaloids which contained heliotridine. It has been shown that actually, a racemic mixture of the two compounds is formed from pyrrolizidine alkaloids containing either retronecine or heliotridine (Kedzierski and Buhler, 1985). The racemic mixture of the two pyrroles is referred to as dehydropyrrolizidine (DHP). The production of dehydroretronecine (most likely DHP) *in vivo* in rats given either of the pyrrolizidine alkaloids monocrotaline (Hsu et al., 1973) or seneciphylline (Kedzierski and Buhler, 1986) has been demonstrated.

Pyrrolic metabolites that react with soluble cell constituents such as thiols, then may be transported out of the liver. It has been shown *in vitro*, that senecionine reacts, via dehydrosenecionine, with glutathione (GSH) to form a soluble DHP-GSH conjugate (Reed et al., 1992). Also *in vitro*, dehydroretronecine reacts with both cysteine and GSH (Robertson et al., 1977). *In vivo* experiments have shown that retrorsine also reacts, via dehydroretrorsine, with glutathione to form a soluble DHP-GSH conjugate (Yan and Huxtable, 1995). Pyrrolic metabolites also react with thiol groups on hemoglobin, forming relatively stable thioethers (Mattocks and Jukes, 1990a, 1990b, 1992; Seawright et al., 1991a). Detection of these pyrrolic thioethers in both blood and tissues has been used as a method of confirming pyrrolizidine alkaloid poisoning (Seawright et al., 1991a; Winter et al., 1990, 1992, 1993).

Pyrrolic metabolites also react with macromolecules such as nucleic acids or proteins, becoming covalently bound (Cheeke and Shull, 1985; Hsu et al., 1975; Swick, 1984). Both of the pyrrolizidine alkaloids seneciphylline and senecionine have been found to bind covalently to DNA in rat liver (Candrian et al., 1985). It has been demonstrated *in vitro*, that dehydroretronecine reacts with nucleosides such as deoxyguanosine (Robertson, 1982), guanosine, adenosine, deoxythymidine, uridine, and with several nucleotides as well (Wickramanayake et al., 1985). Also *in vitro*, dehydromonocrotaline reacts with the nucleosides deoxyadenosine, deoxyguanosine, thymidine and deoxycytidine (Niwa et al., 1991), in addition to causing DNA cross-linking (Wagner et al., 1993). Pyrroles cross-linking with DNA *in vitro* has been observed (Kim et al., 1995; White and Mattocks, 1972), as well as

pyrrolic metabolites bound in the liver of rats given the pyrrolizidine alkaloid retrorsine (Mattocks and White, 1973). Also *in vitro*, dehydroretronecine produces DNA cross-links (Reed et al., 1988). It has been demonstrated *in vivo*, that the pyrrolizidine alkaloids monocrotaline and jacobine both produce DNA cross-links in rats (Petry et al., 1984, 1986). In mice, the pyrrolizidine alkaloids senecionine and seneciphylline both bind covalently to hepatic DNA, RNA and protein (Eastman et al., 1982), and the pyrrolizidine alkaloid riddelliine causes an increase in unscheduled DNA synthesis in mice (Mirsalis et al., 1993). The alkylation of DNA with pyrroles impairs cell division, leading to liver cirrhosis which ultimately leads to death (Cheeke and Shull, 1985).

The formation of pyrroles in the liver from various pyrrolizidine alkaloids generally follows the same pattern as toxicity vs. structure, with the formation of N-oxides following the opposite pattern. Studies in rats have shown that the unesterified aminoalcohols (necine bases) are not metabolized to pyrroles (Mattocks, 1978, 1981). Diesters form about 15 times more pyrroles than monoesters, and cyclic diesters are more efficiently converted to pyrroles than noncyclic diesters (Mattocks, 1978, 1981). It has also been noticed that hydroxylation on the acid moiety decreases pyrrole formation, whereas acetylation can reverse this (Mattocks, 1981). This is consistent with Schoental's (1968) proposal that necic acids with acetoxy groups are more toxic than ones with hydroxyl groups. It has been found that the more toxic the pyrrolizidine alkaloid, the more stable is the corresponding pyrrole (Mattocks, 1978). Two factors which can favor an alkaloids conversion to pyrrolic metabolites are a high lipid:water partition

coefficient and sufficient steric resistance to inhibit ester hydrolysis (Mattocks, 1981). Also in rats, non-cyclic diesters form the highest levels of N-oxides, while macrocyclic diesters yield very low levels of N-oxides (Mattocks and Bird, 1983).

Changes within the cell, including DNA cross-linking and inducement of megalocytosis by various pyrrolizidine alkaloids also follows the same pattern as toxicity vs. structure. In studies using bovine kidney epithelial cell cultures, the macrocyclic α,β-unsaturated pyrrolizidine alkaloids tested induced the most DNA crosslinking and were also the most potent inducers of megalocytosis (Hincks et al., 1991; Kim et al., 1993, 1995). The macrocyclic pyrrolizidine alkaloid monocrotaline which does not contain α,β-unsaturation and non-cyclic diesters (heliosupine and latifoline) were both weak DNA cross-linkers and weak inducers of megalocytosis (Hincks et al., 1991; Kim et al., 1993). The necine base retronecine produced only barely detectable DNA cross-links at high concentrations and did not induce any detectable megalocytosis (Hincks et al., 1991; Kim et al., 1995).

The production of pyrrolic metabolites in various species generally follows the same pattern of species resistance, with susceptible species, such as mice, hamsters and rats producing high levels of pyrroles and resistant species such as guinea pigs producing low levels of pyrroles (Chu and Segall, 1991). The formation of N-oxides generally follows the opposite pattern (Chu and Segall, 1991). Enzyme assisted hydrolysis of pyrrolizidine alkaloids has been identified, with two carboxylesterases being isolated from guinea pig hepatic microsomes which show activity toward the pyrrolizidine alkaloids seneciphylline, monocrotaline, senecionine and integerrimine (Dueker et al., 1992b). In guinea pigs, esterase hydrolysis

accounted for 92% of the metabolized pyrrolizidine alkaloid, whereas rats displayed no esterase activity under similar conditions (Dueker et al., 1992a). The enzyme which converts senecionine to DHP has been shown to be the major enzyme for bioactivation in rats, but not in guinea pigs (Chung and Buhler, 1994). It has also been shown that glutathione-S-transferase, which converts jacobine to a soluble conjugate with glutathione, is active in guinea pigs, but not in rats (Dueker et al., 1994). The resistance of guinea pigs can be attributed to a combination of the low conversion of pyrrolizidine alkaloids to pyrroles, the conversion of pyrrolizidine alkaloids to soluble glutathione conjugates and N-oxides and the enzyme mediated hydrolysis of pyrrolizidine alkaloids (Chu and Segall, 1991; Dueker et al., 1992a, 1992b, 1994).

As previously mentioned, non-esterified retronecine yields low amounts of pyrroles in rat livers (Mattocks, 1981). Likewise, when retronecine pyrrole, which is a weak alkylating agent, was given to rats, liver lesions did not develop (Mattocks, 1970), although *in vitro* it does inhibit DNA synthesis (Armstrong and Zuckerman, 1971). When injected interperitoneally, dehydroheliotridine has caused acute toxicity in rats, as well as chronic damage to the liver (Peterson et al., 1972); it has also been shown to interact with DNA *in vitro* (Black and Jago, 1970). The diacetyl retronecine pyrrole has been shown to produce liver lesions identical to those produced by the pyrroles of the pyrrolizidine alkaloids retrorsine and monocrotaline at similar dose levels (Mattocks, 1970). The author concluded that the differences in the toxicities of various pyrrolizidine alkaloids are due to the different amounts of each that are metabolized to pyrroles, and the different stabilities of the reactive

metabolites within the cell. Mattocks (1970) has also suggested that the structure of the acid moiety is not important at the site of the toxic reaction. Recent work has confirmed that differences in the toxicities of various pyrrolizidine alkaloids is due to the reactivity of the dehydroalkaloid to nucleophillic attack (Yan et al., 1995). The more reactive the dehydroalkaloid, the greater the amount of conjugation with GSH and alkylating with macromolecules, and thus direct toxic effects by the pyrrolizidine alkaloids.

RUMEN METABOLISM

Many herbivores, such as sheep, goats, cattle, deer, and elk are ruminant animals. That is, that rather than a having monogastric digestive system (like humans), they have a segmented digestive system, with the first segment being the rumen. The rumen is a highly reductive, anaerobic, fermentative organ which is buffered by bicarbonate to pH=6.0-6.7 (Hungate, 1988). Digestion begins on ingested plant material in the rumen, both chemically and biologically. The rumen contains a large population of microflora, including bacteria (10^{11} viable cells/mL rumen contents), protozoa (10^5-10^6 ciliate protozoa/mL rumen contents) and anaerobic fungi (10^3-10^5 zoospore/mL rumen contents) (Ogimoto and Imai, 1981; Orpin and Joblin, 1988; Stewart and Bryant, 1988). This microflora performs several functions for the ruminant animal. For example, it is estimated that protozoa are responsible for one quarter to one third of the fiber digestion that occurs (Williams and Coleman, 1988). Among bacteria there are cellulose degraders, starch degraders, pectin degraders, methane producers, and many others (Brock

and Madigan, 1988). The majority of rumen bacteria are obligate anaerobes, meaning that the presence of oxygen is harmful or lethal to them (Brock and Madigan, 1988; Hungate, 1988; Ogimoto and Imai, 1981). There are several factors which can influence the size and composition of the population of microflora, including diurnal variations, diet effects (composition and frequency of feeding), seasonal variation, geographical location and species of ruminant (Dehority and Orpin, 1988).

One of the major pyrrolizidine alkaloid containing plants in Australia is *Heliotropium europaeum*, which contains several pyrrolizidine alkaloids, including europine, heleurine, heliotrine, lasiocarpine and acetyl-lasiocarpine (Bull et al., 1968; Mattocks, 1986). The most abundant pyrrolizidine alkaloids in *H. europaeum*, heliotrine and lasiocarpine, both contain the necine base heliotridine. It has been shown that sheep in Australia contain ruminal microbes which are capable of detoxifying the pyrrolizidine alkaloids heliotrine and lasiocarpine. The conversion of heliotrine to 7α-hydroxy-1-methylene-8α-pyrrolizidine, as shown by pathway A in Figure 1.6, was observed when heliotrine was incubated with rumen liquor (Culvenor et al., 1962; Dick et al., 1963). It has been shown that when lasiocarpine is incubated with sheep rumen contents, 7α-angeloxy-1-methylene-8α-pyrrolizidine is formed as shown by pathway C in Figure 1.6 (Lanigan and Smith, 1970). Lanigan and Smith (1970) also demonstrated that both of the methylene compounds are further converted to 7α-hydroxy-1α-methyl-8α-pyrrolizidine (pathways B and D in Figure 1.6), but they found no evidence to indicate that the necine base ring system gets broken down further (Lanigan, 1970). In separate experiments, the

intermediate 7α-angeloxy-1-methylenepyrrolizidine was tested for hepatotoxicity in rats, and was found to produce no liver damage (Culvenor et al., 1976).

When incubating the pyrrolizidine alkaloid heliotrine with sheep ruminal contents, a lag time of anywhere between 6 and 40 hr has been observed (Dick et al., 1963; Lanigan, 1970; Russell and Smith, 1968). The addition of pyrrolizidine

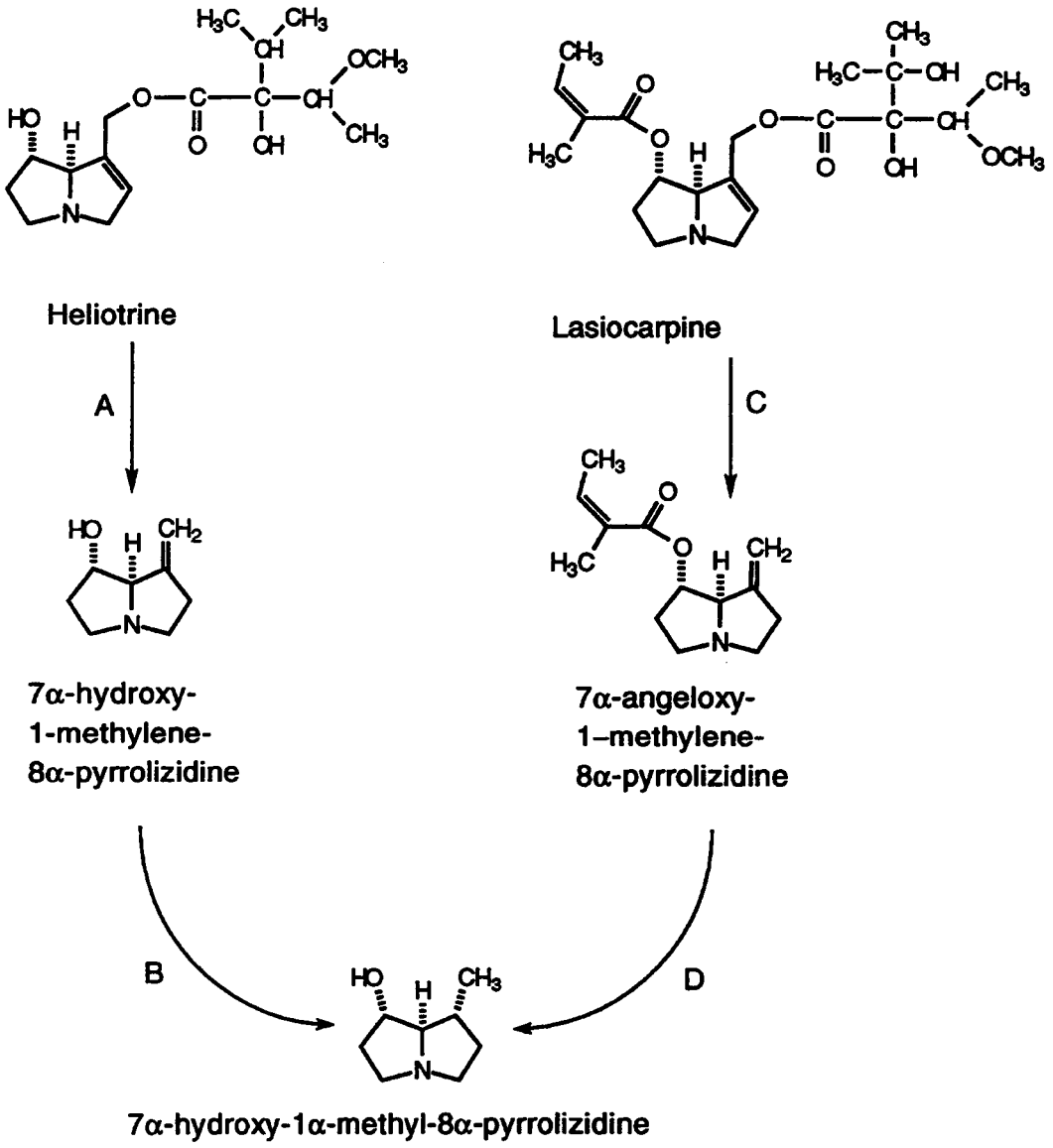


Figure 1.6 Detoxification of pyrrolizidine alkaloids in Australian sheep rumen fluid.

alkaloid containing plants, such as *H. europaeum* to the diet of sheep, selects for the bacteria involved in the detoxification of the pyrrolizidine alkaloids and increases their numbers within the rumen. This greatly increases the rate at which the alkaloids are metabolized (Lanigan, 1970), indicating that the bacteria responsible for detoxifying the pyrrolizidine alkaloids are not prominent species among the normal rumen microflora, but that when their specific substrate becomes available they are able to reproduce and grow rapidly. The detoxification of heliotrine involves a reductive cleavage which requires molecular hydrogen. The normal population of rumen bacteria which produces methane also requires molecular hydrogen. As both metabolic pathways are competing for hydrogen, the addition of methane inhibitors to sheeps diet can stimulate the metabolism of pyrrolizidine alkaloids in the rumen (Culvenor, 1978; Lanigan, 1972).

Two bacteria have been isolated form Australian sheep ruminal contents which are able to convert selected pyrrolizidine alkaloids to their respective methylene compounds. A small Gram-negative coccus isolated from the rumen contents of Australian Merino sheep reductively cleaves heliotrine to heliotric acid and the corresponding 1-methylene compound (7α-hydroxy-1-methylene-8α-pyrrolizidine) when grown in a partially defined media under strict anaerobic conditions (pathway A in Figure 1.6) (Russell and Smith, 1968). Lanigan (1976) isolated a Gram-positive coccus (*Peptostreptococcus heliotrinreducens*, Anonymous, 1983a, 1983b; Ezaki and Yabuuchi, 1986) capable of converting several mono- and diester pyrrolizidine alkaloids (heliotrine, europine, supinine and lasiocarpine) to their corresponding 1-methylene type compounds when grown in

media, although corresponding 1-methyl type compounds were found only when the pyrrolizidine alkaloids were incubated with sheep ruminal fluid. Some macrocyclic diester pyrrolizidine alkaloids (crispatine and monocrotaline) were metabolized by *Peptostreptococcus heliotrinreducens* at a slower rate than the monoesters, while other macrocyclic diester pyrrolizidine alkaloids (anacrotine and retrorsine) were not metabolized at all under similar conditions. Also, the necine base heliotridine and the saturated pyrrolizidine alkaloids cynaustraline and sarracine were not metabolized under similar conditions.

In the northwestern United States pyrrolizidine alkaloid poisoning of livestock is caused by *Senecio jacobaea*, a weed commonly referred to as tansy ragwort. Tansy ragwort was inadvertently introduced to North America in the late 1800s, was first observed in Oregon in 1922, and has continued to spread throughout the Pacific Northwest (Muth, 1968; Snyder, 1972). Cases of pyrrolizidine alkaloid poisoning were first confirmed in the 1950s, and since that time, losses due to pyrrolizidine alkaloid poisoning have risen steadily (Snyder, 1972). It takes only a small amount of tansy ragwort, 2% of their body weight over a 20-day period, to be toxic to cattle (Johnson, 1978). The two most abundant pyrrolizidine alkaloids found in tansy ragwort are jacobine and seneciphylline, with senecionine, jacoline, jaconine, and jacozine also being present in lesser amounts (Bull et al., 1968; Mattocks, 1986). All of these pyrrolizidine alkaloids contain the necine base retronecine (Mattocks, 1986).

In the United States, resistance of sheep to pyrrolizidine alkaloid poisoning has been observed, and in places infested with *Senecio* spp., sheep have been

used as a biological control agent. Using sheep to graze an area before allowing cattle into the area has reduced the losses due to *Senecio* poisoning (Dollahite, 1972; Muth, 1968). Intense grazing of tansy ragwort infested pasture by sheep, in addition to eliminating established plants, also reduces seed production, thus resulting in an overall reduction of the tansy ragwort population (Sharrow and Mosher, 1982).

Initial experiments to determine if the sheep in the United States owe their resistance to pyrrolizidine alkaloid poisoning from tansy ragwort to ruminal microbes yielded negative results. Experiments were conducted in which ground tansy ragwort was incubated with strained rumen fluid from sheep or cattle, and then the resulting material was incorporated into the diet of rats (Shull et al., 1976). Autoclaved rumen fluid incubated with tansy ragwort from either cattle or sheep provided protection from pyrrolizidine alkaloid poisoning, as did the cattle rumen fluid incubated with tansy ragwort, but the sheep rumen fluid incubated with tansy ragwort did not provide protection. Further experiments were done in a similar manner with sheep ruminal fluid from animals exposed to tansy ragwort in their diet (Swick et al., 1983). These results also indicated that there was no difference in pyrrolizidine alkaloid toxicity from untreated tansy ragwort and tansy ragwort incubated with sheep ruminal fluid. Also in these incubations, methylene intermediates similar to those identified as metabolites of heliotrine and lasiocarpine in incubations with sheep ruminal fluid were searched for, but not found.

Craig et al. (1986) were the first to demonstrate that protection against pyrrolizidine alkaloid poisoning occurred within the digestive tract of sheep. In these

experiments sheep and calves both had a chronic indwelling catheter surgically implanted in the portal vein of the liver. Through this catheter pyrrolizidine alkaloids isolated from tansy ragwort were introduced directly to the liver, thus by-passing the entire digestive tract. All of the animals which received a pyrrolizidine alkaloid infusion, both sheep and calves, developed clinical, enzymatic and histopathologic signs of pyrrolizidine alkaloid poisoning, at comparable doses of pyrrolizidine alkaloid. Sheep given twice the dose of pyrrolizidine alkaloid orally did not show any of the symptoms of pyrrolizidine alkaloid poisoning. The authors concluded that this was due to either a protective mechanism in the rumen or a lack of absorption and/or metabolism of the alkaloid in the gastrointestinal tract.

Subsequent work has shown the ability of sheep ruminal microbes to detoxify the pyrrolizidine alkaloids contained in tansy ragwort (Craig et al., 1992; Wachenheim et al., 1992a, 1992b). When unstrained sheep ruminal fluid is incubated with pyrrolizidine alkaloids isolated from tansy ragwort at a concentration of 100 µg/mL, the pyrrolizidine alkaloids disappear in less than 24 hr, while in sterile controls, the pyrrolizidine alkaloid concentration remains constant throughout the entire incubation period (Craig et al., 1992; Wachenheim et al., 1992a). Pyrrolizidine alkaloids were observed to be metabolized under methanogenic conditions but not under denitrifying conditions (Craig et al., 1992). It has been concluded that the pyrrolizidine alkaloids are metabolized by a consortium of small bacteria, with gram-positive bacteria being critical members (Craig et al., 1992; Wachenheim et al., 1992b).

In addition to sheep rumen fluid, the ability of goat and cow rumen fluid to detoxify pyrrolizidine alkaloids has also been evaluated (Wachenheim et al., 1992a). With pyrrolizidine alkaloids isolated from tansy ragwort, the rate of pyrrolizidine alkaloid biotransformation was nearly the same in sheep and goat ruminal fluid, but the rate in cow rumen fluid was 5-6 times slower. This slower rate of pyrrolizidine alkaloid metabolism is most likely a result of a smaller population of pyrrolizidine alkaloid degrading bacteria/mL of rumen fluid within the cow vs. the sheep or goat. The authors estimated that in cows, pyrrolizidine alkaloid biotransforming bacteria constitute 0.1% of the overall rumen bacterial population, whereas in sheep and goats it is about 0.3%. It could be concluded that the larger number of pyrrolizidine alkaloid degrading bacteria allows for increased rates of pyrrolizidine alkaloid metabolism and therefore increased protection from pyrrolizidine alkaloid poisoning (Wachenheim et al., 1992a).

As previously mentioned, work by other groups has not been able to show that detoxification of pyrrolizidine alkaloids occurs in sheep ruminal fluid (Shull et al., 1976; Swick et al., 1983), while work in our laboratory has been able to provide consistent evidence of pyrrolizidine alkaloid detoxification by sheep ruminal fluid. Several factors could be responsible for the success of the latter group, including utilization of ruminal solids, anaerobic techniques, blending of ruminal contents, agitation during incubation, uniform sampling times (shortly after feeding), pre-exposure of animals to pyrrolizidine alkaloids and individual differences among animals (Wachenheim et al., 1992a, 1992b). Some of the animals in the experiments done by other groups were pre-exposed to tansy ragwort, but at very

high levels (50% tansy ragwort in their diet) (Swick et al., 1983). This is consistent with our findings that higher levels of pyrrolizidine alkaloids within the rumen fluid requires longer incubation times for pyrrolizidine alkaloid disappearance to be observed, and that there may be a toxic threshold above which metabolism of pyrrolizidine alkaloids in sheep ruminal fluid cannot be demonstrated (Craig et al., 1992).

In our laboratory attempts which have been made to isolate and identify the microbes responsible for metabolizing the pyrrolizidine alkaloids have resulted in a mixed culture of 4-6 anaerobic microbes (L4M2) which is capable of metabolizing the pyrrolizidine alkaloids isolated from tansy ragwort. When these microbes are separated and grown as individual pure cultures, none of them have demonstrated the ability to metabolize the pyrrolizidine alkaloids; only when grown as a mixture do they degrade pyrrolizidine alkaloids.

THESIS OVERVIEW

Recent research in this laboratory has focused on the mechanism of pyrrolizidine alkaloid metabolism, both in incubations with whole rumen fluid and with the pyrrolizidine alkaloid metabolizing mixed culture. Metabolism of the pyrrolizidine alkaloids may involve a small structural change which renders them unavailable for the conversion in the liver to the toxic pyrrole form. It is also possible that more complete metabolism is occurring, including degradation of the necine base. Indirect evidence has indicated that the necine base is degraded. The mass spectra of pyrrolizidine alkaloids have mass/charge ratio signals (m/z)

which are characteristic of the particular necine base present in the pyrrolizidine alkaloid. Pyrrolizidine alkaloids which contain saturated necine bases generally have signals at 82, 95-97, 122-123, and 138-140 (Bull et al., 1968; Mattocks, 1986). Pyrrolizidine alkaloids which contain necine bases with a 1,2 double bond (such as retronecine) generally have signals 2 units less, at 80, 93-95, 119-121, and 136-139 (Bull et al., 1968; Mattocks, 1986). The largest signals in the mass spectra of the pyrrolizidine alkaloids contained in tansy ragwort are 93, 120, and 136. If the metabolism of these pyrrolizidine alkaloids involves degradation of the ring system, the metabolites would not have the characteristic signals of the necine base ring system. Preliminary studies based on this premise have indicated that metabolites containing a necine base ring system are not being produced.

In an attempt to conclusively identify metabolites of the pyrrolizidine alkaloids, two possible metabolic pathways involving the necine base were investigated (Figure 1.7). The first possible metabolic pathway involves hydrolysis of the pyrrolizidine alkaloids with the production of retronecine as an end-product or a transient intermediate (pathway A in Figure 1.7). The second possible

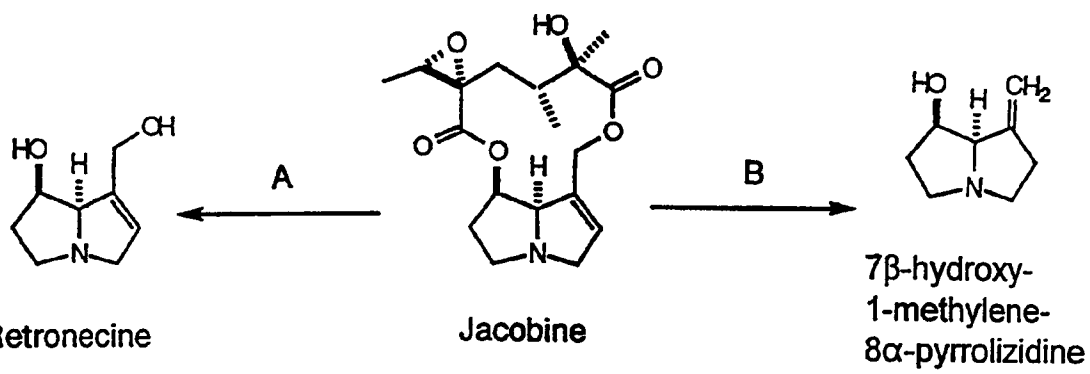


Figure 1.7 Two possible metabolic pathways

metabolic pathway investigated involves production of the necine base which has been converted to a methylene compound, similar to those produced during the metabolism of heliotrine and lasiocarpine by *Peptostreptococcus heliotrinreducens*, as described previously (pathway B in Figure 1.7).

Hydrolysis of pyrrolizidine alkaloids in the rumen would result in the necine base retronecine, a product consistent with the lack of toxicological effects observed in sheep. Retronecine would be absorbed and transported to the liver, but since the mixed function oxidase systems show very little activity towards retronecine, it would not be converted to toxic pyrroles but instead excreted in the urine (Mattocks, 1978, 1981). The mixed culture of pyrrolizidine alkaloid metabolizing microbes contains anywhere from four to six microbes, each of which may play a part in the metabolism. One or more organisms could be hydrolyzing the ester linkages, producing the necic acid and the necine base retronecine. Other organisms in the mixed culture could be further metabolizing retronecine. If this is the case, retronecine may be observed as a transient intermediate during the metabolism of pyrrolizidine alkaloids. Retronecine will not be observed if its' rate of metabolism is significantly faster than the rate of pyrrolizidine alkaloid metabolism. Also, retronecine should be metabolized independently of the parent pyrrolizidine alkaloids. In an effort to confirm this, it was decided that a labeled compound would be useful in tracking and identifying the metabolites directly.

Few pyrrolizidine alkaloids are available commercially, and none are available commercially with a label of any kind. Labelled pyrrolizidine alkaloids have been obtained by growing pyrrolizidine alkaloid producing plants in a growth

chamber saturated with $^{14}CO_2$, and then isolating the labelled pyrrolizidine alkaloids from the plant material (Dueker et al., 1992a; Eastman et al., 1982; Estep et al., 1991; Segall et al., 1983). Tritium labelled pyrrolizidine alkaloids have been obtained by feeding pyrrolizidine alkaloid producing plants nutrients containing [2,3-^{3}H]putrescine hydrochloride and then isolating the labelled pyrrolizidine alkaloids from the plant material (Candrian et al., 1985). Other precursors which have been tested are ornithine (both ^{3}H and ^{14}C labelled) and spermine (^{14}C labelled) (Reed et al., 1985). These labelling methods take a considerable amount of time, specialized knowledge and equipment, and generally yield < 2% recovery of label into the desired compounds (Reed et al., 1985).

Necine bases, although not available commercially, are accessible. In particular, retronecine can be isolated from the commercially available pyrrolizidine alkaloid monocrotaline, and subsequently labelled at the C-9 position (Hsu and Allen, 1975; Mattocks, 1977; Mattocks, 1982). For use in determining if retronecine is a transient intermediate during pyrrolizidine alkaloid metabolism, the necine base retronecine was isolated and labelled for use in subsequent experiments. Details of its purification and labelling are included in this study in Chapter 2. The commercially available pyrrolizidine alkaloid monocrotaline was hydrolyzed to yield retronecine. Procedures for the hydrolysis were similar to those in the literature (Hoskins and Crout, 1977) but the cleanup method was improved upon. The retronecine obtained was then oxidized and subsequently reduced, using a deuterated reducing agent, which yielded retronecine deuterated in the C-9 position. Methods of oxidation used previously yielded several side products and resulted in

a low overall percent yield of the desired products (Hsu and Allen, 1975; Mattocks, 1982). The research presented here utilized a new application of the Dess-Martin oxidizing agent periodinane (Dess and Martin, 1983, 1991) for the purpose of oxidizing retronecine. This resulted in less side products being formed and a higher recovery than had been previously reported in the literature.

A method for the determination of retronecine from biological matrices was developed. Details of the method and its development are included in this study in Chapter 3. The procedure included derivatization with N-heptafluorobutyryl-imidazole (HFBI) followed by GC/FID analysis and confirmation by GC/MS. The method works for samples from whole rumen fluid as well as for samples from bacterial growth media. Also presented in this study in Chapters 3 and 4 are the results of experiments which demonstrated that hydrolysis is not a significant mechanism in the metabolism of pyrrolizidine alkaloids. Incubations were conducted with both sheep whole rumen fluid and the pyrrolizidine alkaloid metabolizing mixed culture. Analysis indicated that although the pyrrolizidine alkaloids were observed to disappear over time, retronecine was not detected as an intermediate in any of the samples. Also, retronecine was not metabolized under these conditions.

As mentioned previously, the second possible metabolic pathway investigated involves the production of the necine base which has been converted to a methylene compound (pathway B in Figure 1.7). These products would be similar to those produced during the metabolism of heliotrine and lasiocarpine by *Peptostreptococcus heliotrinreducens*. Details of this study are included in Chapter

5. This organism (available via the American Type Culture Collection) was initially grown on monocrotaline, which was completely metabolized in two to three weeks. From these cultures, the corresponding methylene intermediate was identified with GC/MS analysis. This organism was also grown (in individual cultures) on heliotrine or lasiocarpine, from which the corresponding methylene intermediates were identified with GC/MS analysis. *Peptostreptococcus heliotrinreducens* did not metabolize jacobine or seneciphylline. In similar experiments with the mixed culture L4M2 which metabolizes jacobine or seneciphylline, heliotrine and lasiocarpine were metabolized to 1-methylene compounds (identified with GC/MS). These products were identical to those produced from heliotrine and lasiocarpine by *P. heliotrinreducens*. L4M2 also produced very low levels of the corresponding 1-methylene compounds from jacobine and seneciphylline, although more extensive metabolism is occurring.

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Chapter 2

IMPROVED LABELLING METHODS FOR C9-^{2}H-RETRONECINE

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ABSTRACT

The necine base retronecine (**1a**), common to numerous toxic pyrrolizidine alkaloids, has previously been labelled with ^{3}H at C-9 for use in metabolic studies. The current method for labelling retronecine is inefficient, and we report here an improved method which utilizes the Dess-Martin periodinane reagent. Characterization of the resulting aldehyde (**2**) is described. The overall yield of C9-^{2}H-retronecine (**1b**) is 71% (from **1a**).

INTRODUCTION

Toxic plants containing pyrrolizidine alkaloids are common throughout the world and are responsible for the death of livestock as well as humans (Bull et al., 1968; Kingsbury, 1964; Mattocks, 1986; Snyder, 1972). All pyrrolizidine alkaloids contain an amine portion, known as a necine base, that is usually attached through one or more ester linkages to a necic acid portion. There are some 6,000 plants that contain a total of at least 180 known pyrrolizidine alkaloids (Bull et al., 1968; Mattocks, 1986; World Health Organization, 1988). The most toxic of these are cyclic diesters, also known as macrolactone pyrrolizidine alkaloids, usually containing the unsaturated necine base retronecine (**1a**) (Bull et al., 1968; Mattocks, 1981; Mattocks, 1986; World Health Organization, 1988).

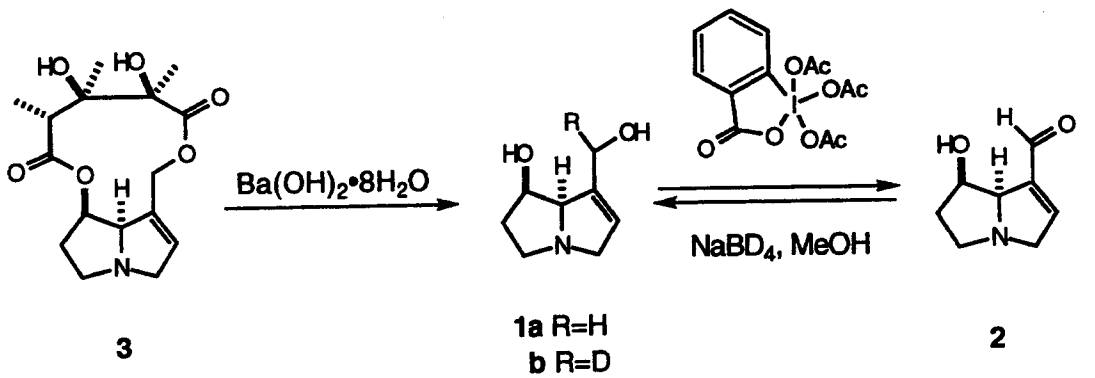
Irreversible liver cirrhosis occurs in cattle when they ingest pyrrolizidine alkaloid containing plants at as little as 5% of their body weight (Bull et al., 1968; Craig et al., 1991; Cheeke, 1984; Dollahite, 1972; Ford et al., 1968; Thorpe and

Ford, 1968). In contrast, the ingestion of these same plants by sheep at 200-300% of their body weight produces no such effect (Cheeke, 1984; Dollahite, 1972; Kingsbury, 1964). The resistance of sheep to these toxins has been attributed to a consortium of bacteria contained within sheep rumen, that detoxify pyrrolizidine alkaloids before they come into contact with the liver (Craig et al., 1986, 1992; Lanigan, 1976; Wachenheim et al., 1992a, 1992b). We required retronecine for our research in order to determine how sheep ruminal bacteria detoxify pyrrolizidine alkaloids.

Studies involving pyrrolizidine alkaloids are often hindered by the difficulty in obtaining large quantities of the pure compounds (Craig et al., 1986; Eastman et al., 1982; Mattocks, 1981, 1977b; Segall et al., 1983). For this reason, semisynthetic derivatives of pyrrolizidine alkaloids, both labelled and unlabelled, have been used in several metabolic studies to simulate the toxic effects of pyrrolizidine alkaloids and to elucidate the origin of their toxicity (Cheeke, 1984; Craig et al., 1991; Dollahite, 1972; Eastman et al., 1982; Ford et al., 1968; Mattocks, 1977b; Thorpe and Ford, 1968). Since the necine base has been implicated in the toxicity of pyrrolizidine alkaloids, several studies have employed the more accessible necine base retronecine (Cheeke, 1984; Craig et al., 1991; Dollahite, 1972; Ford et al., 1968; Mattocks, 1977b; Thorpe and Ford, 1968).

The current method for labelling retronecine is inefficient (10-30% overall yield) (Hsu and Allen, 1975; Mattocks, 1982). The only published method involves a selective manganese dioxide oxidation (Hsu and Allen, 1975; Mattocks, 1977a, 1977b, 1982; Piper et al., 1981; Shumaker et al., 1978) followed by reduction of the

resultant aldehyde with a labelled reducing reagent, such as $LiAl^3H_4$ (Hsu and Allen, 1975) or NaB^3H_4 (Mattocks, 1977b, 1982; Piper et al., 1981; Shumaker, et al., 1978). The manganese dioxide oxidation affords a variety of undesired side products and the isolation is further complicated because the desired aldehyde (**2**) can not be stored or purified (Mattocks, 1977a). We report an improved method for isolating retronecine (**1**) and for deuterating retronecine at C-9 by utilizing a more selective method of oxidation (Figure 2.1).



3 **1a** R=H **b** R=D **2**

Figure 2.1 Reaction scheme for the conversion of monocrotaline (**3**) to retronecine (**1a**), followed by oxidation to the aldehyde (**2**) and reduction to deuterated retronecine (**1b**).

EXPERIMENTAL

Melting points were measured on a Büchi melting point apparatus and are uncorrected. Infrared spectra were recorded on a Nicolet 5DBX FT-IR spectrophotometer. Optical rotations were measured in 1-dm cells (1 mL capacity) on a Perkin Elmer Model 243 polarimeter at ambient temperature. Nuclear magnetic resonance spectra were recorded on a Bruker AM-300 spectrometer in

D_2O or $CDCl_3$ for ^1H and ^{13}C nmr, and in $CHCl_3$ for ^2H nmr. Chemical shifts are given in ppm relative to the solvent peak, either HOD in D_2O or $CHCl_3$ in $CDCl_3$ for the ^1H and ^{13}C nmr, or natural abundance $CDCl_3$ in $CHCl_3$ for the ^2H nmr. Mass spectra and exact mass determinations were obtained on a Varian MAT 311 spectrometer.

Retronecine (**1a**)

A mixture of a saturated aqueous solution of $Ba(OH)_2$ prepared from $Ba(OH)_2 \bullet 8H_2O$ (4.0 g, 13 mmol) and water (20 mL), and crystalline monocrotaline (1.9667 g, 6.04 mmol) was stirred for 2 h at 40-50 °C, and then refluxed for 2 h. After cooling to room temperature, solid CO_2 was added, and the resultant mixture was then kept at room temperature for 16 h. The solution was filtered through filter paper, and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography on silica gel ($CHCl_3$:MeOH:Et_3N (13:5:2)), which yielded 0.8523 g (91%) of a light tan solid. Recrystallization from hot acetone produced pure retronecine as white crystals with mp 108 °C. $[\alpha]^{20}_D$ +52.7° (c=0.67, EtOH). Ir v(film): 3400, 2940, 2850, 1635 cm^{-1}. ^1H Nmr (D_2O) δ 1.84 (2H, br m), 2.60 (1H, br m), 3.13 (1H, br m), 3.29 (1H, ddd, J=2.0, 4.9, 15.5 Hz), 3.72 (1H, dt, J=3.5, 15.4 Hz), 4.12 (3H, br m), 4.28 (1H, br m), 5.63 (1H, s). ^{13}C Nmr (D_2O) δ 35, 53, 58, 61, 71, 76, 125, 137. High resolution mass spectra calculated for $C_8H_{13}NO_2$ 155.0946, found 155.09460.

[9-^{2}H]-Retronecine (**1b**)

To a stirred dichloromethane solution (5 mL) of Dess-Martin periodinane (305.8 mg, 0.721 mmol) and CF_3CO_2H (60 µL, 0.616 mmol) was added a dichloromethane solution (25 mL) of retronecine (100.6 mg, 0.648 mmol). The resultant mixture was stirred for 1 h, after which dry ether (100 mL) was added. The resultant precipitate was filtered and washed successively with ether. The volume of the filtrate was reduced below 15 °C under reduced pressure to approximately 3 mL to afford a solution of the crude, unstable, aldehyde (**2**) which was used without purification. Ir v(film): 3400, 1680 cm^{-1}. ^{1}H Nmr ($CDCl_3$) δ 2.25 (3H, br m), 3.14 (1H, br m), 3.94 (2H, br m), 4.78 (2H, br m), 5.15 (1H, d, J=1.7 Hz), 6.81 (1H, q, J=2.0, 4.1 Hz), 9.80 (1H, s). High resolution mass spectra calculated for $C_8H_{11}NO_2$ 153.0790, found 153.07900.

Methanol (10 mL) was added to the crude aldehyde solution (≈3 mL) and the mixture was cooled to 0 °C, followed by the addition of NaB^2H_4 (175 mg, 4.18 mmol). The reaction was monitored by tlc (silica gel, $CHCl_3$:MeOH:Et_3N (13:5:2)), until all of the starting material was consumed (≈30 min). The solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (silica gel, $CHCl_3$:MeOH:Et_3N (13:5:2)), to yield a light tan solid (71.9 mg, 71% from **1a**). Ir v(film): 3400, 2940, 1640 cm^{-1}. ^{1}H Nmr (D_2O) δ 1.85 (2H, br m), 2.61 (1H, br m), 3.14 (1H, br m), 3.29 (1H, dt, J=5.0, 15.5 Hz), 3.73 (1H, dd, J=1.6, 15.5 Hz), 4.10 (2H, br m), 4.29 (1H, br m), 5.64 (1H, s). ^{13}C Nmr (D_2O) δ 35, 53, 58 (t), 61, 71, 76, 125, 137. ^{2}H Nmr ($CHCl_3$) δ 4.18 (1D, s), 4.38 (1D, s). High resolution mass spectra calculated for $C_8H_{12}DNO_2$ 156.1009, found 156.10090.

RESULTS AND DISCUSSION

Monocrotaline is efficiently hydrolyzed with $Ba(OH)_2$ as previously reported, but its purification involves a tedious isolation procedure (Hoskins and Crout, 1977). The crude product mixture obtained after hydrolysis and treatment with CO_2 is most efficiently purified by column chromatography on silica gel using a ternary solvent system ($CHCl_3$:MeOH:Et_3N). With this new protocol, we have obtained pure retronecine in up to 95% yield on a 5-gram scale.

Having obtained large quantities of retronecine, we next developed an efficient method for preparing C9-^{2}H-retronecine. We reasoned that a chemoselective oxidation of retronecine would be more efficient and might be achieved using the Dess-Martin periodinane reagent. This reagent could potentially differentiate between the two alcohol functions because of their difference in steric environments. In addition, the enhanced reactivity of the Dess-Martin periodinane with benzylic alcohols (Dess and Martin, 1983, 1991) suggested that the rate of reaction with the allylic alcohol moiety may be faster than with the alcohol function at C7.

In practice, oxidation of retronecine with Dess-Martin periodinane proceeded cleanly and quickly (less than 1 h). Our experiments indicate that oxidizing retronecine with periodinane yields two products, the major product being the desired aldehyde (**2**). The aldehyde (**2**) has been reported previously, although the respective nmr signals have not been unambiguously assigned (shown in Figure 2.2). Table 2.1 contains the complete chemical shift assignments for the aldehyde (**2**), as well as those for compounds (**1a**) and (**1b**), that correspond well with recently

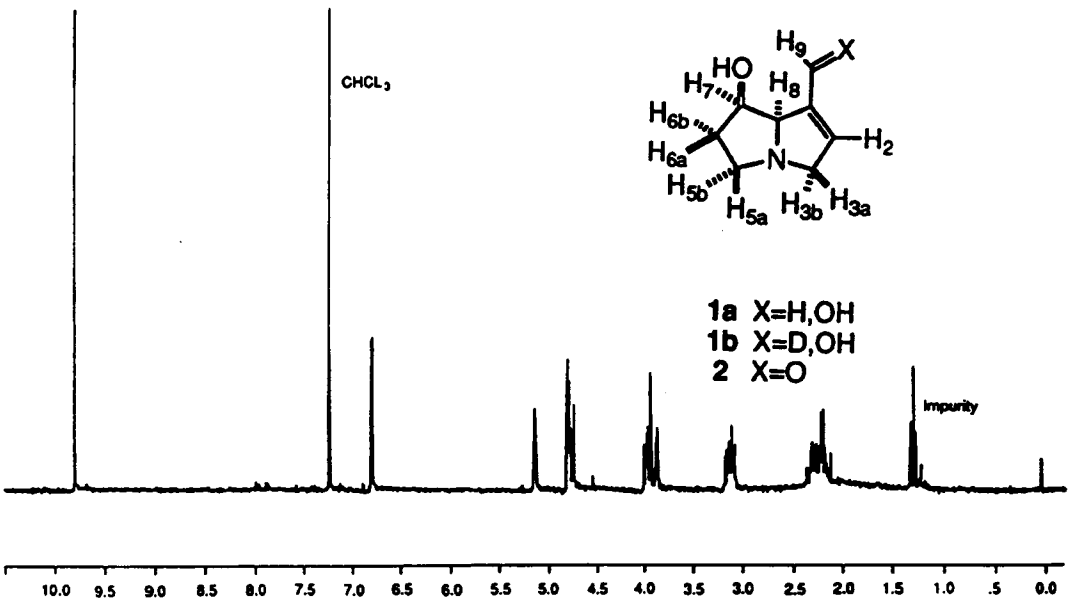


Figure 2.2 ^{1}H nmr of compound (**2**).

Table 2.1 ^{1}H nmr chemical shifts in ppm (δ).

	1a[a]	**1b**[a]	**2**[b]
2-H	5.63, s[c]	5.64, s	6.81, q[d]
3-H_a	3.72, dt[e]	3.73, dd[f]	4.78, br m[g]
3-H_b	3.29, ddd[h]	3.29, dt	3.94, br m
5-H_a	3.13, br m	3.14, br m	3.94, br m
5-H_b	2.60, br m	2.61, br m	3.14, br m
6-$H_{a,b}$	1.84, br m	1.85, br m	2.25, br m
7-H	4.28, br m	4.29, br m	4.78, br m
8-H	4.12, br m	4.10, br m	5.15, d[i]
9-$H_{a,b}$	4.12, br m	4.10, br m	9.80, s

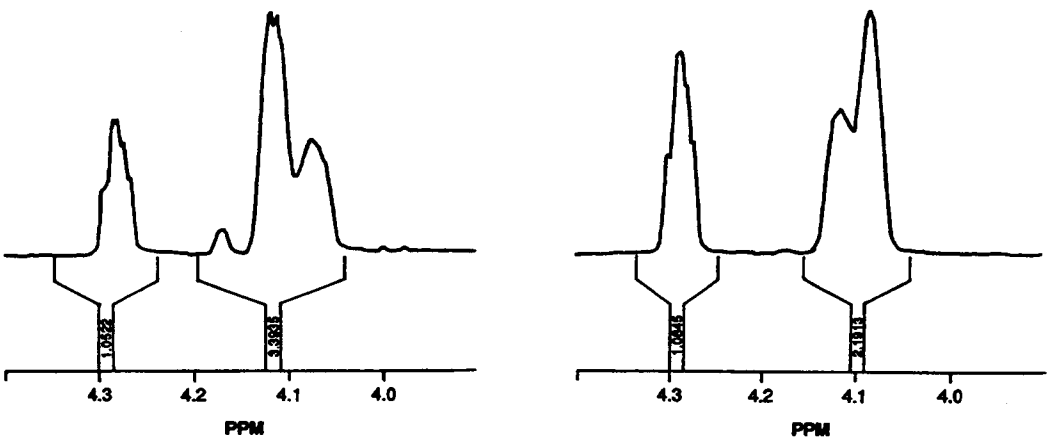
[a]Taken in D_2O. [b]Taken in $CDCl_3$. [c]s is singlet [d]q is quadruplet
[e]dt is doublet triplet [f]dd is doublet doublet
[g]br m is broad multiplet [h]ddd is doublet doublet doublet [i]d is doublet

published nmr data (Röder et al., 1992). The overlap between protons 7-H and 3-H_a and protons 3-H_b and 5-H_a in compound (**2**) prevented the coupling constants from being determined. Further characterization of **2** was complicated by instability under various conditions, including but not limited to, heat, silica gel, and storage at greater than 0 °C. For compounds (**1a**) and (**1b**), many of the signals were insufficiently resolved to allow extraction of coupling constants, although the large coupling between 3-H_a and 3-H_b was clearly visible (see Experimental section).

The reduction of aldehyde (**2**) closely followed earlier methods (Mattocks, 1982; Piper et al., 1981; Shumaker et al., 1978). We have, however, found that the reduction can be readily performed in a MeOH-Et_2O solvent mixture, avoiding isolation and retarding decomposition of the rather unstable aldehyde (**2**). Treatment of **2** with NaB^2H_4 affords C9-^{2}H-retronecine in 71% overall yield (from **1a** to **1b**). Figure 2.3 shows the proton nmr spectra for compounds (**1a**) and (**1b**) in the region affected by the deuterium substitution. The signal centered at 4.3 ppm is due to 7-H, while the signal centered at 4.1 ppm is from the three protons 8-H and 9-H. The integration in the 3.9-4.2 ppm region clearly indicates the presence of three protons in **1a**, but only two protons in **1b**. The deuterium nmr of compound (**1b**) consists of two singlets with equal integration. This occurs because the reducing agent (NaB^2H_4) is not stereoselective and gives rise to both the R and S configurations at C-9 in approximately equal amounts.

We anticipate using the deuterium labelled retronecine to investigate the detoxification of pyrrolizidine alkaloids by sheep ruminal bacteria. Detection of the deuterium label in the degradation products will allow us to determine the

degradation pathway(s). The greater efficiency involved in labelling retronecine will greatly facilitate our studies of the biological degradation of retronecine, and will also facilitate other workers in this area.



1a **1b**

Figure 2.3 ^{1}H nmr detail of compounds (**1a**) and (**1b**).

ACKNOWLEDGMENTS

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Chapter 3

A ROUTINE METHOD FOR THE DETERMINATION OF RETRONECINE

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ABSTRACT

A method for the routine determination of the necine base retronecine from biological matrices is described, using gas chromatography for quantification. The biological matrices studied included blended sheep whole rumen fluid and bacterial growth media. The structurally similar compound 2,6-dimethoxypyridine was utilized as an internal standard. Prior to gas chromatography, the bis(heptafluorobutyrate) derivatives of both compounds were formed. The relative percent recoveries of retronecine and the internal standard were 73% and 82%, respectively. The detection limit of retronecine was found to be 0.09 µg/mL in blended whole rumen fluid and 0.02 µg/mL in bacterial growth media. The precision of the peak area ratio (retronecine to internal standard) was 10% from blended whole rumen fluid, and 14% from bacterial growth media. This method was used to analyze samples from viable cultures incubated with retronecine.

INTRODUCTION

Over 300 unique pyrrolizidine alkaloids have been isolated and identified from thousands of different plant species, many of which are hepatotoxic to both livestock and humans (Bull et al., 1968; Culvenor, 1980; Logie et al., 1994; Mattocks, 1986). Pyrrolizidine alkaloids consist of a necine base attached through one or more ester linkages to a necic acid. The most

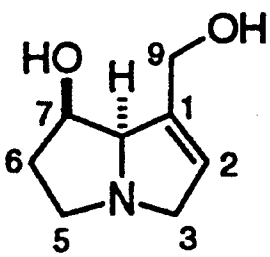

Figure 3.1 Structure of retronecine.

common necine base is retronecine (Figure 3.1), with approximately 40% of all structurally elucidated pyrrolizidine alkaloids containing retronecine (Logie et al., 1994). The parent alkaloids are non-toxic, but they are metabolized in the liver by several pathways, some of which produce non-toxic metabolites (necic acid and necine base or N-oxides) and some of which produce toxic metabolites (pyrrolic compounds; Segall et al., 1991; Swick, 1984).

Cattle are particularly susceptible to pyrrolizidine alkaloid poisoning while sheep and goats are quite resistant (Cheeke, 1984; Dollahite, 1972; Hooper, 1978; Kingsbury, 1964). The resistance of sheep and goats to these toxins has been attributed to bacterial metabolism within the rumen which detoxifies the pyrrolizidine alkaloids before they are absorbed into the bloodstream and transported to the liver (Craig et al., 1986, 1992; Dick et al., 1963; Lanigan, 1970). Work in Australia has focused on sheep which are resistant to pyrrolizidine alkaloid poisoning by the common plant *Heliotropium europaeum*. Rumen fluid from sheep exposed to *H. europaeum* detoxifies pyrrolizidine alkaloids by cleaving the ester linkages and saturating the ring system (Dick et al., 1963; Lanigan and Smith, 1970). No further degradation of the necine base has been observed (Lanigan, 1970). Work in this laboratory has focused on sheep which are resistant to pyrrolizidine alkaloid poisoning by the common plant *Senecio jacobaea* (tansy ragwort). Tansy ragwort contains several cyclic diester pyrrolizidine alkaloids which contain the necine base retronecine (Bull et al., 1968; Mattocks, 1986). To aid in these metabolic studies, it was necessary to determine retronecine from liquid culture media, in the µg/mL range without significant interferences.

Many of the studies on the liver metabolism of pyrrolizidine alkaloids include assays for retronecine which are generally either preparative or qualitative in nature (Dueker et al. 1992a; Eastman and Segall, 1982; Mattocks, 1981). There are several studies which utilize ^{14}C-labeled pyrrolizidine alkaloids in which products are typically separated with semi-preparative HPLC. Individual fractions are then used to qualitatively determine each product, and liquid scintillation counting is used to quantitate the total amount of ^{14}C-labeled products recovered (Dueker et al., 1992a, 1992b; Eastman and Segall, 1982). Hydrolysis rates are often determined by quantitating remaining pyrrolizidine alkaloid and the necic acid produced, but not the necine base (Chu and Segall, 1991; Dueker et al., 1992b). None of these methods are quantitative or have low enough detection limits to be applicable to our metabolic studies. Only recently, has a quantitative assay for retronecine from microsomal studies been described (Dueker et al., 1995), but it does not utilize an internal standard and is not optimized for sheep whole rumen fluid. The method presented here has been optimized for sheep whole rumen fluid and 2,6-dimethoxypyridine was selected as an appropriate internal standard. This compound is structurally similar to retronecine, and has similar reactivity under the selected conditions. The bis(heptafluorobutyrate) derivatives of both compounds were formed and analyzed by gas chromatography.

EXPERIMENTAL

Chemicals

All solvents were HPLC grade or better and were purchased from commercial sources. Type I reagent grade filtered water was produced in a Milli-Q water system (Millipore, Bedford, MA). The derivatization reagents and the compounds listed in Tables 3.1 and 3.2 were purchased from commercial sources. Retronecine was isolated and purified as previously described (Hovermale et al., 1994). Stock solutions of retronecine were prepared at a concentration of 10 mg/mL in either methanol or phosphate buffer (pH=7) and diluted as necessary into water, basal growth media or blended whole rumen fluid. The basal growth media was a modification of MPN-PA medium; modifications included the omission of hemin and volatile fatty acids except for sodium acetate and the addition of sodium formate (5.0 g/L), tryptone (1.0 g/L) and yeast extract (2.0 g/L) (Wachenheim et al., 1992). Sheep whole rumen fluid was blended with a modification of McDougall buffer (25:75) (McDougall, 1948; Wachenheim et al., 1992). Working standards were prepared in both basal growth media and blended whole rumen fluid and ranged from 0 to 100 µg/mL. Stock solutions of 2,6-dimethoxypyridine (internal standard) were prepared at a concentration of 10 mg/mL in methanol. The working internal standard solution was prepared daily by diluting to 1 mg/mL in water.

Table 3.1 Compounds tested as possible internal standards with two derivatization reagents, HFBI and HFBA, which did form derivatives.

Name	Derivatization Reagent	Number of Peaks Observed (retention time, min)	Comments (about peaks)
4-acetamidophenol	HFBI	1 (9.90)	very small
	HFBA	3 (9.74, 10.02, 10.03)	
4-aminophenol hydrochloride	HFBI	2 (9.86, 11.20)	very small
	HFBA	1 (9.76)	
1,3-benzenediol	HFBI	0	
	HFBA	1 (6.85)	small
2-*t*-butyl-1,4-dihydroxybenzene	HFBI	2 (11.45, 12.39)	very small
	HFBA	3 (9.05, 10.97, 11.10)	double peaks
2,3-diaminotoluene	HFBI	1 (11.51)	very small
	HFBA	1 (11.35)	very small
2,6-dimethoxypyridine	HFBI	1 (9.82)	
	HFBA	1 (9.81)	
3-pyrrolidino-1,2-propanediol	HFBI	1 (8.52)	small
	HFBA	0	
1,4-dihydroxy-2,3,5-trimethylbenzene	HFBI	3 (9.51, 11.27, 11.96)	very small
	HFBA	2 (9.44, 11.37)	small

Table 3.2 List of compounds tested as possible internal standards which did not form derivatives with either derivatization reagent, HFBI or HFBA.

2,6-di-*t*-butyl-4-methylphenol
2,4-diaminophenol dihydrochloride
2,4-diaminotoluene
2,6-diaminotoluene
3,4-diaminotoluene
2,3-dihydroxypyridine
2-methoxypyridine
phenol

Sample Preparation

Experimental samples were typically analyzed in duplicate 1-mL aliquots. Optimization studies typically utilized 1-mL aliquots of water, basal growth media, or blended whole rumen fluid spiked with retronecine (100 µg/mL). To each, a glass bead and 100 µL of the working internal standard solution were added and vortexed. Samples were dried under the flow of nitrogen, with heat applied (70 °C) and with vortexing of the samples half way through the drying process. After acetonitrile had been added (2 mL), the samples were vortexed and sonicated to dissolve and/or suspend residue. The derivatization reagent heptafluorobutyrylimidazole (HFBI) was added (300 µL), and the samples vortexed and heated at 70 °C for 15 min in a block heater. The samples were centrifuged at 1520 RCF for 5 min, and the supernatant was then analyzed.

Samples for analysis by GC/MS were prepared in a manner similar to that described herein except for the following modifications. Internal standard was not always added, and toluene was used in place of acetonitrile. To remove excess derivatization reagent after heating, 1 mL of water was added and the samples were vortexed and centrifuged. The organic layer was then analyzed.

Instrumentation

Gas chromatography was performed on an Rt_x-5 capillary column (30-m x 0.25-mm, 0.25-µm film thickness, Restek Corp., Bellefonte, PA) using a Perkin-Elmer Autosystem gas chromatograph (Perkin-Elmer Corp., Norwalk, CT) equipped

with a flame ionization detector and a split-splitless injector operated in the splitless mode (injector temp. 200 °C, detector temp. 350 °C, temp. program: 60 °C (1 min), 25 °C/min to 150 °C (6 min), 25 °C/min to 250 °C (2 min)). Helium was used as the carrier gas. Data were collected at a rate of 3.448 points/s using an PE Nelson 970 interface box and Turbochrom chromatography software running on a personal computer (Perkin-Elmer Corp., PE Nelson Div., San Jose, CA).

Gas chromatography-mass spectrometry (GC/MS) using electron ionization (EI, 70 eV) was performed on either a Finnigan GC (Finnigan Corp., Austin, TX) or a HP model 5890 GC (Hewlett-Packard Co., Palo Alto, CA) interfaced with a Finnigan 5100 quadrupole mass spectrometer. Chromatographic conditions were as described above. Samples were injected by a HP model 7673A automatic injector. The transfer line and manifold were held at 250 °C and 100 °C, respectively. The scan range was 45-620 amu (1.15 s/scan). Data were collected via personal computer running Galaxy 2000 software (LGC, San Jose, CA).

RESULTS AND DISCUSSION

Analytical procedure

Samples must be completely dry upon the addition of the HFBI, as water destroys the derivatization reagent. Samples of blended whole rumen fluid are sometimes resistant to drying by the formation of a thin skin on the surface which can trap small amounts of water beneath it. The addition of the glass bead and

vortexing once during the drying step provides the opportunity for the surface skin to be broken so that samples dry completely and homogeneously.

A typical chromatogram of retronecine prepared from blended whole rumen fluid illustrates that excess derivatization reagent and by-products elute in three different areas (Figure 3.2). The two large fronted peaks vary in size and exact retention time with individual samples, with the two peaks of interest eluting after all excess derivatization reagent and by-products. Occasionally in samples with little

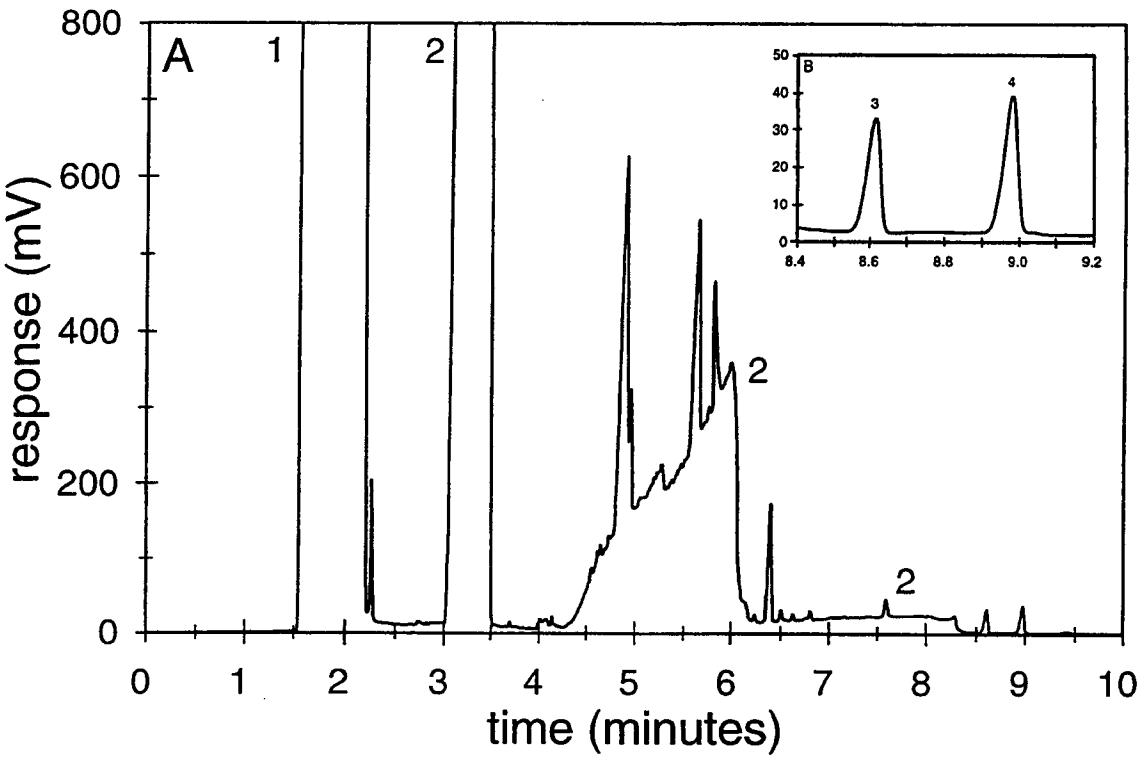

Figure 3.2 GC/FID chromatogram of retronecine (100 µg/mL) prepared from 1 mL of blended whole rumen fluid. A) Entire chromatogram showing the solvent peak (1) and excess derivatization reagent and by-products (2). B) Expanded view in the region of the analyte showing the bis(heptafluorobutyrate) derivative of 2,6-dimethoxypyridine (3) and the bis(heptafluorobutyrate) of retronecine (4). GC temp. program: 60 °C (1 min), 25 °C/min to 150 °C (6 min), 25 °C/min to 250 °C (2 min). Injector temp. 200 °C, detector temp. 350 °C.

or no retronecine, excess reagent results in an elevated baseline in the region of the analyte and the internal standard.

Selected samples were analyzed by GC/MS to confirm peak identity. The mass spectrum for the bis(heptafluorobutyrate) derivative of retronecine (Figure 3.3A) is consistent with those published for this compound (Deinzer et al., 1978). The bis(heptafluorobutyrate) derivative of the internal standard was also confirmed by GC/MS (Figure 3.3B).

Influence of derivatization reagent

Five derivatization reagents which would acylate polar groups such as hydroxyls and amines were investigated, and HFBI was found to be the best choice. The other derivatization reagents were trifluoroacetic acid anhydride (TFAA), N-trifluoroacetylimidazole (TFAI), acetic acid anhydride (AAA) and heptafluorobutyric acid anhydride (HFBA). The matrix of most interest in these experiments, whole rumen fluid, is a complicated matrix containing many potentially interfering compounds, as well as particulate matter. Experiments testing the different derivatization reagents generally followed the sample preparation method described in the experimental. Variations included using toluene and/or acetonitrile, lower temperatures during heating, or longer heating times. In order to reduce the number of steps and potential loss of analyte, excess derivatization reagent was not removed from the samples except in the case of AAA.

Under none of the conditions tested were any derivatives formed when using AAA as the derivatization reagent. The bis(trifluoroacetate) derivative was formed

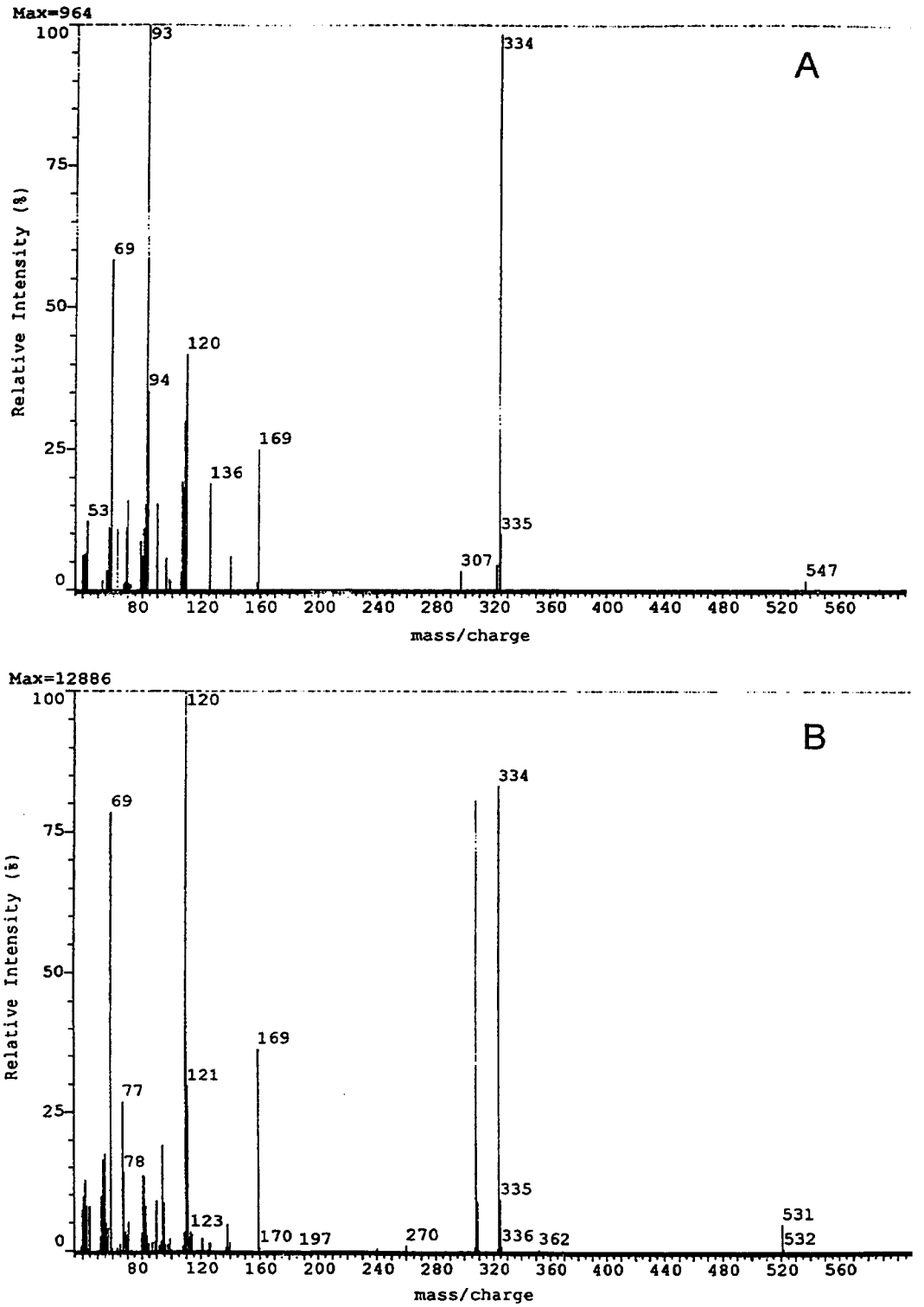

Figure 3.3 Mass spectra of derivatives. A) Bis(heptafluorobutyrate) derivative of retronecine. MW 547. B) Bis(heptafluorobutyrate) derivative of 2,6-dimethoxypyridine. MW 531.

by both TFAA and TFAI, with confirmation by GC/MS. In samples of blank rumen fluid prepared with TFAI, a small peak was present at 8.24 min which coeluted with the bis(trifluoroacetate) derivative of retronecine which eluted at 8.28 min. The bis(heptafluorobutyrate) derivative of retronecine, being a higher molecular weight compound, eluted later at 9.11 min, and did not interfere with any peak seen in blank rumen fluid. HFBI was chosen as the optimum derivatization reagent, and excess HFBI and byproducts are observed in the typical chromatograms.

Choice of internal standard

Numerous different compounds were tested as possible internal standards with two derivatization reagents, HFBI and HFBA. Compounds which formed derivatives are included in Table 3.1, and compounds which did not form derivatives are listed in Table 3.2. All of the chosen compounds had at least one of the key structural characteristics of retronecine (heterocyclic ring system, amine, hydroxyl groups, double bond). Experiments testing the different potential internal standards generally followed the sample preparation method described in the experimental. Variations included using less derivatization reagent, no heating, or longer heating times.

Several factors led to the decision that the most suitable internal standard would be 2,6-dimethoxypyridine. When reacting with the imidazole, the 2,6-dimethoxypyridine yielded one clean peak that eluted near retronecine, but did not overlap with it, or any of the peaks normally present in blended whole rumen fluid. At a concentration of 100 µg/mL, 2,6-dimethoxypyridine yields a peak nearly

equivalent in area to that of retronecine at this concentration. It contains several of the key structural elements in retronecine, including two derivatizable hydroxyl groups and a tertiary amine within a heterocyclic ring system. Also, both compounds have at least one hydroxymethyl group which is adjacent to a double bond. GC/MS confirmed the structure of the bis(heptafluorobutyrate) derivative of 2,6-dimethoxypyridine.

Different solvents

Several different solvents were studied in addition to acetonitrile, including acetone, ethyl acetate, dioxane, pyridine, dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO). Experiments testing the different solvents generally followed the sample preparation method described in the experimental. Variations included less derivatization reagent, longer and shorter heating times, smaller samples, and less solvent. The solvents ethyl acetate and acetonitrile both yielded large, well shaped peaks for both compounds. As many of the initial experiments had been conducted with acetonitrile, it was decided to continue using acetonitrile to maintain continuity.

Heating time

With 0.5 mL acetonitrile and 200 µL of HFBI, the influence of no heating (ambient temperature) and heating at 70 °C for 15-120 min was investigated. Heating increased the peak areas, but heating longer than 15 min did not yield significantly larger peak areas.

Solvent and derivatization reagent volume

A series of experiments was run to determine the optimum volume of both acetonitrile and HFBI. The volume of acetonitrile varied from 1-3 mL and the volume of HFBI varied from 200-500 µL. Shown in Table 3.3 are the peak areas of retronecine and the internal standard when prepared from 1 mL samples of spiked blended rumen fluid. As whole rumen fluid is a complex matrix that may contain other compounds with derivatizable groups, it was anticipated that an excess of HFBI might be required.

Increasing the volume of acetonitrile typically resulted in smaller peak areas due to sample dilution. However, when increasing the volume of acetonitrile from 1 to 2 mL, the peak areas would be expected to decrease about 50%; the data in Table 3.3 shows that with 300 µL of HFBI or greater, peak area decreases only 10 - 20%. This indicates that increasing the acetonitrile volume enhances the reaction and yields a greater amount of product. When using 2 mL of acetonitrile, increasing the HFBI volume from 200 to 300 µL results in larger peak areas, but above 300 µL of HFBI the peak areas appear to stabilize. At the higher volumes of HFBI, excess derivatization reagent and by-products become larger and sometimes interfere with the peaks of interest. Thus it was determined that using 2 mL of acetonitrile and 300 µL of HFBI is an acceptable compromise between dilution and product formation.

Table 3.3 Peak areas of retronecine (ret) and the internal standard (int std) when prepared with varying volumes of acetonitrile (ACN) and HFBI.

ACN vol (mL):	1		2		3	
peak area:	ret	int std	ret	int std	ret	int std
HFBI vol (µL)						
200	194,510	178,469	105,354	104,882		
300	141,100	126,167	114,516	115,459	94,790	95,028
400			124,520	111,285	81,132	81,846
500			122,181	112,888	83,112	83,792

GC conditions identical to those in Figure 3.2, except the injector temp. was 180 °C.

Quantification

Quantification of retronecine in samples was based on a least squares fit of the peak area ratio of the analyte to internal standard versus analyte concentration. The working standards in both basal growth media and blended whole rumen fluid were prepared and chromatographed as described previously. A typical equation for the linear regression of working standards in blended whole rumen fluid is as follows: $y = 8.74 \times 10^{-3}(x) + 1.47 \times 10^{-3}$.

Detection limit

The detection limit, calculated from a 1 µg/mL sample of retronecine in water, was determined to be 0.02 µg/mL (1.7-s peak width at half height); the detection

limit, calculated from a 5 µg/mL sample of retronecine in blended whole rumen fluid, was determined to be 0.09 µg/mL (2.0-s peak width at half height).

Methods for determining the detection limit of a method utilizing an internal standard are not well described. These detection limits were calculated using $2Nw/A_R$, where N is the noise in the peak area ratio, w is the sample weight, and A_R is the peak area ratio (Poole and Schuette, 1984). The noise in the peak area ratio (N) was calculated using $(N/A_R)^2 = (s_1/A_1)^2 + (s_2/A_2)^2$ which is derived using propagation of error techniques (Shoemaker et al., 1989). The variables A_1 and A_2 are the peak areas of retronecine and the internal standard, respectively, and s_1 and s_2 are the estimates of the standard deviations of the peak areas of retronecine and the internal standard, respectively. As the detection limit is approached, A_1 approaches zero while A_2 remains relatively constant. Thus $(s_1/A_1)^2$ becomes very large compared to $(s_2/A_2)^2$, and N can be approximated by $N = s_1/A_2$. As the detection limit is approached, the standard deviation of the mean of the total area of retronecine approaches the standard deviation of the mean of the baseline, thus s_1 can be estimated using $s_1 = (s/\sqrt{n})\sqrt{2}$, where s is the standard deviation in the baseline and n is the number of data points used to determine the peak area.

Recovery

The relative percent recoveries of retronecine and the internal standard were 73% and 82%, respectively (n = 20). The relative percent recovery was calculated from the ratio of each component's peak area in blended whole rumen fluid to its peak area in water. Replicate samples (1 mL each) were prepared and

chromatographed as described previously. Corrections based on the relative recovery were not used when quantitating samples.

Precision

The relative standard deviations of the peak areas of retronecine and the internal standard prepared from water (1 mL each) were 7% and 15%, respectively. The corresponding RSD of the peak area ratio was 14% (n = 20). The RSD of the peak areas of retronecine and the internal standard prepared from blended whole rumen fluid (1 mL each) were 10% and 12%, respectively. The corresponding RSD of the peak area ratio was 10% (n = 20). The RSD of the peak area ratio from blended whole rumen fluid of 10% was considered acceptable for quantitation.

Analysis of Incubation Samples

Retronecine was incubated with cultures inoculated with either blended sheep whole rumen fluid or a pyrrolizidine alkaloid degrading enrichment (L4M2). Duplicate bottles contained retronecine at initial levels of 50-100 µg/mL. Samples were taken and frozen from all bottles at time zero, and throughout the incubation period (2 days or 2 weeks). Samples (1 mL each) were prepared and analyzed in duplicate as described previously. The results of the analysis for retronecine are shown in Figure 3.4. In both cases the concentration of retronecine did not change appreciably over the incubation period. Over the 48-hr incubation period, the RSD of the concentration of retronecine in the blended sheep whole rumen fluid in

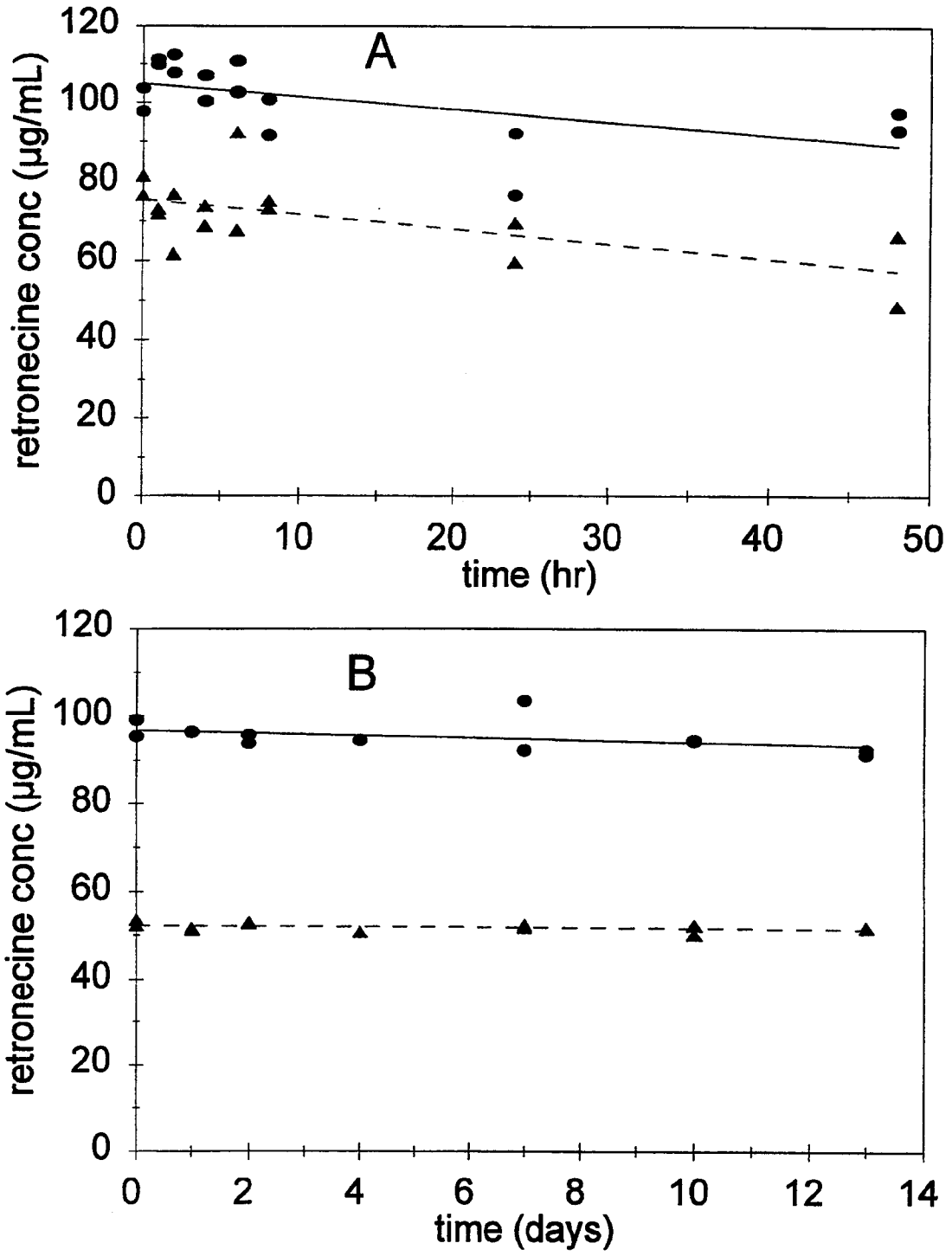

Figure 3.4 Results of retronecine analysis. Duplicate bottles individual data points (●, ▲) and the linear regression (———, – – –). A) Blended sheep whole rumen fluid incubated for two days. B) Basal media inoculated with L4M2 and incubated for two weeks.

duplicate bottles was 13% and 9%; this is not significantly different than the RSD of the method determined in blended whole rumen fluid which is 10%. The slopes of the linear regressions through the individual data points were -0.38 and -0.33 µg/mL/hr. Over the 2-week incubation period, the RSD of the concentration of retronecine in the basal growth media in duplicate bottles was 3% and 2%; this is significantly less than the RSD of the method determined in water which is 14%. The slopes of the linear regressions through the individual data points were -0.27 and -0.07 µg/mL/day. Under these conditions, retronecine was not degraded by sheep whole rumen fluid or by the enrichment L4M2.

CONCLUSIONS

This method enables the determination of retronecine from biological matrices on a routine basis. The technique has been developed specifically for liquid culture media with regard to decreasing sample preparation time and complexity, and increasing reproducibility. This method is currently being used to investigate the detoxification by sheep ruminal bacteria of pyrrolizidine alkaloids isolated from tansy ragwort . Studies are being conducted with both blended whole rumen fluid and the bacterial enrichment L4M2. The ability to detect retronecine from these biological matrices at low levels will aid in determining if hydrolysis contributes significantly to the detoxification of pyrrolizidine alkaloids by ruminal microbes. Using this analytical method, the experiments presented in Chapter 4 indicate that hydrolysis is not occurring within the rumen.

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Chapter 4

METABOLISM OF RETRONECINE AND PYRROLIZIDINE ALKALOIDS BY OVINE RUMINAL BACTERIA

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ABSTRACT

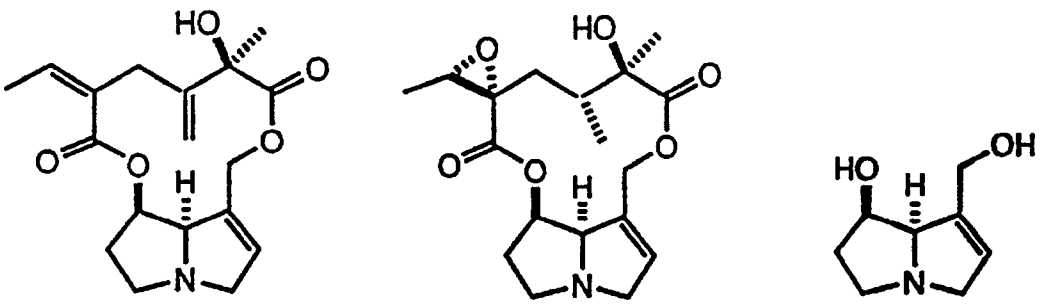
Macrocyclic pyrrolizidine alkaloids are produced naturally by *Senecio jacobaea* which are toxic to several species of livestock, including cattle and horses. Sheep, however, typically display resistance to pyrrolizidine alkaloid poisoning. This resistance to pyrrolizidine alkaloid toxicosis has been attributed to metabolism of pyrrolizidine alkaloids within the rumen by anaerobic microbes. In this study the metabolism of pyrrolizidine alkaloids by ovine ruminal microbes was investigated with the specific focus being the potential intermediate retronecine, a non-esterified necine base. Metabolism of retronecine independent of the macrocyclic pyrrolizidine alkaloids was also investigated. Experiments were conducted with both blended ovine whole rumen fluid and with a mixed culture of pyrrolizidine alkaloid-metabolizing microbes derived from ovine rumen fluid. Retronecine was not observed as a transient intermediate under these conditions, and also it was not metabolized independent of the macrocyclic pyrrolizidine alkaloids.

INTRODUCTION

Pyrrolizidine alkaloids are produced in numerous plants throughout the world. Many of the isolated and identified pyrrolizidine alkaloids (over 300) are toxic to several species, including cattle, horses, rats, and humans (Culvenor, 1980; Mattocks, 1986; Segall et al., 1991). Other species, such as sheep and goats, have demonstrated resistance to pyrrolizidine alkaloid toxicosis. In fact, sheep are used as biological control agents in areas heavily infested with pyrrolizidine alkaloid

containing plants (Cheeke, 1984; Dollahite, 1972; Hooper, 1978). In the Pacific Northwestern region of the United States, tansy ragwort (*Senecio jacobaea*) is a pyrrolizidine alkaloid containing plant of economic importance. Tansy ragwort contains several macrocyclic pyrrolizidine alkaloids which cause irreversible liver cirrhosis in cattle, while sheep are typically not affected. It has been demonstrated that metabolism of the pyrrolizidine alkaloids in tansy ragwort by anaerobic ruminal microorganisms is responsible for protecting sheep from toxicosis (Craig et al., 1986, 1992; Wachenheim et al., 1992a, 1992b).

All of the pyrrolizidine alkaloids in tansy ragwort are macrocyclic diesters of the necine base retronecine (Mattocks, 1986), with the two most abundant pyrrolizidine alkaloids being jacobine and seneciphylline (Figure 4.1). The parent alkaloids are non-toxic, but once they are absorbed into the bloodstream and transported to the liver they can be converted by the mixed function oxidase systems to toxic pyrroles (Mattocks and White, 1971; Segall et al., 1991). The pyrroles then bind to proteins and macromolecules causing liver lesions and



Seneciphylline Jacobine Retronecine

Figure 4.1 Structures of the two most predominant pyrrolizidine alkaloids in tansy ragwort, seneciphylline and jacobine, and the necine base retronecine.

ultimately cirrhosis (Cheeke and Shull, 1985; Hsu et al., 1975; Kim et al., 1995; Swick 1984; White and Mattocks, 1972). Hydrolysis of pyrrolizidine alkaloids in the rumen would result in the necine base retronecine, a product consistent with the lack of toxicological effects observed in sheep. If retronecine is produced in the rumen it would be absorbed and transported to the liver, but since the mixed function oxidase systems show very little activity towards retronecine, it would not be converted to toxic pyrroles but instead excreted in the urine (Mattocks, 1978, 1981).

Recent work in this laboratory has been aimed at determining the ruminal metabolic pathway. To this end, our laboratory has utilized an anaerobic mixed culture derived from ovine rumen fluid (identified as L4M2) which metabolizes the pyrrolizidine alkaloids isolated from tansy ragwort. It is likely that this mixed culture contains anywhere from three to six microbes, each of which may play a part in the pyrrolizidine alkaloid metabolism. One or more organisms could be hydrolyzing the pyrrolizidine alkaloids to the necic acid and the necine base retronecine; however, preliminary work with this culture has indicated that metabolites containing a necine base ring system are not being produced. It is possible that other organisms in the mixed culture could be further metabolizing retronecine. If this is the case, retronecine may be observed only as a transient intermediate during the metabolism of pyrrolizidine alkaloids, but also retronecine itself should also be metabolized, independent of the macrocyclic pyrrolizidine alkaloids.

This study has two main objectives: 1) to determine if retronecine is produced during the metabolism of the pyrrolizidine alkaloids isolated from tansy ragwort,

either as an end-product or as a transient intermediate; 2) to determine if retronecine is degraded independently of the macrocyclic pyrrolizidine alkaloids. These experiments utilized both ovine whole rumen fluid and the mixed culture, L4M2. Based on previously defined methods (Craig et al., 1992; Wachenheim et al., 1992a), pyrrolizidine alkaloids isolated from tansy ragwort were metabolized by sheep ruminal microbes, and the presence or absence of retronecine was determined at intermediate time points. Also, attempts were made to metabolize retronecine directly with sheep ruminal microbes, both alone and in combination with the pyrrolizidine alkaloids isolated from tansy ragwort.

MATERIALS AND METHODS

Media and growth conditions

Rumen contents were obtained from rumen-cannulated sheep 2-4 hr after feeding and transported anaerobically to the laboratory. All subsequent procedures were performed anaerobically. The ruminal contents were blended with a modification of McDougall Buffer (25:75) at high speed for 1 min, then dispensed into serum bottles (100 mL in 200-mL serum bottles), and stock solutions (10 mg/mL) of either pyrrolizidine alkaloids isolated from *Senecio jacobaea* and/or retronecine were added for a final concentration of 50-100 µg/mL. The stock solutions were prepared in 1% phosphoric acid adjusted to pH 7.0 with sodium hydroxide. The bottles were incubated at 37 °C with shaking, and sample aliquots were removed at appropriate time intervals and frozen until analysis. Incubations

were performed in duplicate or triplicate. Autoclaved serum bottles served as sterile controls, and serum bottles without pyrrolizidine alkaloids or retronecine added served as viable controls.

The mixed culture L4M2 which metabolizes the pyrrolizidine alkaloids in *Senecio jacobaea* has been derived from ovine rumen fluid in this laboratory (Johnston, 1995). L4M2 was grown in a basal growth media which is a modification of previously defined MPN-PA medium; modifications included the omission of hemin and volatile fatty acids except for sodium acetate and the addition of sodium formate (5.0 g/L), tryptone (1.0 g/L) and yeast extract (2.0 g/L) (Wachenheim et al., 1992a). Stock solutions of pyrrolizidine alkaloids or retronecine were added as described above to cultures inoculated with the L4M2 culture on the previous day. The bottles were incubated at 37 °C with shaking, and sample aliquots were removed at appropriate time intervals and frozen until analysis. Incubations were performed in duplicate. Uninoculated serum bottles served as sterile controls, and serum bottles without pyrrolizidine alkaloids or retronecine served as viable controls.

Pyrrolizidine Alkaloid Analysis

Pyrrolizidine alkaloids were determined by TLC analysis (Wachenheim et al., 1992a). Samples (1 mL) were mixed with 5 **M** NaOH (100 µL) and dichloromethane (500 µL). Samples were centrifuged for 5-15 min as necessary to separate the layers. The dichloromethane was transferred to a clean glass tube (12x75 mm) and then removed under vacuum at 43 °C. Residue was reconstituted in 20 µL of dichloromethane and spotted on HPKF silica gel TLC plates (Whatman, Clifton, NJ).

Chromatograms were developed in a chloroform-methanol-propionic acid (36:9:5) solvent. Visualization of alkaloids was by sequential spraying with Dragendorff spray reagent (Sigma Co., St. Louis, MO) and 5% sodium nitrite. The R_f values of jacobine (reddish brown), seneciphylline (dark purple) and senecionine (orange) were 0.66, 0.72 and 0.76, respectively. The detection limit of total pyrrolizidine alkaloids was estimated to be 2 µg/mL (based on visual detection), although distinct colors were only visible down to 6 µg/mL.

Retronecine Analysis

A recently developed method for the determination of retronecine was utilized with subsequent GC/FID or GC/MS analysis (Hovermale and Craig, 1998; Chapter 3). Briefly, 2,6-dimethoxypyridine was added to duplicate aliquots (1 mL) of each sample and then dried (70 °C). The bis(heptafluorobutyrate) derivatives were formed using N-heptafluorobutyrylimidazole in acetonitrile (70 °C, 15 min). The supernatant could by analyzed directly by GC/FID. Samples prepared for analysis by GC/MS were prepared using toluene in place of acetonitrile, and excess derivatization reagent was removed before analysis. Chromatography conditions were as previously described (Hovermale and Craig, 1998). The detection limit was reported to be 0.09 µg/mL in blended whole rumen fluid and 0.02 µg/mL in basal growth media.

RESULTS

Metabolism by blended ovine whole rumen fluid

In the cultures of viable whole rumen fluid in which pyrrolizidine alkaloids were added without retronecine, the pyrrolizidine alkaloids were metabolized to undetectable levels in less than 24 hr; whereas, in the autoclaved controls, the pyrrolizidine alkaloids were present throughout the 48-hr incubation period. This is consistent with previously published results for pyrrolizidine alkaloid metabolism (Craig et al., 1992; Wachenheim et al., 1992a). Retronecine was not detected from any of these cultures throughout the 48-hr incubation period. Controls which had no pyrrolizidine alkaloids added (both viable and sterile) had none present at any of the time points tested. Under these conditions retronecine was not detected as an intermediate during pyrrolizidine alkaloid metabolism.

In the cultures of viable whole rumen fluid in which only retronecine was added, the concentration of retronecine did not change appreciably over the 48-hr incubation period (presented in part, Hovermale and Craig, 1998). In the cultures of viable whole rumen fluid in which pyrrolizidine alkaloids and retronecine were both added, the macrocyclic pyrrolizidine alkaloids were metabolized to undetectable levels in less than 24 hr, and the concentration of retronecine did not change appreciably over the 48-hr incubation period (Figure 4.2). The relative standard deviation (RSD) for determining the concentration of retronecine at 100 µg/mL was previously determined to be 10% (Hovermale and Craig, 1998; Chapter 3); over the 48-hr incubation period, the RSD of the concentration of retronecine in

triplicate bottles was found to be 6%, 13% and 10%. The slopes of the linear regressions through the individual data points (Figure 4.2) were -0.10, -0.37, and -0.52 µg/mL/hr. In sterile controls in which retronecine was added, either with or without pyrrolizidine alkaloids, the concentration of retronecine also did not change appreciably over the 48-hr incubation period (RSD = 6%, slope = 0.05 µg/mL/hr). Under these conditions retronecine was not degraded by the ovine whole rumen fluid.

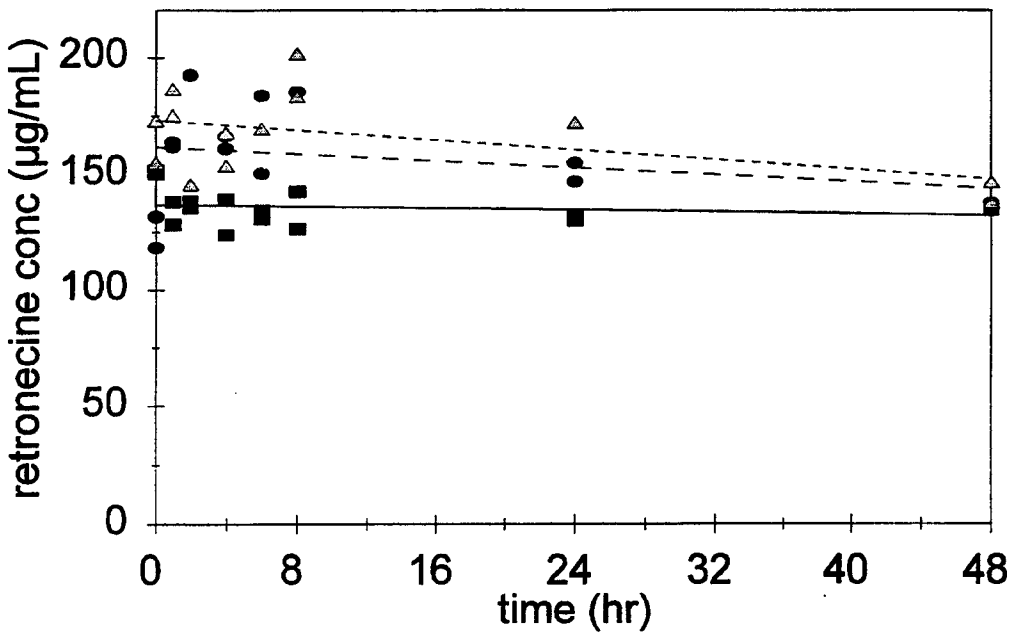


Figure 4.2 Results of retronecine analysis from incubation bottles of blended sheep whole rumen fluid initially containing both retronecine and macrocyclic pyrrolizidine alkaloids. Triplicate bottles individual data points (■, ●, ▲) and the linear regression (——, — — —, - - - - -).

Metabolism by the mixed culture L4M2

In the cultures of viable L4M2 in which pyrrolizidine alkaloids were added, the pyrrolizidine alkaloids were metabolized to undetectable levels in less than 7 days; whereas, in the uninoculated control, the pyrrolizidine alkaloids were present throughout the 2-week incubation period. In these cultures (both viable and sterile), retronecine was not detected for the first 4 days of incubation, but for the samples after 7, 10 and 13 days of incubation, very low levels of retronecine were detected (less than 5 µg/mL, compared to 100 µg/mL of pyrrolizidine alkaloids initially added). The presence of retronecine in these samples was confirmed by GC/MS analysis. The exact source of this retronecine is unclear, but because it appears in both viable and sterile cultures, it is likely that the source is not due to microbial metabolism.

For controls to which no macrocyclic pyrrolizidine alkaloids were added (both viable and sterile), none were detected in any of the samples tested. Also for the cultures to which retronecine was added, no pyrrolizidine alkaloids were detected in any of the samples tested. Under these conditions, a significant amount of retronecine above the sterile control was not detected as an intermediate during pyrrolizidine alkaloid metabolism.

In the cultures of viable L4M2 to which retronecine was added, the concentration did not change appreciably over the 2-week incubation period (presented in part, Hovermale and Craig, 1998). In the controls to which no retronecine or pyrrolizidine alkaloids were added (both viable and sterile), no

retronecine was detected throughout the 2-week incubation period. Under these conditions retronecine was not degraded by the mixed culture L4M2.

DISCUSSION

The mean retention time of material in the gut of sheep has been reported to be 47.4 ± 26.5 hr (Stevens, 1988). Turnover times for rumen particulate matter in sheep ranges from 19 to 53 hr, with most values being slightly over 24 hr (Dehority, 1997). For ruminal metabolism to be beneficial to the sheep, it must occur within this time period. To account for possible animal to animal variation, these experiments with blended whole ovine rumen fluid were all conducted for 48 hr. Metabolism of pyrrolizidine alkaloids by blended ovine whole rumen fluid was observed to occur is less than 24 hr, consistent with previous work (Craig et al., 1992; Wachenheim et al., 1992a). If hydrolysis of the pyrrolizidine alkaloids was occurring, then the concentration of retronecine should increase within 24 hr, after which time its concentration should either stabilize if it is not further metabolized, or decrease if it is further metabolized. This is based on the assumption that the hydrolysis of macrocyclic pyrrolizidine alkaloids occurs at about the same rate or significantly faster than that of retronecine. If the metabolism of retronecine is significantly faster than that of the macrocyclic pyrrolizidine alkaloids, then retronecine may not be detected as a transient intermediate. After comparing the RSD of the retronecine concentrations in the blended whole rumen fluid to that previously determined for the method it was determined that the concentration of retronecine did not change significantly over the 48-hr incubation period. In the

cultures of blended ovine whole rumen fluid with only retronecine added, again the concentration of retronecine did not change significantly over the 48-hr incubation period.

In these studies with the mixed culture L4M2, 7 days were required to metabolize pyrrolizidine alkaloids. Very low levels of retronecine were observed in these cultures, but because it was not greater than that in the sterile controls, it is most likely not due to microbial metabolism. In the cultures of L4M2 with only retronecine added, its concentration did not change significantly over the 2-week incubation period. The fact that L4M2 requires a longer time to metabolize the macrocyclic pyrrolizidine alkaloids than blended whole ovine rumen fluid indicates that ideal conditions simulating the rumen environment have not yet been achieved. Growth conditions are currently being improved and will greatly facilitate further studies.

Two main results suggest that hydrolysis of macrocyclic pyrrolizidine alkaloids is not occurring within the sheep rumen. First, retronecine was not observed to appear as an end-product when macrocyclic pyrrolizidine alkaloids were metabolized. Second, retronecine was not metabolized independently of the macrocyclic pyrrolizidine alkaloids. The metabolism of pyrrolizidine alkaloids in the rumen which protects sheep from their toxicity must involve other reaction pathways. Further studies are focused on identifying the metabolites containing the necine base portion of the pyrrolizidine alkaloids. Because toxicity in the liver is directly related to the conversion of the necine base to a pyrrole, it is likely that detoxification mechanisms in the rumen modify this moiety of the molecule.

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Chapter 5

METABOLISM OF PYRROLIZIDINE ALKALOIDS BY *PEPTOSTREPTOCOCCUS HELIOTRINREDUCENS* AND A MIXED CULTURE DERIVED FROM OVINE RUMINAL FLUID

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ABSTRACT

A mixed culture of ovine ruminal microbes metabolizes the macrocyclic pyrrolizidine alkaloids present in the plant *Senecio jacobaea*, including jacobine and seneciphylline. Previous attempts to identify metabolites of these alkaloids have not been successful. The objective of this study was to compare the metabolism of pyrrolizidine alkaloids by the mixed culture of ovine ruminal microbes to the metabolism of pyrrolizidine alkaloids by the known organism *Peptostreptococcus heliotrinreducens*. *P. heliotrinreducens* metabolizes the pyrrolizidine alkaloids heliotrine and lasiocarpine to 7α-hydroxy-1-methylene-8α-pyrrolizidine and 7α-angelyl-1-methylene-8α-pyrrolizidine, respectively. This organism does not metabolize the pyrrolizidine alkaloids jacobine or seneciphylline. The mixed culture of ovine ruminal microbes also metabolizes heliotrine and lasiocarpine to identical methylene compounds. This mixed culture also metabolizes jacobine and seneciphylline, with production of very low levels of the corresponding 1-methylene compounds. Samples were analyzed with TLC and GC/MS.

INTRODUCTION

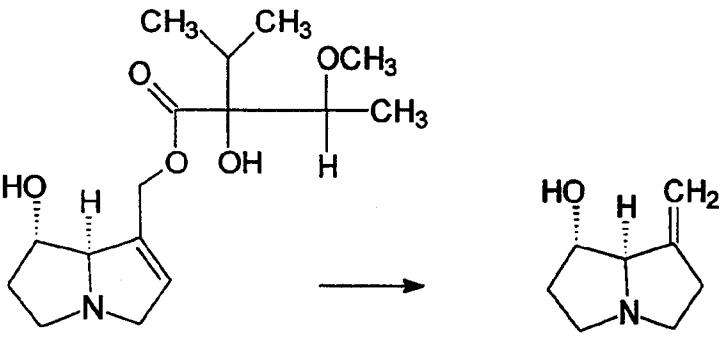
Sheep in the Pacific Northwestern United States are considered to be resistant to pyrrolizidine alkaloid poisoning, especially by the plant *Senecio jacobaea* (tansy ragwort) (Dollahite, 1972; Hooper, 1978; Muth, 1968). All of the pyrrolizidine alkaloids in tansy ragwort are macrocyclic diesters of the necine base retronecine, with the two most abundant being jacobine and seneciphylline

(Mattocks, 1986). It has been demonstrated that the pyrrolizidine alkaloids in tansy ragwort are metabolized in ovine ruminal fluid (Craig et al., 1992; Wachenheim et al., 1992). A mixed culture of anaerobic microbes which metabolizes the pyrrolizidine alkaloids jacobine and seneciphylline has been derived from the rumen fluid of sheep maintained on tansy ragwort. This mixed culture has been named L4M2 and contains anywhere from three to six microbes. It has not been possible to isolate a pure culture which metabolizes jacobine or seneciphylline.

Previous attempts to conclusively identify metabolites produced by the ruminal metabolism of jacobine and seneciphylline have been unsuccessful (Hovermale and Craig, 1998; Chapter 3; Chapter 4). Most pyrrolizidine alkaloids are not available commercially and must be isolated from plant material. Labelled pyrrolizidine alkaloids are even more difficult obtain, with the most efficient method being to grow pyrrolizidine alkaloid producing plants in an atmosphere of $^{14}CO_2$ or by using some other labelled precursors (Lamé et al., 1996; Reed et al., 1985). This requires a considerable amount of time and specialized knowledge and equipment. Generally yields are less than 2% recovery of label into the desired compounds (Reed et al., 1985). Thus, using labelled compounds to identify metabolic products was not considered a viable option.

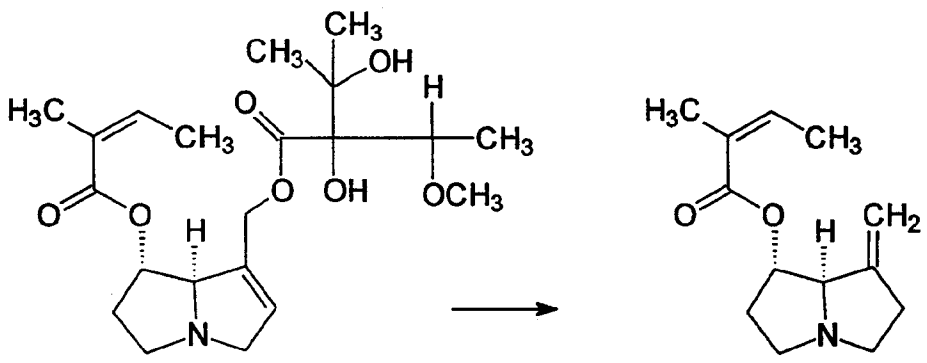
In this study the metabolism of various pyrrolizidine alkaloids by L4M2 was compared to the metabolism of these pyrrolizidine alkaloids by the organism *Peptostreptococcus heliotrinreducens*. *P. heliotrinreducens* was originally isolated from Australian sheep rumen contents and is known to metabolize several mono- and diester pyrrolizidine alkaloids to compounds with a 1-methylene group external to the pyrrolizidine ring (Figure 5.1) (Lanigan, 1976). *P. heliotrinreducens*

metabolizes a few macrocyclic pyrrolizidine alkaloids more slowly than it does the mono- and diesters; some macrocyclic pyrrolizidine alkaloids are not metabolized at all (Lanigan, 1976). In this study, both *P. heliotrinreducens* and L4M2 were grown on heliotrine, lasiocarpine, monocrotaline and a mixture of pyrrolizidine alkaloids isolated from tansy ragwort. Samples from the cultures were analyzed for pyrrolizidine alkaloids and methylene products using TLC and GC/MS.



Heliotrine

7α-hydroxy-1-methylene-8α-pyrrolizidine



Lasiocarpine

7α-angelyl-1-methylene-8α-pyrrolizidine

Figure 5.1 Pyrrolizidine alkaloids metabolized by *Peptostreptococcus heliotrinreducens* to 1-methylene compounds.

MATERIALS AND METHODS

Organism, media and growth conditions

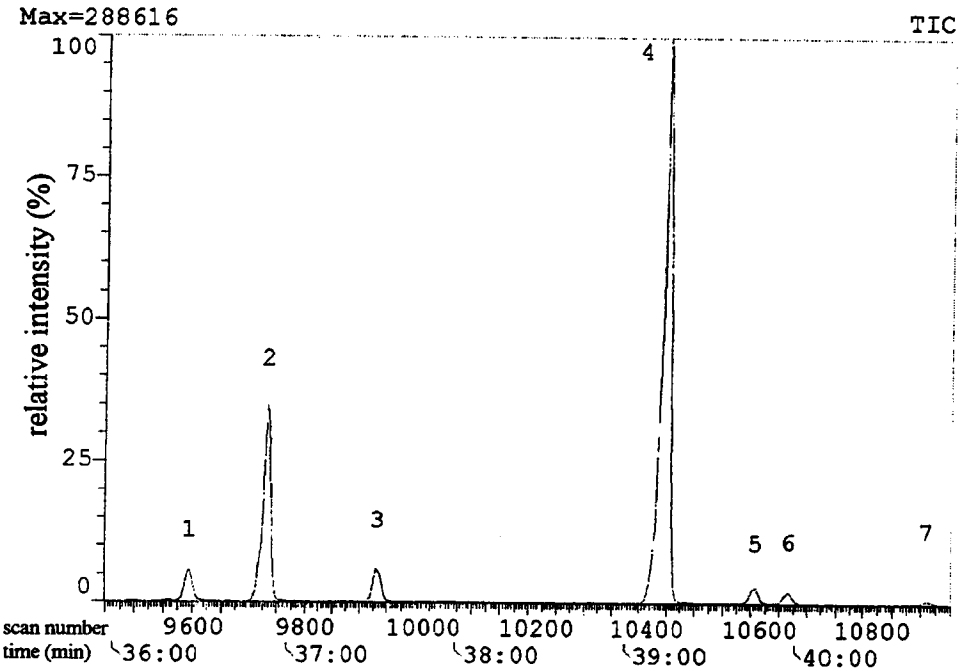
Peptostreptococcus heliotrinreducens was purchased from the American Type Culture Collection (Strain #29202) and grown in TYM medium in balch tubes under anaerobic conditions at 38 °C (Lanigan, 1976). Cultures were maintained by serial transfers on a daily basis. Parallel cultures were grown with different pyrrolizidine alkaloids as substrates. The anaerobic mixed culture derived from ovine rumen fluid (L4M2) was maintained on E medium in hungate tubes under anaerobic conditions at 38 °C with serial transfers on a daily basis. Parallel cultures were grown with different pyrrolizidine alkaloids as substrates. Samples were removed from the cultures at appropriate time intervals and analyzed for pyrrolizidine alkaloids and methylene products.

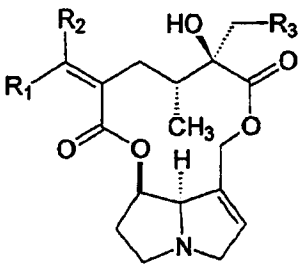
The E medium had the following composition: mineral I solution, 40 mL/L; mineral II solution, 40 mL/L; major volatile fatty acid (VFA) solution, 11.4 mL/L; supplemental VFA solution, 11.4 mL/L; trace metal solution, 11.4 mL/L; 0.1% resazurin, 1.1 mL/L; 0.01% hemin (in 0.05 **M** NaOH), 1.1 mL/L; clarified rumen fluid, 100 mL/L; 8% sodium carbonate (w/v), 50 mL/L; 2.5% cysteine hydrochloride (w/v), 10 mL/L; and 2.5% sodium sulfide (w/v), 6 mL/L. The headspace gas was CO_2. The pH was adjusted to 7.0. The solutions of sodium carbonate, cysteine hydrochloride and sodium sulfide were prepared as previously described (Bryant and Robinson, 1961) and added after autoclaving as filter-sterilized solutions. Mineral solution I contained K_2HPO_4, 6 g/L (Bryant and Robinson, 1961). Mineral

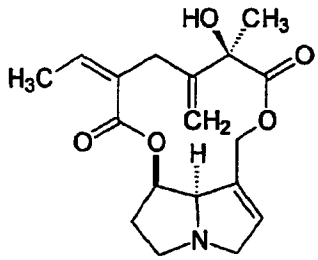
solution II had the following components: NaCl, 12 g/L; KH_2PO_4, 6 g/L; $(NH_4)_2SO_4$, 12 g/L; $MgSO_4 \cdot 7H_2O$, 1.0 g/L; and $CaCl_2 \cdot 2H_2O$, 1.0 g/L (Bryant and Robinson, 1961). The major VFA solution had the following components: sodium acetate, 20.82 g/L; sodium propionate, 9.6 g/L; and sodium butyrate, 5.5 g/L (Leedle and Hespell, 1980). The supplemental VFA solution had the following components: isobutyric acid, 10 mL/L; 2-methylbutyric acid, 10 mL/L; isovaleric acid, 10 mL/L; and valeric acid, 10 mL/L (Leedle and Hespell, 1980). The pH of both VFA solutions was adjusted to 7.5 using NaOH. The trace metals solution had the following components: Na_2EDTA, 0.43 g/L; $FeSO_4 \cdot 7H_2O$, 0.20 g/L; $MnSO_4 \cdot H_2O$, 0.17 g/L; H_3BO_3, 0.03 g/L; $CoCl_2 \cdot 6H_2O$, 0.012 g/L; $ZnSO_4 \cdot 7H_2O$, 0.01 g/L; $NaMoO_4$, 0.0025 g/L; $NiCl_2 \cdot 6H_2O$, 0.002 g/L; and $CuCl_2 \cdot 2H_2O$, 0.001 g/L (Pfennig, 1965).

Chemicals

A crude mixture of pyrrolizidine alkaloids was isolated from *Senecio jacobaea* (tansy ragwort) as previously described (Liddell and Logie, 1993). This mixture contains mostly jacobine and seneciphylline, although senecionine, integerrimine, jacozine, jacoline, and retrorsine are all present (Figure 5.2). Integerrimine, which is an isomer of senecionine, has not been reported previously in *S. jacobaea* and may be an artifact of the extraction procedure; isomerization may be occurring in the methanol extract. Monocrotaline was purchased from Sigma. Heliotrine and lasiocarpine were gifts from Dr. Edgar, CSIRO, Australia. The lasiocarpine was originally isolated prior to 1993, and currently consists of a mixture of angelyl and tiglyl isomers, as well as two breakdown products (Figure 5.3). The spontaneous

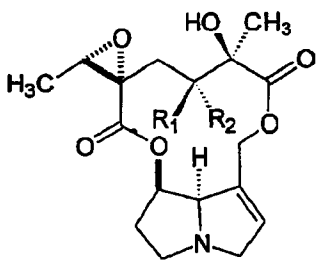


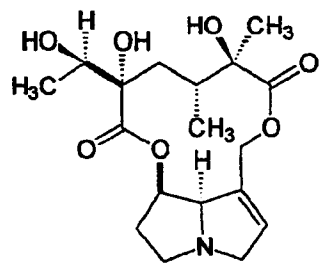




	R_1	R_2	R_3	
(1)	CH_3	H	H	Senecionine
(3)	H	CH_3	H	Integerrimine
(7)	CH_3	H	OH	Retrorsine

(2) Seneciphylline

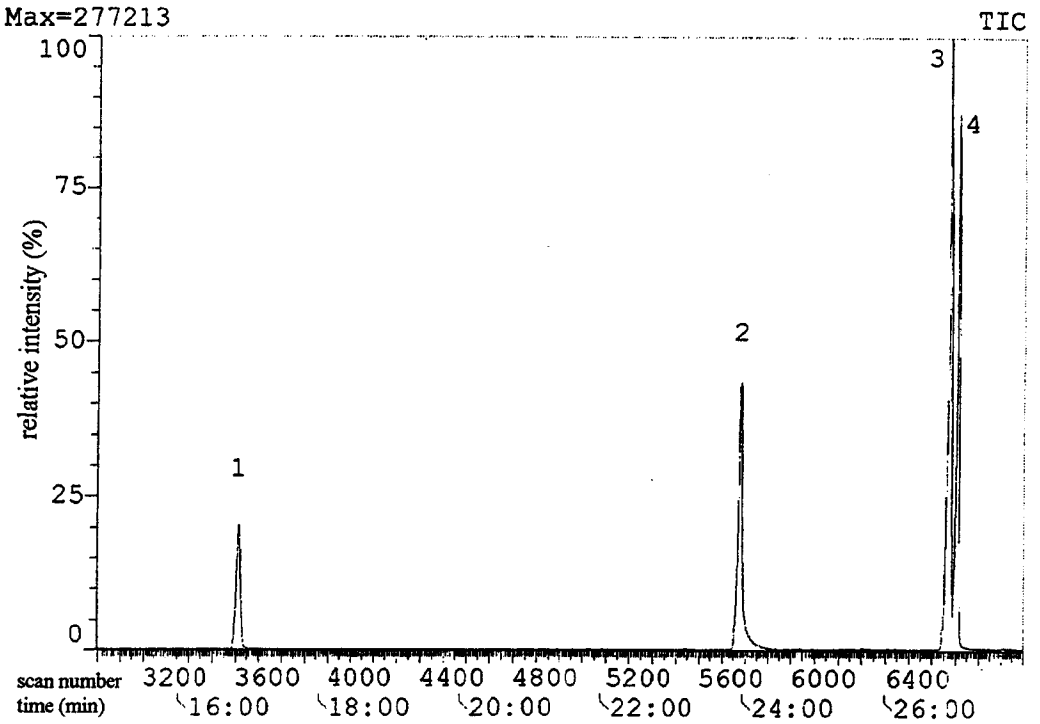


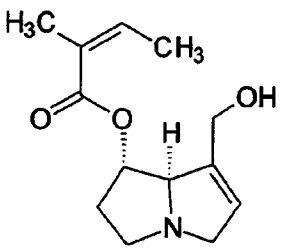


	R_1	R_2	
(4)	H	CH_3	Jacobine
(5)	-CH_2-		Jacozine

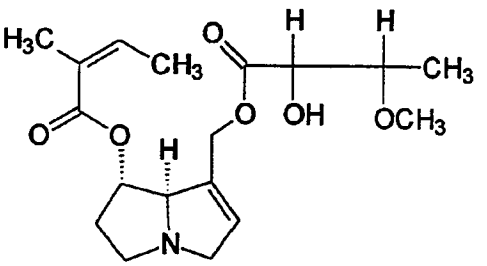
(6) Jacoline

Figure 5.2 GC/MS total ion chromatogram of pyrrolizidine alkaloid mixture isolated from *Senecio jacobaea* (tansy ragwort). Chromatographic conditions described in the text under temperature program 1.

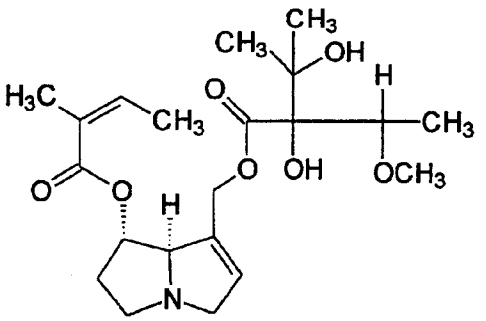




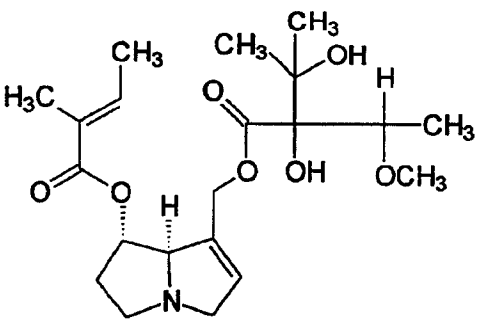
(1) 7-angelyl-heliotridine



(2)



(3) Lasiocarpine



(4) tiglyl isomer of lasiocarpine

Figure 5.3 GC/MS total ion chromatogram of lasiocarpine isomers (3, 4) and proposed breakdown products (1, 2). Chromatographic conditions described in the text under temperature program 3.

isomerization from angelyl to tiglyl esters has been observed for both mono- and diesters (Stelljes et al., 1991). Although the mass spectra for the angelyl and tiglyl isomers of lasiocarpine were indistinguishable, identification (peaks 3 and 4 in Figure 5.3) was based upon the known elution order; angelyl isomers elute prior to tiglyl isomers (Stelljes et al., 1991). Stock solutions of individual pyrrolizidine alkaloids or the tansy pyrrolizidine alkaloid mixture were prepared in phosphate buffer (pH=6.8) and added to the growth media for a final concentration of 50 - 100 µg/mL. Stock solutions of individual pyrrolizidine alkaloids or the tansy pyrrolizidine alkaloid mixture were prepared at a concentration of 10 mg/mL in methanol and diluted into water to prepare the working standards. Each set of 4 working standards ranged from 10 to 160 µg/mL and was used to quantitate samples analyzed by GC/MS as described below.

TLC analysis

This method was modified from previously reported methods (Chapter 4; Wachenheim et al., 1992a, 1992b). Samples (0.5 mL) were mixed with 5 **M** NaOH (50 µL) and dichloromethane (1 mL). Samples were centrifuged (5 min) to separate the layers. The dichloromethane was transferred to a clean glass tube (12x75 mm), and then removed under vacuum at 43 °C. The residue was reconstituted in methanol (20 µL) and spotted in duplicate (2 x 10 µL) onto HPKF silica gel TLC plates (Whatman). The plates were developed in either an acidic solvent system (A) or a basic solvent system (B). Solvent system A consisted of chloroform-methanol-propionic acid (36:9:5); solvent system B consisted of chloroform-

methanol-ammonium hydroxide (85:14:1). Individual plates were then visualized either with spray system C or spray system D. System C consisted of sequential spraying with 1% *o*-chloranil in toluene and Ehrlichs spray reagent (Sigma), with heating at 80 °C after each spraying; system D consisted of sequential spraying with Dragendorff spray reagent (Sigma) and 5% aqueous sodium nitrite. The R_f values of the pyrrolizidine alkaloids in both solvent systems are shown in Table 5.1, and the detection limits (based on visual detection) of the pyrrolizidine alkaloids with both spray systems are shown in Table 5.2.

Table 5.1 R_f values for pyrrolizidine alkaloids developed in solvent systems A and B.

	A	B
heliotrine	0.47	0.25
lasiocarpine	0.67	0.71
monocrotaline	[a]ND	0.42
tansy PA mixture		
jacobine	0.70	0.59
seneciphylline	0.80	0.62

[a]ND Not determined.

GC/MS analysis

Samples (1.0 - 3.0 mL) were mixed with concentrated NH_4OH (100 µL) and then extracted with dichloromethane (2 mL each). The dichloromethane was combined and then removed under vacuum at 43 °C. The residue was reconstituted in ethyl acetate or toluene (200 - 500 µL). Samples were

Table 5.2 Detection limits for pyrrolizidine alkaloids visualized with spray systems C and D (µg/mL).

	C	D
heliotrine [a]	6	4
lasiocarpine [b]	10	8
monocrotaline [a]	8	4
total PA from tansy [b]	10	6

[a]Determined from plates developed in solvent system B.
[b]Determined from plates developed in solvent system A.

chromatographed on an Rt_x-5MS capillary column (30-m x 0.25-mm, 0.5-µm film thickness, Restek) installed in an HP 5890 GC (Hewlett-Packard) interfaced with a Finnigan 5100 quadrupole mass spectrometer (EI, 70 eV) (Finnigan). Samples were injected by an HP model 7673A automatic injector into a split-splitless injector operated in the splitless mode (injector temp. 200 °C, transfer line temp. 280 °C, manifold temp. 100 °C). Three different oven programs were used (1, 2 and 3). Program 1: 100 °C (3 min), 5 °C/min to 280 °C (5 min). Program 2: 100 °C (3 min) 10 °C/min to 280 °C (8.5 min). Program 3: 150 °C (3 min) 5 °C/min to 280 °C (4.5 min), with a transfer line temp. of 250 °C. The scan range was 45-420 amu (0.19 s/scan). Data were collected via personal computer running Galaxy 2000 software (LGC).

Derivative formation

Mass spectral data are consistent with the hypothesis that heliotrine is converted to 7α-hydroxy-1-methylene-8α-pyrrolizidine (Figure 5.1). The metabolite produced in cultures grown on heliotrine was converted to the heptafluorobutyrate derivative as follows. Selected samples from cultures of both *P. heliotrinreducens* and L4M2 grown on heliotrine were extracted, as described under GC/MS analysis, and reconstituted in toluene (1.0 mL). The derivatization reagent heptafluorobutyrylimidazole (HFBI) was added (100 µL), and the samples vortexed and heated at 70 °C for 30 min in a block heater. To remove excess derivatization reagent, 1 mL of water was added and the samples were vortexed and centrifuged at 1520 RCF for 5 min. The organic layer was then concentrated (to 100 µL) and analyzed via GC/MS with temperature program 1. Shown in Figure 5.4 are the mass spectra of this compound before and after derivatization. Shown are data from cultures of *P. heliotrinreducens* grown on heliotrine; cultures of L4M2 grown on heliotrine yielded equivalent results.

Preparative TLC

Mass spectral data are consistent with the hypothesis that lasiocarpine is converted to 7α-angelyl-1-methylene-8α-pyrrolizidine (Figure 5.1). The metabolite produced in cultures grown on lasiocarpine was isolated using preparative TLC of selected cultures of both *P. heliotrinreducens* and L4M2 grown on lasiocarpine. Samples (5 - 10 mL) were mixed with 5 **M** NaOH (0.5 - 1.0 mL) and extracted with

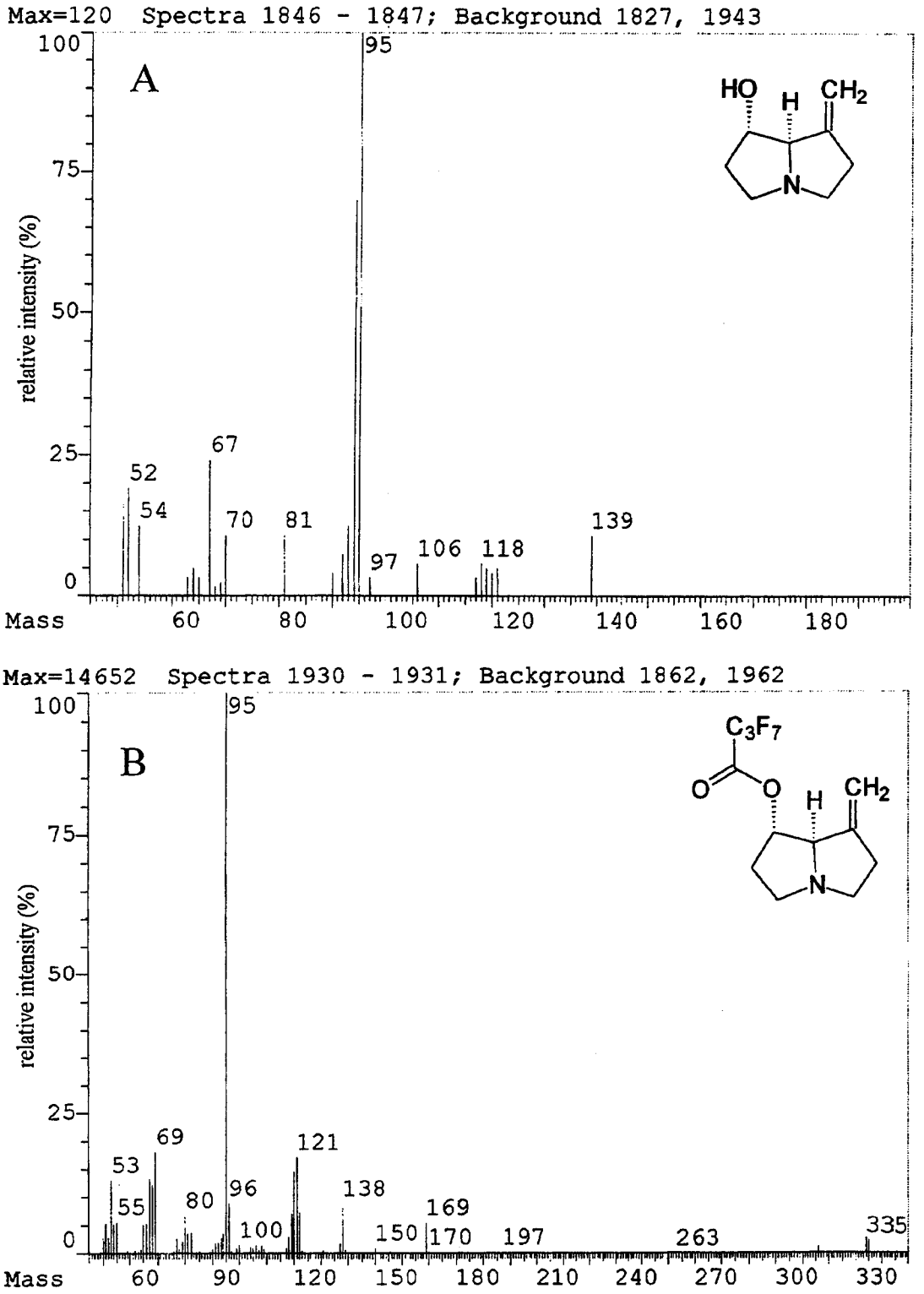


Figure 5.4 Mass spectra. A) 7α-hydroxy-1-methylene-8α-pyrrolizidine. MW 139. B) Heptafluorobutyrate derivative of 7α-hydroxy-1-methylene-8α-pyrrolizidine. MW 335.

dichloromethane (10 - 15 mL). The dichloromethane was then removed under vacuum. The residue was dissolved in methanol and streaked onto HPKF silica gel TLC plates (Whatman). After the plates were developed in solvent system A, they were completely covered except for 1 cm, which was visualized with spray system D. After location of the product, the silica on the unsprayed portion of the plate was scraped off of the glass plate (R_f = 0.68 - 0.80) and placed on top of a plug of glass wool in a Pasteur pipet. The product was eluted with 3 1-mL aliquots of chloroform-methanol (3:1) followed by 1 mL of methanol. The solvent was removed under vacuum; the residue was reconstituted in toluene and analyzed by GC/MS with temperature program 2. A small amount of 7α-tiglyl-1-methylene-8α-pyrrolizidine was also identified (Figure 5.5). The mass spectra for the angelyl and tiglyl isomers were indistinguishable and are consistent with that previously reported for the 7β-angelyl- isomer (Logie et al., 1997). Identification was based upon the known chromatographic elution order (angelyl isomers elute before tiglyl isomers) (Stelljes et al., 1991). The data shown in Figure 5.5 is from cultures of *P. heliotrinreducens* grown on lasiocarpine; cultures of L4M2 grown on lasiocarpine yielded equivalent results.

RESULTS AND DISCUSSION

TLC analysis was used as a rapid method to monitor each cultures ability to metabolize pyrrolizidine alkaloids, and also to determine if methylene compounds were being produced. The majority of the pyrrolizidine alkaloids typically chromatographed better in the basic solvent system than in the acidic solvent, but

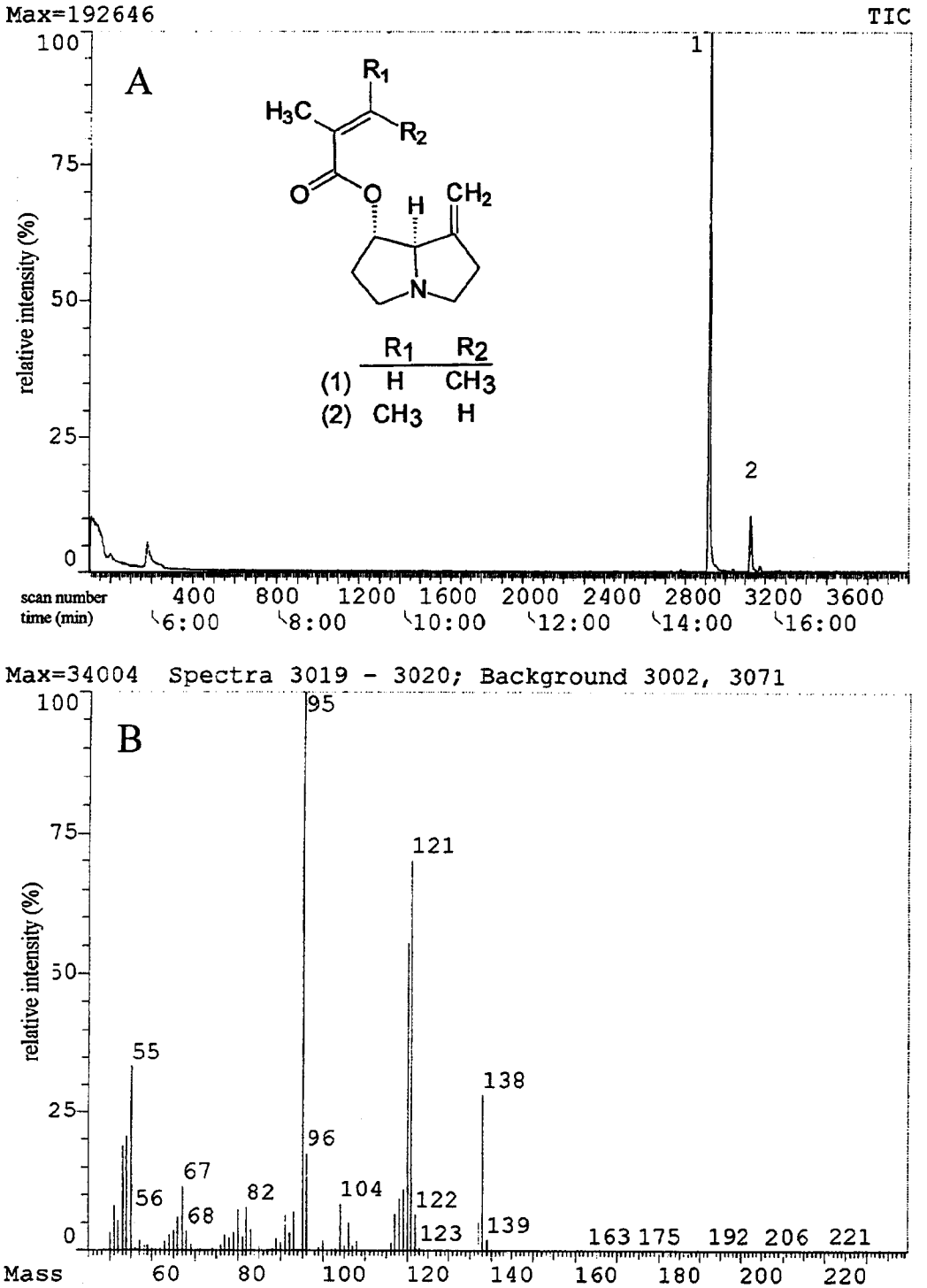


Figure 5.5 Analysis results of preparative TLC. A) GC/MS total ion chromatogram showing 7α-angelyl-1-methylene-8α-pyrrolizidine (1) and 7α-tiglyl-1-methylene-8α-pyrrolizidine (2). Chromatographic conditions described in the text under temperature program 2. B) Mass spectra of compound (1). MW 221.

the tansy pyrrolizidine alkaloids did not fully separate in the basic solvent system and lasiocarpine separated from its methylene product better in the acidic system. The pyrrolizidine alkaloids used in this study were visible with both spray systems. However, since system C is selective for unsaturated pyrrolizidines and system D will visualize any amine, any methylene compounds formed were visible only with system D. Shown in Table 5.3 are the R_f values for the methylene products. The TLC analysis did not detect any 1-methylene compounds in the cultures of L4M2 grown on monocrotaline or tansy pyrrolizidine alkaloids, or in the cultures of *P. heliotrinreducens* grown on tansy pyrrolizidine alkaloids. As monocrotaline has β-stereochemistry at C7, it is presumed that it's 1-methylene product does as well.

Table 5.3 R_f values for 1-methylene products developed in solvent systems A and B.

	A	B
7α-angelyl-1-methylene-8α-pyrrolizidine[a]	0.75	0.74
7α-hydroxy-1-methylene-8α-pyrrolizidine[b]	[d]ND	0.10
7β-hydroxy-1-methylene-8α-pyrrolizidine[c]	[d]ND	0.10

[a]Produced in cultures of both *P. heliotrinreducens* and L4M2 grown on lasiocarpine.
[b]Produced in cultures of both *P. heliotrinreducens* and L4M2 grown on heliotrine.
[c]Produced in cultures of *P. heliotrinreducens* grown on monocrotaline.
[d]ND Not determined.

Samples taken from one culture over time were analyzed and quantitated with GC/MS to confirm the disappearance of the pyrrolizidine alkaloids over time and the appearance of the 1-methylene products over time. Shown in Figure 5.6

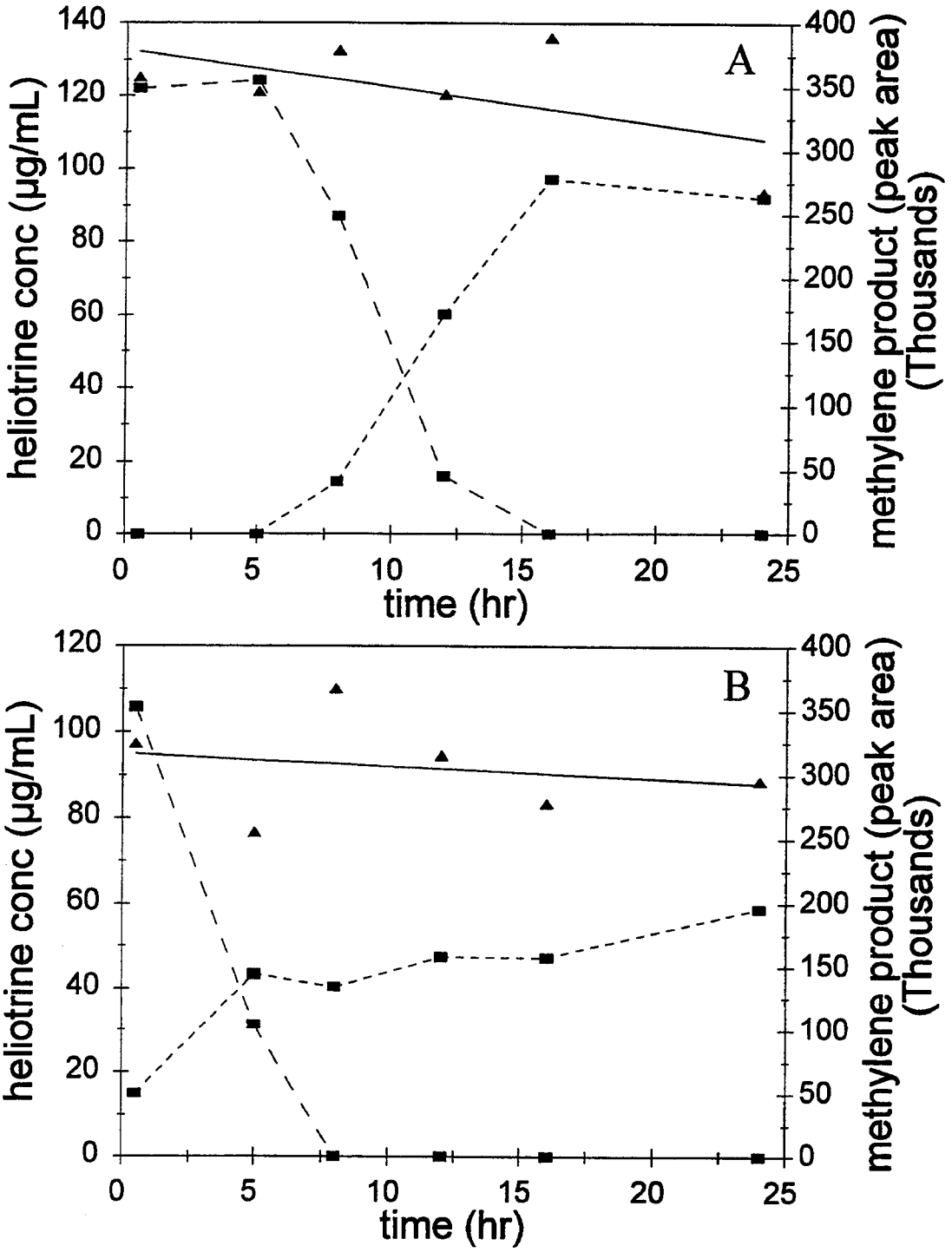


Figure 5.6 GC/MS analysis results from cultures growing on heliotrine. A) *P. heliotrinreducens*. B) L4M2. Heliotrine concentration results from one sterile culture (▲) and the linear regression (————) and one viable culture (— — ■ — —). Methylene product peak area results from the same viable culture (- - - - ■- - - -).

are the results from the cultures grown on heliotrine. *P. heliotrinreducens* metabolized the heliotrine completely within 16 hr, with production of 7α-hydroxy-1-methylene-8α-pyrrolizidine reaching a maximum in 16 hr and then leveling off. A lag time of about 5 hr was observed in both the metabolism of the heliotrine and in the production of the 1-methylene product. The L4M2 culture metabolized the heliotrine completely within 8 hr, with production of 7α-hydroxy-1-methylene-8α-pyrrolizidine stabilizing after about 5 hr. In the sterile controls for both cultures, the heliotrine concentration remained relatively constant throughout the 24-hr period; the slopes were -0.99 and -0.27 μg/mL/hr in the cultures of *P. heliotrinreducens* and L4M2, respectively. No 1-methylene compounds were detected at any time in the sterile controls.

Shown in Figure 5.7 are the results from the cultures grown on lasiocarpine. The lasiocarpine concentration represents the total of all 4 compounds present in the initial material (Figure 5.3). The *P. heliotrinreducens* metabolized the lasiocarpine completely within 16 hr, with production of both 7α-angelyl-1-methylene-8α-pyrrolizidine and 7α-tiglyl-1-methylene-8α-pyrrolizidine reaching a plateau in 16 hr. A lag time of about 5 hr was observed in both the metabolism of the lasiocarpine and in the production of the 1-methylene products. The L4M2 culture metabolized the lasiocarpine completely within 5 hr, with production of both 7α-angelyl-1-methylene-8α-pyrrolizidine and 7α-tiglyl-1-methylene-8α-pyrrolizidine reaching a maximum in 8 hr and then leveling off. In the sterile controls for both cultures, the lasiocarpine concentration remained relatively constant throughout the 24-hr period; the slopes were 0.74 and 0.28 μg/mL/hr in the cultures of *P.*

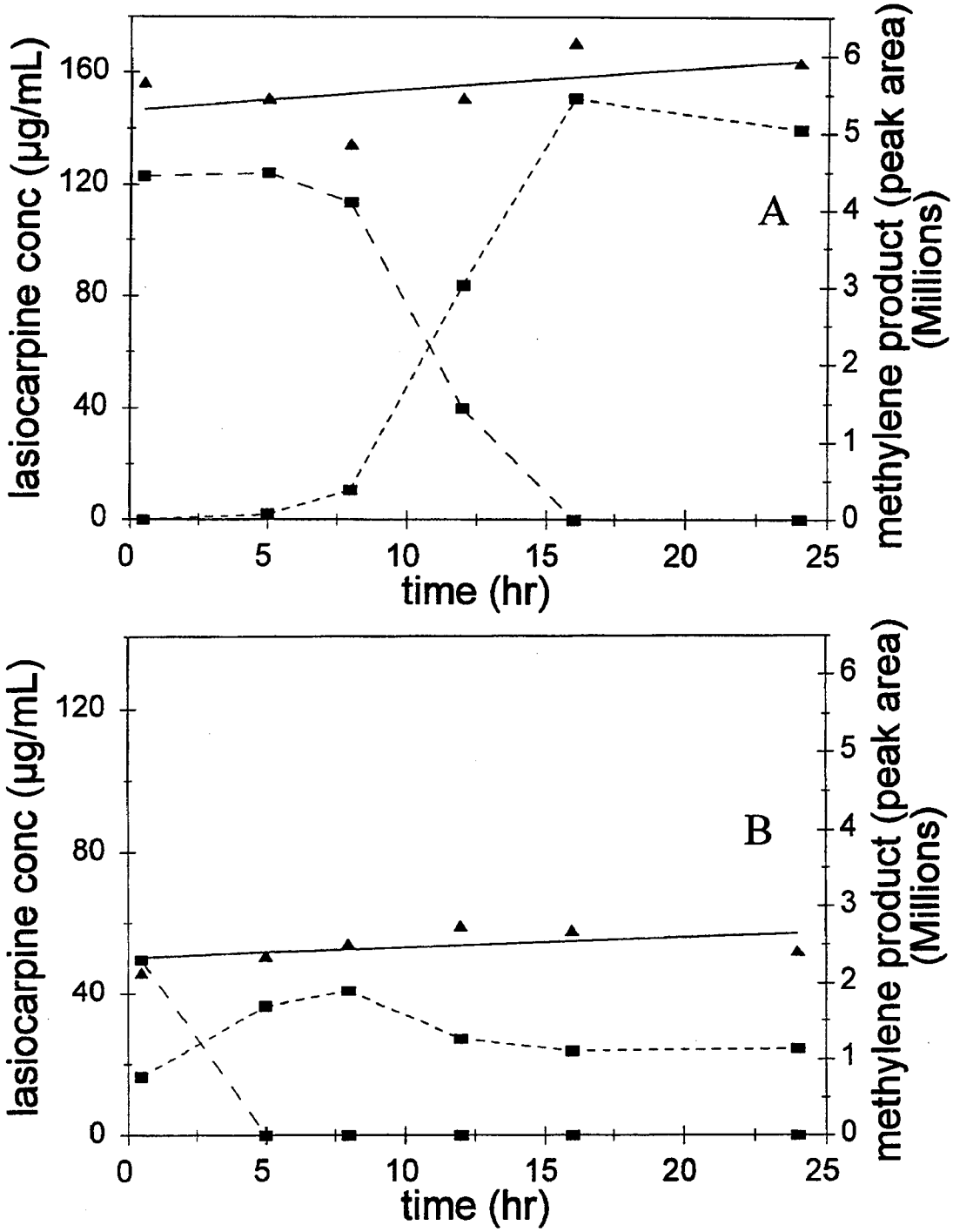


Figure 5.7 GC/MS analysis results from cultures growing on lasiocarpine. A) *P. heliotrinreducens*. B) L4M2. Lasiocarpine concentration results from one sterile culture (▲) and the linear regression (————) and one viable culture (— — ■ — —). Methylene product peak area results from the same viable culture (- - - - ■ - - - -).

heliotrinreducens and L4M2, respectively. No 1-methylene compounds were detected at any time in the sterile controls.

Shown in Figure 5.8 are the results from the *P. heliotrinreducens* and L4M2 cultures grown on tansy pyrrolizidine alkaloids. The tansy pyrrolizidine alkaloids concentration represents the total of all 7 compounds present in the initial material (Figure 5.2). The cultures of *P. heliotrinreducens* did not metabolize the tansy pyrrolizidine alkaloids within the 24-hr period; the slopes were -0.38 and -0.04 µg/mL/hr in the viable culture and the sterile control, respectively. No 1-methylene compounds were detected in any of the samples at any time. Analysis of *P. heliotrinreducens* cultures that were allowed to incubate for longer periods (up to 12 days) also indicated that no metabolism of these pyrrolizidine alkaloids occurred. The inability of *P. heliotrinreducens* to metabolize the macrocyclic pyrrolizidine alkaloids isolated from tansy ragwort is consistent with previous results (Lanigan, 1976).

With L4M2, the tansy pyrrolizidine alkaloids were completely metabolized within 12 hr, with production of a small amount of 7β-hydroxy-1-methylene-8α-pyrrolizidine increasing throughout the 24-hr period (Figure 5.8B). The mass spectra for the 7α- and 7β-hydroxy-1-methylene-8α-pyrrolizidine compounds are essentially identical (Figure 5.4A). All of the pyrrolizidine alkaloids in the mixture isolated from tansy have β-stereochemistry at C7 (Figure 5.2); therefore, it is presumed that the 1-methylene product does as well. In the sterile controls for L4M2, the concentration of tansy pyrrolizidine alkaloids remained relatively

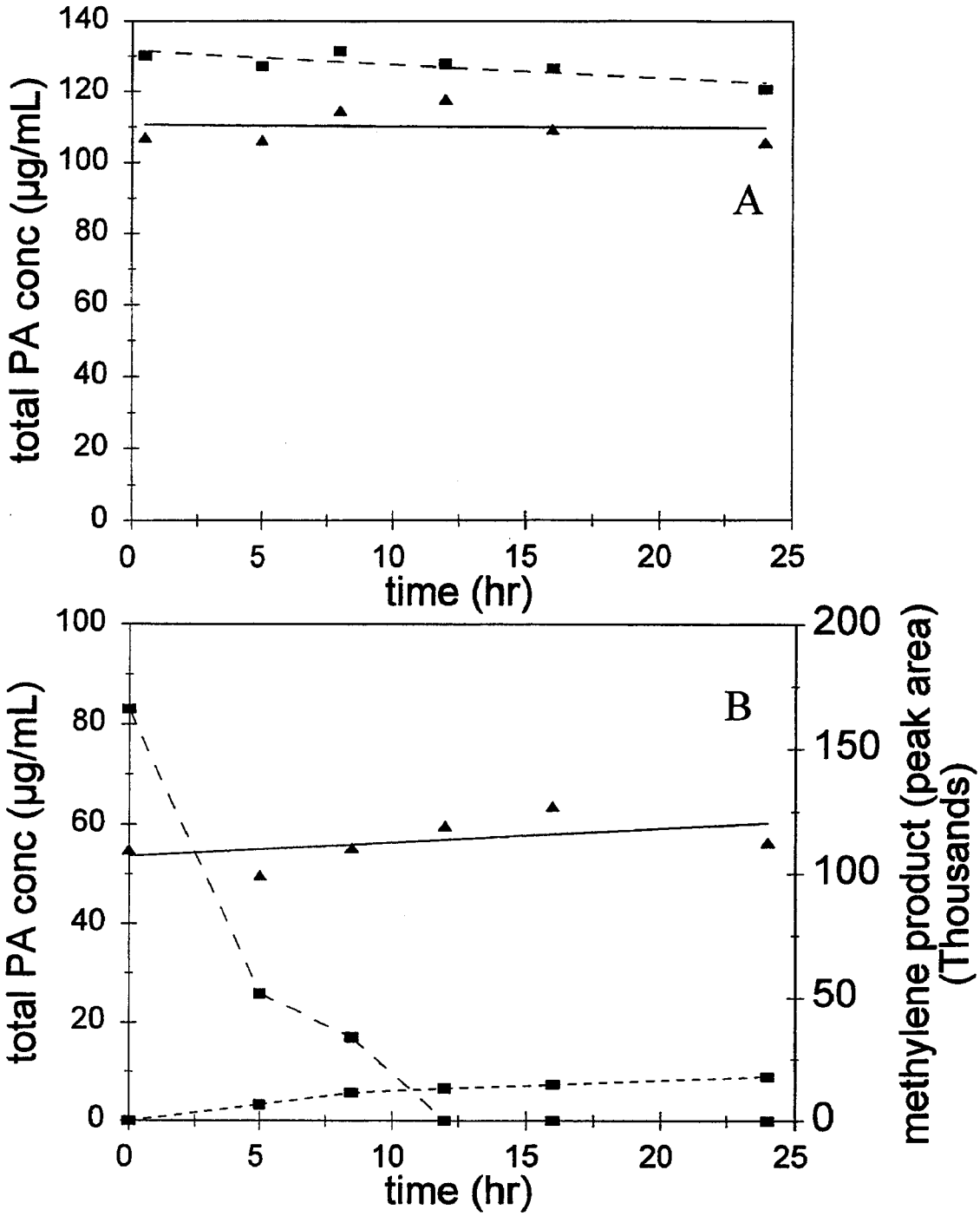


Figure 5.8 GC/MS analysis results from cultures growing on tansy pyrrolizidine alkaloids. A) *P. heliotrinreducens*. Total pyrrolizidine alkaloid concentration results from one viable culture (■) and one sterile culture (▲) and the linear regressions (— — — —, ————). B) L4M2. Total pyrrolizidine alkaloid concentration results from one sterile culture (▲) and the linear regression (————) and one viable culture (— — ■ — —). Methylene product peak area results from the same viable culture (- - - - ■ - - - -).

constant throughout the 24-hr period (slope = 0.27 µg/mL/hr) and no 1-methylene compounds were detected at any time.

Shown in Figure 5.9 are the results from the *P. heliotrinreducens* and L4M2 cultures grown on monocrotaline. The cultures of *P. heliotrinreducens* did not metabolize the monocrotaline within the 24-hr period; the slopes were 0.56 and -0.33 µg/mL/hr in the viable culture and the sterile control, respectively. Trace amounts of 7β-hydroxy-1-methylene-8α-pyrrolizidine were detected in the viable cultures at 16 and 24 hr. Analysis of *P. heliotrinreducens* cultures that were allowed to incubate for longer periods indicated that metabolism of monocrotaline occurred with production of higher amounts of 7β-hydroxy-1-methylene-8α-pyrrolizidine, although inconsistently; complete metabolism of monocrotaline required anywhere from 6 to 23 days, and not all replicates metabolized monocrotaline.

For the L4M2 culture, monocrotaline was completely metabolized within 12 hr, with production of 7β-hydroxy-1-methylene-8α-pyrrolizidine (Figure 5.9B). Due to the large degree of scatter in the results of the product peak area, it is impossible to determine from this data if the amount of product is stable or if it is changing. However, this data was useful in confirming the β-stereochemistry of the product at C7. These samples were analyzed using the same GC program as the samples from the cultures grown on heliotrine (temperature program 2). The 7α-hydroxy-1-methylene-8α-pyrrolizidine produced in the cultures grown on heliotrine eluted at 9.10 min (standard deviation = 0.02 min). The 7β-hydroxy-1-methylene-8α-pyrrolizidine produced in the cultures grown on monocrotaline eluted at 7.85 min (standard deviation = 0.01 min). As previously mentioned, the mass spectra of

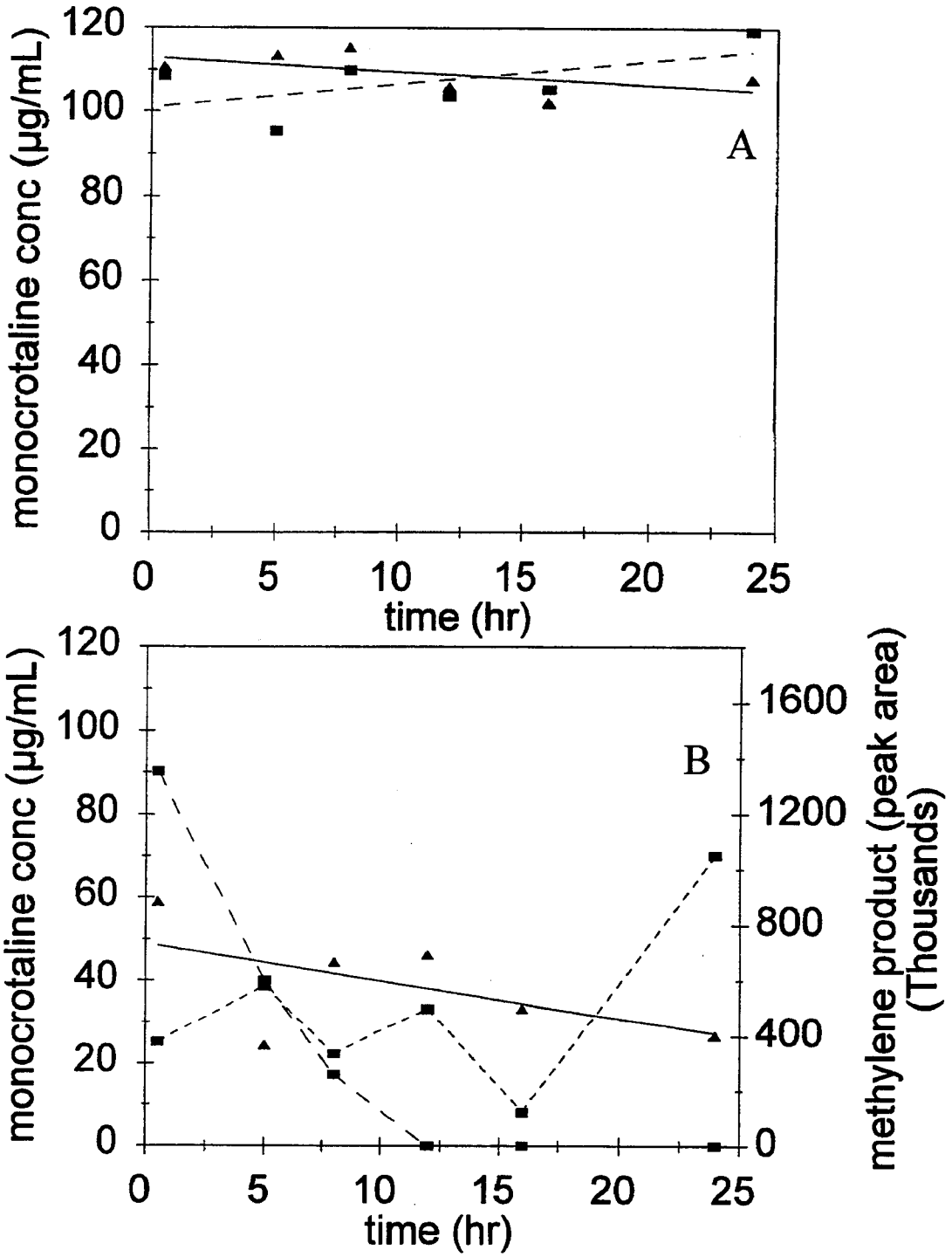


Figure 5.9 GC/MS analysis results from cultures growing on monocrotaline. A) *P. heliotrinreducens*. Monocrotaline concentration results from one viable culture (■) and one sterile culture (▲) and the linear regressions (— — — —, ————). B) L4M2. Monocrotaline concentration results from one sterile culture (▲) and the linear regression (————) and one viable culture (— — ■ — —). Methylene product peak area results from the same viable culture (- - - - ■ - - - -).

these two compounds is essentially identical; thus, the significant difference in retention time indicates different stereochemistry. Identification of the early eluting peak as the β- isomer is based upon the known chromatographic elution order of necine bases; the GC retention time of heliotridine (C7-α and C8-α) is greater than that of retronecine (C7-β and C8-α) (Stelljes et al., 1991). In the sterile controls for L4M2, the concentration of monocrotaline remained relatively constant throughout the 24-hr period (slope = -0.91 μg/mL/hr) and no 1-methylene compounds were detected at any time.

To summarize, the experimental data are consistent with the predicted metabolism of heliotrine (a mono-ester) and lasiocarpine (a diester) to the 1-methylene compounds by both *P. heliotrinreducens* and the mixed culture L4M2 (Figure 5.1). The macrocyclic pyrrolizidine alkaloids isolated from tansy ragwort were not metabolized by *P. heliotrinreducens*; these same pyrrolizidine alkaloids were metabolized rapidly by L4M2 with production of very low levels of the corresponding 1-methylene compound. The macrocyclic pyrrolizidine alkaloid monocrotaline was metabolized by *P. heliotrinreducens* inconsistently (either slowly or not at all); L4M2 metabolized monocrotaline rapidly with production of the corresponding 1-methylene compound.

The mixed culture L4M2 has displayed more variety than the single organism *P. heliotrinreducens*, both in the type of pyrrolizidine alkaloids metabolized and in the final products. Although *P. heliotrinreducens* shows a preference for mono- or di-ester pyrrolizidine alkaloids, it still requires a lag time before effective metabolism occurs. This is in contrast to L4M2, for which a lag time was not observed during

metabolism of any of the pyrrolizidine alkaloids. Because all of the mono- and di-ester pyrrolizidine alkaloids used in this study had α-stereochemistry at C7, and all of the macrocyclic pyrrolizidine alkaloids used had β-stereochemistry at C7, it is not clear which feature is more important in terms of metabolism by *P. heliotrinreducens*. In all of the cases in which *P. heliotrinreducens* metabolized pyrrolizidine alkaloids, experimental data were consistent with the predicted 1-methylene compounds which were produced as end-products.

L4M2 appears to have produced 1-methylene compounds as end-products in some cases, but possibly as intermediates in others. L4M2 grown on heliotrine seems to produce 7α-hydroxy-1-methylene-8α-pyrrolizidine as an end-product; within the 24-hr period monitored its concentration appears to stabilize. L4M2 grown on lasiocarpine seems to produce 7α-angelyl-1-methylene-8α-pyrrolizidine as an end product, although it is unclear if the maximum peak area observed at 8 hr is due to further metabolism or random error in the assay. This same trend was observed in duplicate cultures.

Experimental data indicates that L4M2 grown on the tansy pyrrolizidine alkaloids produces 7β-hydroxy-1-methylene-8α-pyrrolizidine, although at much lower levels than in any of the other cultures; its peak area is at least 10 times less than that of the 7α- isomer produced in cultures of L4M2 grown on heliotrine. Due to the very low levels produced it is difficult to conclude if the concentration of 7β-hydroxy-1-methylene-8α-pyrrolizidine is increasing throughout the 24-hr period, or if it has reached a maximum at 12 hr and then stabilized. Either way, metabolism

of these pyrrolizidine alkaloids by L4M2 appears to produce 1-methylene compounds as intermediates.

Currently ongoing experiments in this laboratory are directed at identifying individual organisms present in the L4M2 group using 16S rRNA methodology. Preliminary results indicate that L4M2 does not include an organism belonging to the *Peptostreptococcus* genus. This implies that pyrrolizidine alkaloid metabolizing behavior is not unique to one genus and may be common to several different genera.

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Chapter 6

SUMMARY

The work presented herein centered around providing direct evidence of the specific metabolites of the macrocyclic pyrrolizidine alkaloids jacobine and seneciphylline produced during incubation with ovine ruminal microorganisms. Preliminary work had indicated that metabolites containing a necine base ring system were not being produced. Two specific metabolic pathways involving the necine base ring system were investigated. The first pathway studied focused on hydrolysis of jacobine and seneciphylline followed by metabolism of the necine base. Hydrolysis was not observed in either sheep whole rumen fluid or with the mixed culture L4M2, nor was metabolism of the necine base independent of the parent alkaloids observed. The second pathway studied involved the production of compounds in which the necine base had been modified by the conversion of the 1,2-double bond to an external methylene group. This pathway yielded interesting results, with jacobine and seneciphylline being converted to 1-methylene compounds by the mixed culture L4M2, although with low yields. L4M2 also converted heliotrine and lasiocarpine to 1-methylene compounds as end products. L4M2 has demonstrated the ability to metabolize a greater variety of pyrrolizidine alkaloids than the single organism *Peptostreptococcus heliotrinreducens*. As the mono- and di-ester pyrrolizidine alkaloids used in this study all had α-stereochemistry at C7 and the macrocyclic pyrrolizidine alkaloids all had β-

stereochemistry at C7, it is not clear which structural feature determines the extent of metabolism by *P. heliotrinreducens*. Neither feature affected the extent of metabolism by L4M2, although they are likely influencing the end product formation. Although L4M2 was originally derived from the ovine rumen fluid of sheep maintained on a diet of *Senecio jacobaea*, it is likely that these animals would be resistant to a variety of toxic plants which contained the pyrrolizidine alkaloids monocrotaline, heliotrine and lasiocarpine.

Future experiments should include the determination of error (relative standard deviation) in the GC/MS analysis of the various pyrrolizidine alkaloids, and if possible in the GC/MS analysis of the methylene product. Error in these assays could be reduced by the utilization of an injection internal standard. Data on the methylene product in this study is difficult to interpret; it is not always possible to distinguish between a plateau or a maximum in the amount of methylene compound produced. Making a derivative of the methylene product may improve this assay, especially at low levels, but it would then be difficult to obtain data on the parent pyrrolizidine alkaloid concentration in the same analysis. Quantitation of the methylene product was not an available option in this study because a standard was not available. Heliotrine and lasiocarpine are not available in quantities large enough to isolate a large amount of product. Synthesis of methylene compounds may be an option, although separation from co-products is difficult and yields are low.

Further studies could also investigate the influence of stereochemistry and structure on pyrrolizidine alkaloid metabolism by L4M2. Using mono- or diester

pyrrolizidine alkaloids with β-stereochemistry at C7 would help determine the influence of structure on the metabolites. As almost all macrocyclic pyrrolizidine alkaloids have β-stereochemistry at C7, the study of pyrrolizidine alkaloids with α-stereochemistry at C7 would be limited to mono- and diester pyrrolizidine alkaloids.

Confirmation that 1-methylene compounds are intermediates, would verify that ruminal metabolism detoxifies pyrrolizidine alkaloids. The 1,2-double bond in the necine base is necessary for pyrrolizidine alkaloid toxicity because it enhances the conversion to the toxic pyrroles. The 1-methylene compounds do not form pyrroles with the oxidizing reagent *o*-chloranil and likely would not form pyrroles in the liver either. Although *P. heliotrinreducens* converts selected pyrrolizidine alkaloids to 1-methylene compounds, ovine rumen fluid from Australian sheep will further metabolize these compounds, converting the methylene to a methyl group. It should be determined if L4M2, which may utilize 1-methylene compounds as intermediates in the metabolism of jacobine and seneciphylline, is producing compounds with a 1-methyl group.

Currently ongoing experiments in this laboratory are directed at identifying the individual microorganisms present in the L4M2 group using 16S rRNA methodology. Preliminary results indicate that L4M2 does not include an organism belonging to the *Peptostreptococcus* genus. This implies that pyrrolizidine alkaloid metabolizing behavior is not unique to one genus and may be common to several different genera.

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