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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 a 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. A mixed culture of ovine ruminal microbes also metabolized heliotrine and lasiocarpine to identical methylene compounds. This mixed culture also metabolized jacobine and seneciphylline, with the production of very low levels of the corresponding 1-methylene compounds. Samples were analyzed by TLC and GC/MS.

Keywords: Pyrrolizidine alkaloids; Ruminal metabolism

1. 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) [1–3]. Cattle and horses who ingest over 5% of their body weight over time die of hepatic cirrhosis 2–24 months after ingestion [1,4–7]. 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 [8]. It has been demonstrated that the pyrrolizidine alkaloids in tansy ragwort are metabolized in ovine ruminal fluid [9,10]. A mixed culture of anaerobic microbes that 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 that metabolizes jacobine or seneciphylline.

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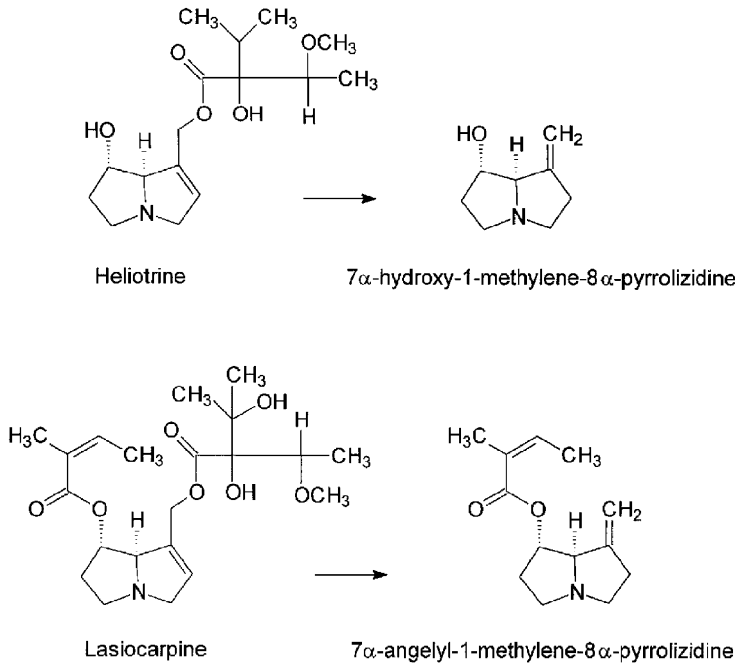

Fig. 1. Pyrrolizidine alkaloids metabolized by *Peptostreptococcus heliotrinreducens* to 1-methylene compounds.

Previous attempts to conclusively identify metabolites produced by the ruminal metabolism of jacobine and seneciphylline have been unsuccessful [11,12]. Most pyrrolizidine alkaloids are not commercially available and must be isolated from plant material. Labeled 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 using some other labeled precursors [13,14]. 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 [14]. Thus, using labeled 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 (Fig. 1) [15]. *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 [15]. In this study both *P. heliotrinreducens* and L4M2 were incubated with heliotrine, lasiocarpine, monocrotaline or a mixture of pyrrolizidine alkaloids isolated from tansy ragwort. Samples from the cultures were analyzed for parent compounds and for any methylene metabolic products using TLC and GC/MS.

2. Experimental

2.1. 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 [15]. 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 time intervals of 0, 5, 8, 12, 16 and 24 h and analyzed for pyrrolizidine alkaloids and methylene products. On occasion, incubations were allowed to proceed for up to 23 days.

The E medium had the following composition (ml/l): mineral I solution, 40; mineral II solution, 40; major volatile fatty acid (VFA) solution, 11.4; supplemental VFA solution, 11.4; trace metal solution, 11.4; 0.1% resazurin, 1.1; 0.01% hemin (in 0.05 M NaOH), 1.1; clarified rumen fluid, 100; 8% (w/v) sodium carbonate, 50; 2.5% (w/v) cysteine hydrochloride, 10; and 2.5% (w/v) sodium sulfide, 6. 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 [16] and added after autoclaving as filter-sterilized solutions. Mineral solution I contained K_2HPO_4, 6 g/l [16]. Mineral solution II had the following components (g/l): NaCl, 12; KH_2PO_4, 6; $(NH_4)_2SO_4$, 12; $MgSO_4 \cdot 7H_2O$, 1.0; and $CaCl_2 \cdot 2H_2O$, 1.0 [16]. The major VFA solution had the following components (g/l): sodium acetate, 20.82; sodium propionate, 9.6; and sodium butyrate, 5.5 [17]. The supplemental VFA solution had the following components (ml/l): isobutyric acid, 10; 2-methylbutyric acid, 10; isovaleric acid, 10; and valeric acid, 10 [17]. The pH of both VFA solutions was adjusted to 7.5 using NaOH. The trace metals solution had the following components (g/l): Na_2EDTA, 0.43; $FeSO_4 \cdot 7H_2O$, 0.20; $MnSO_4 \cdot H_2O$, 0.17; H_3BO_3, 0.03; $CoCl_2 \cdot 6H_2O$, 0.012; $ZnSO_4 \cdot 7H_2O$, 0.01; $NaMoO_4$, 0.0025; $NiCl_2 \cdot 6H_2O$, 0.002; and $CuCl_2 \cdot 2H_2O$, 0.001 [18,19].

2.2. Chemicals

A crude mixture of pyrrolizidine alkaloids was isolated from *Senecio jacobaea* (tansy ragwort) as previously described [20]. This mixture contains mostly jacobine and seneciphylline, although senecionine, integerrimine, jacozine, jacoline and retrorsine are all present (Fig. 2). Integerrimine, which is an isomer of senecionine, has not been previously reported in *S. jacobaea* and may be an artifact of the extraction procedure; isomerization may occur 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 (Fig. 3). The spontaneous isomerization from angelyl to tiglyl esters has been observed for both mono- and diesters [21]. Although the mass spectra for the angelyl and tiglyl isomers of lasiocarpine were indistinguishable, identification (peaks 3 and 4 in Fig. 3) was based upon the known elution order; angelyl isomers elute prior to tiglyl isomers [21]. 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 four working standards ranged from 10 to 160 μg/ml and was used to quantitate samples analyzed by GC/MS as described below.

2.3. TLC analysis

This method was modified from previously reported methods [10,11,22]. In brief, 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 (12×75 mm), and then removed under vacuum at 43 °C. The residue was reconstituted in methanol (20 μl) and spotted in duplicate (2×10 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

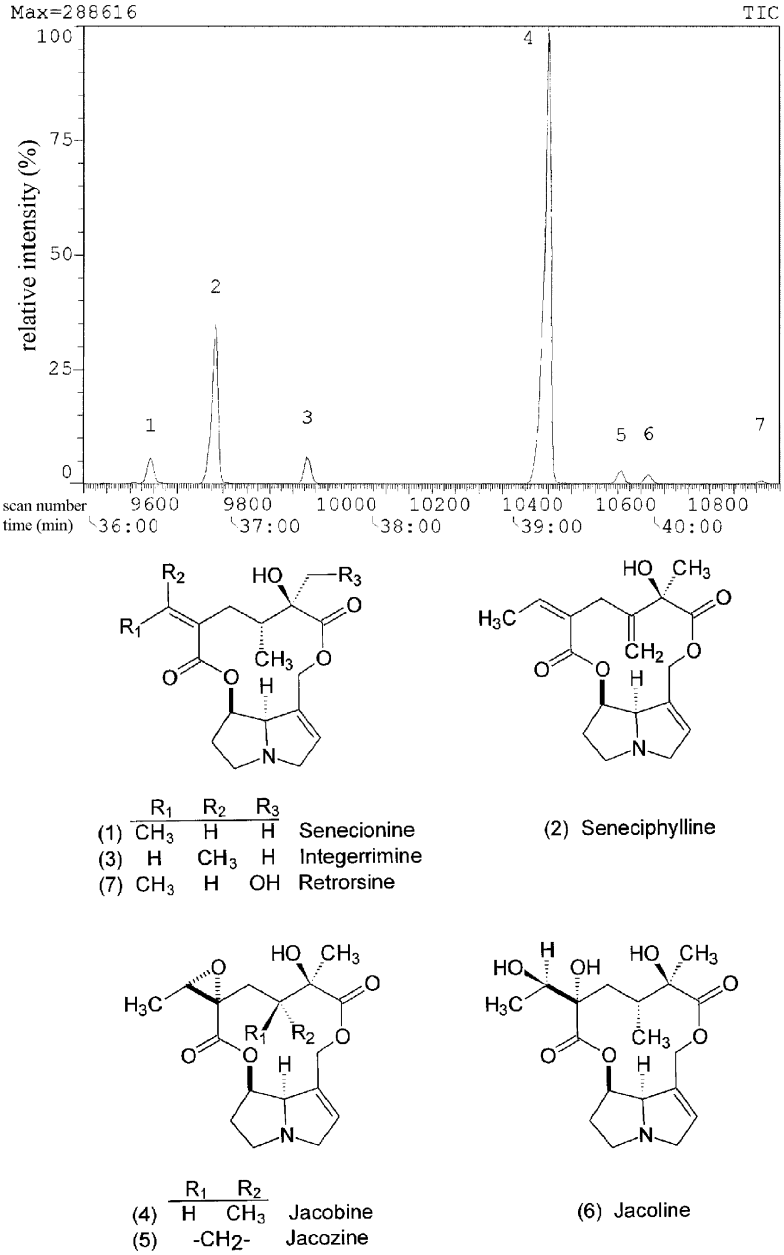

Fig. 2. GC/MS total ion chromatogram of pyrrolizidine alkaloid mixture isolated from *Senecio jacobaea* (tansy ragwort). Chromatographic conditions described in the text under temperature program 1.

(36:9:5); solvent system B consisted of chloroform–methanol–ammonium hydroxide (85:14:1). Individual plates were then visualized with either spray system C or spray system D. System C consisted of sequential spraying with 1% *o*-chloranil in toluene and Ehrlichs spray reagent (Sigma),

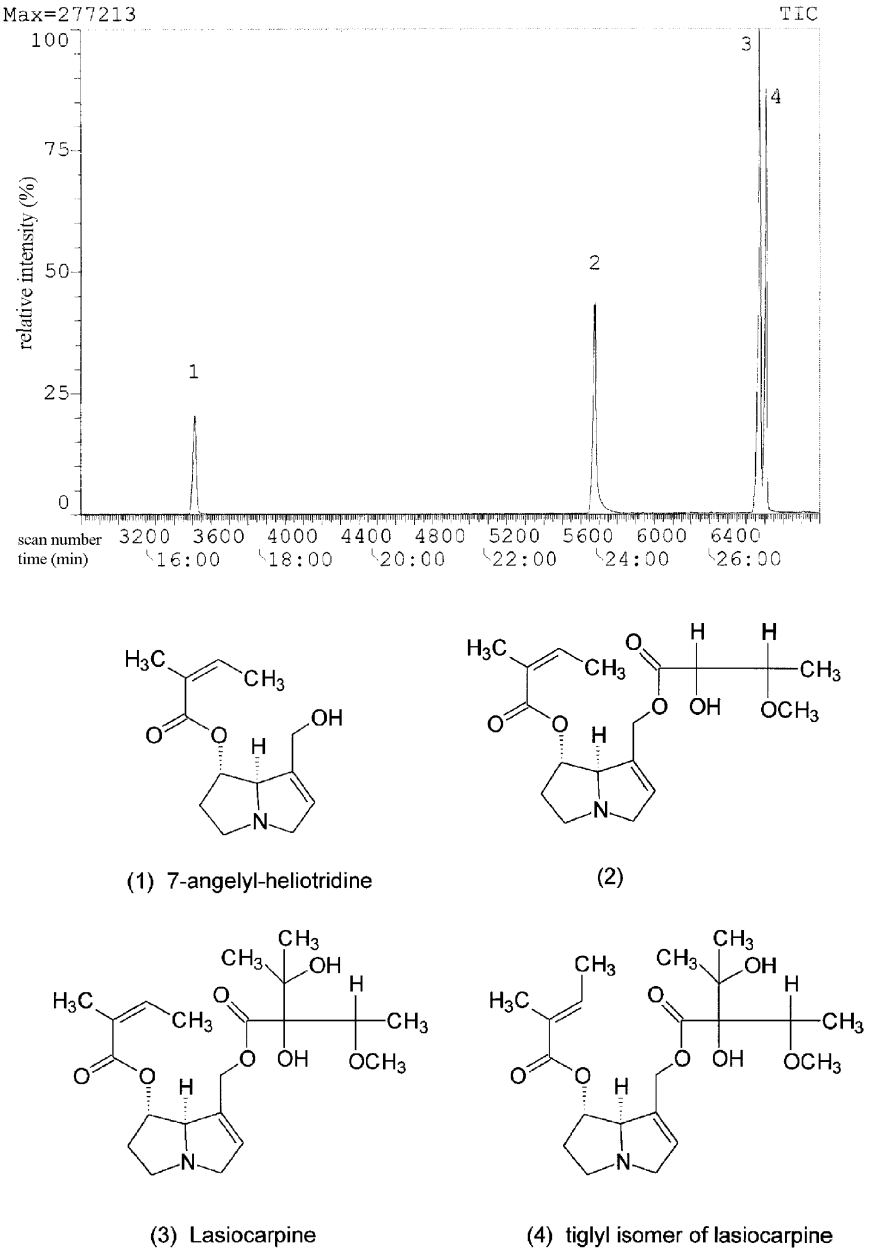

Fig. 3. GC/MS total ion chromatogram of lasiocarpine isomers (**3,4**) and proposed breakdown products (**1,2**). Chromatographic conditions described in the text under temperature program 3.

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 1, and the detection limits (based on visual

Table 1
R_f values for pyrrolizidine alkaloids developed in solvent systems A and B

	R_f value	
	A	B
Heliotrine	0.47	0.25
Lasiocarpine	0.67	0.71
Monocrotaline	ND	0.42
Tansy PA mixture		
Jacobine	0.70	0.59
Seneciphylline	0.80	0.62

ND, not determined.

detection) of the pyrrolizidine alkaloids with both spray systems are shown in Table 2.

2.4. 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 chromatographed on an Rt_x-5MS capillary column (30 m×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 temperature 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).

2.5. Derivative formation

Mass spectral data are consistent with the hypothesis that heliotrine is converted to 7α-hydroxy-1-methylene-8α-pyrrolizidine (Fig. 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. Fig. 4 shows the mass spectra of this compound before and after derivatization. Data are shown for cultures of *P. heliotrinreducens* grown on heliotrine; cultures of L4M2 grown on heliotrine yielded equivalent results.

2.6. Preparative TLC

Mass spectral data are consistent with the hypothesis that lasiocarpine is converted to 7α-angelyl-1-methylene-8α-pyrrolizidine (Fig. 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 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

Table 2
Detection limits for pyrrolizidine alkaloids visualized with spray systems C and D

	Detection limit (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.

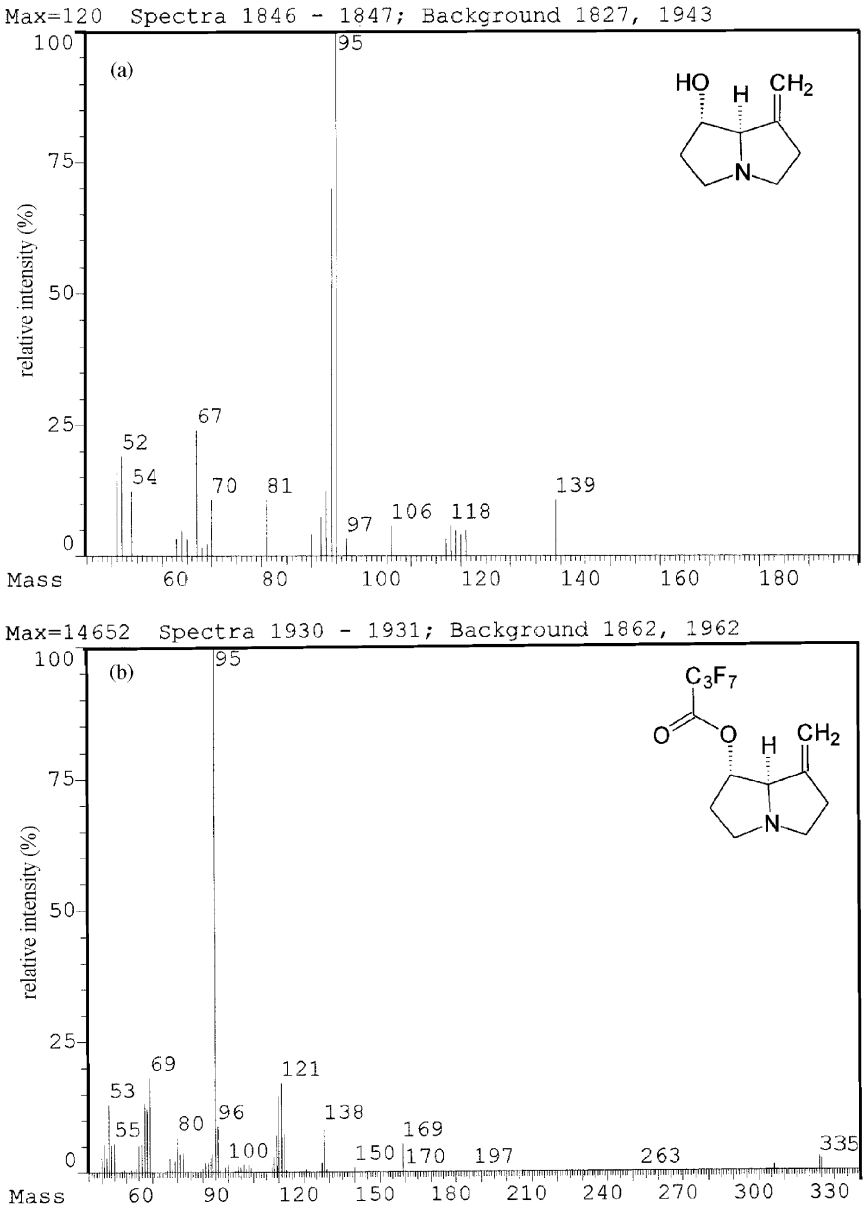

Fig. 4. Mass spectra: (a) 7α-hydroxy-1-methylene-8α-pyrrolizidine (MW=139). (b) Heptafluorobutyrate derivative of 7α-hydroxy-1-8α-pyrrolizidine (MW=335).

(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 the glass plate (R_f=0.68–0.80) and placed on top of a plug of glass wool in a Pasteur pipette. The product was eluted with three 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 pro-

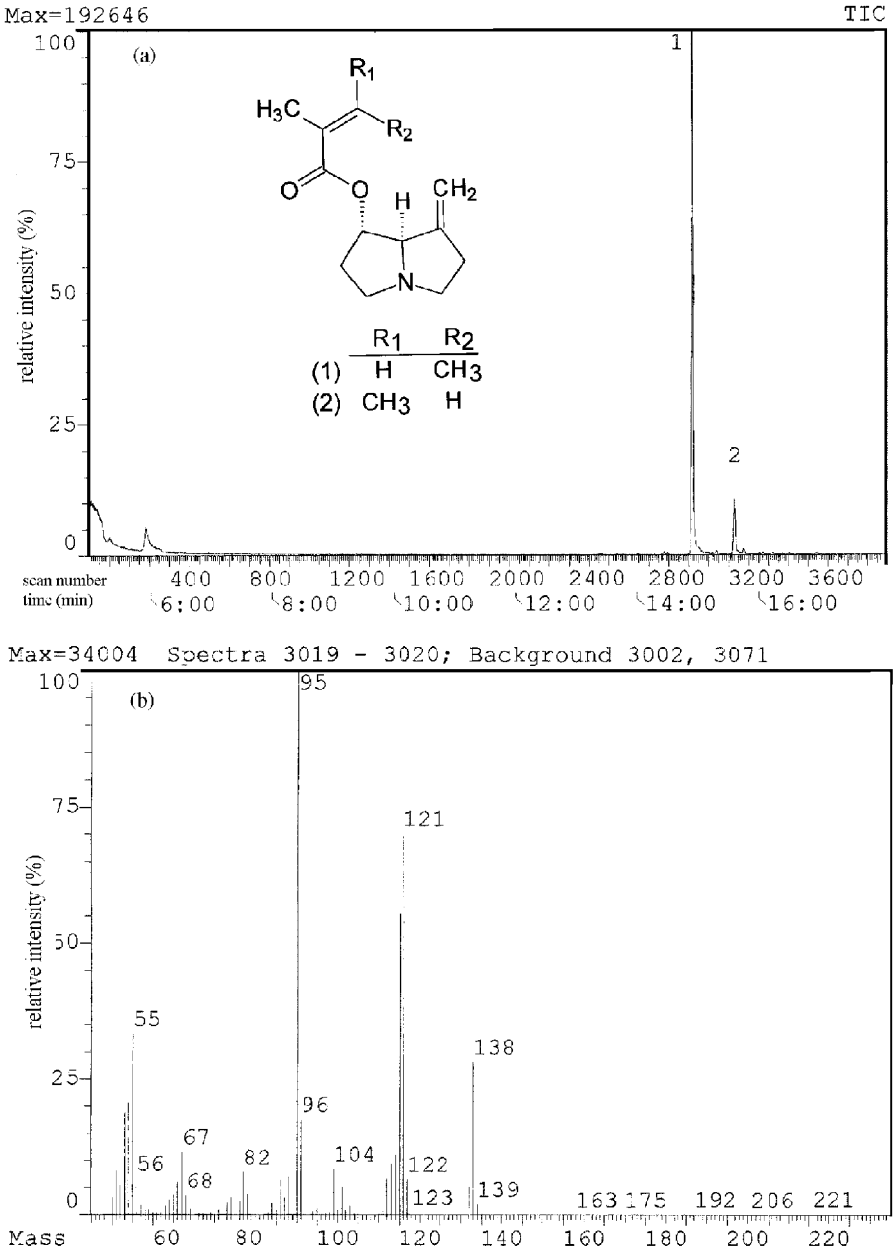

Fig. 5. Analysis results of preparative TLC. (a) GC/MS total ion chromatogram showing 7α-angelyl-1-methylene-8α-pyrrolizidine (1) and 7α-tiglyl-1-methylene-8α-pyrrolizidine (2). Chromatographic conditions described in the text under temperature program 2. (b) Mass spectra of compound (1) (MW=221).

gram 2. A small amount of 7α-tiglyl-1-methylene-8α-pyrrolizidine was also identified (Fig. 5). The mass spectra for the angelyl and tiglyl isomers were indistinguishable and are consistent with that previously reported for the 7β-angelyl isomer [23]. Identification was based upon the known chromat-

Table 3
R_f values for 1-methylene products developed in solvent systems A and B

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

ND, not determined.
[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.

ographic elution order (angelyl isomers elute before tiglyl isomers) [21]. The data shown in Fig. 5 are from cultures of *P. heliotrinreducens* grown on lasiocarpine; cultures of L4M2 grown on lasiocarpine yielded equivalent results.

3. Results and discussion

TLC analysis was used as a rapid method to monitor the ability of each culture to metabolize pyrrolizidine alkaloids and to determine if methylene compounds were being produced. The majority of the pyrrolizidine alkaloids typically chromatographed better in the basic than in the acidic solvent system, but 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. System C is selective for unsaturated pyrrolizidines and system D visualizes any amine. Any methylene compounds formed were only visible with system D. Table 3 shows 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 the corresponding 1-methylene product does as well.

Samples taken from one culture over time were analyzed and quantitated with GC/MS to confirm disappearance of the pyrrolizidine alkaloids and the appearance of the 1-methylene products over time. Fig. 6 shows results from the cultures grown on heliotrine. *P. heliotrinreducens* completely metabolized the heliotrine within 16 h, with production of 7α-hydroxy-1-methylene-8α-pyrrolizidine reaching a maximum in 16 h and then leveling off. A lag time of approximately 5 h was observed for both metabolism of the heliotrine and production of the 1-methylene product. The L4M2 culture completely metabolized the heliotrine within 8 h, with production of 7α-hydroxy-1-methylene-8α-pyrrolizidine stabilizing after approximately 5 h. In the sterile controls for both cultures, the heliotrine concentration remained relatively constant

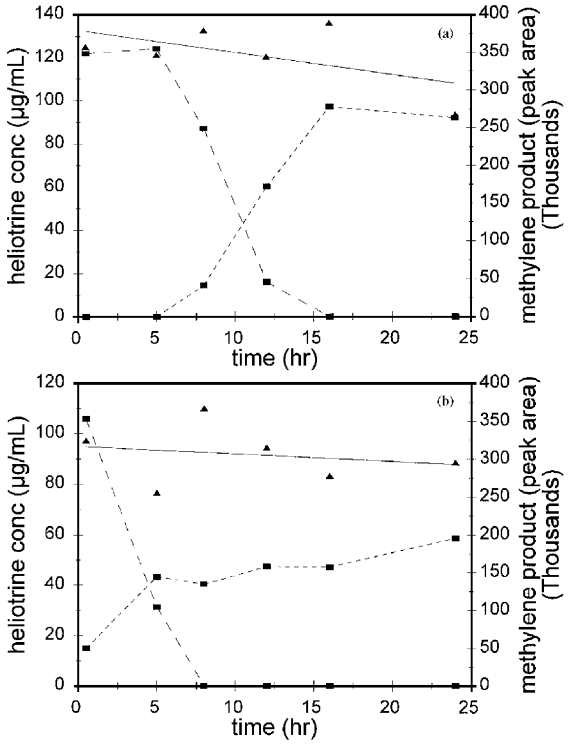

Fig. 6. GC/MS analysis results from cultures growing on heliotrine: (a) *P. heliotrinreducens*; and (b) L4M2. Heliotrine concentration results from one sterile culture (▲) and the linear regression (——) and one viable culture (– –■– –). Methylene product peak area results from the same viable culture (----■----).

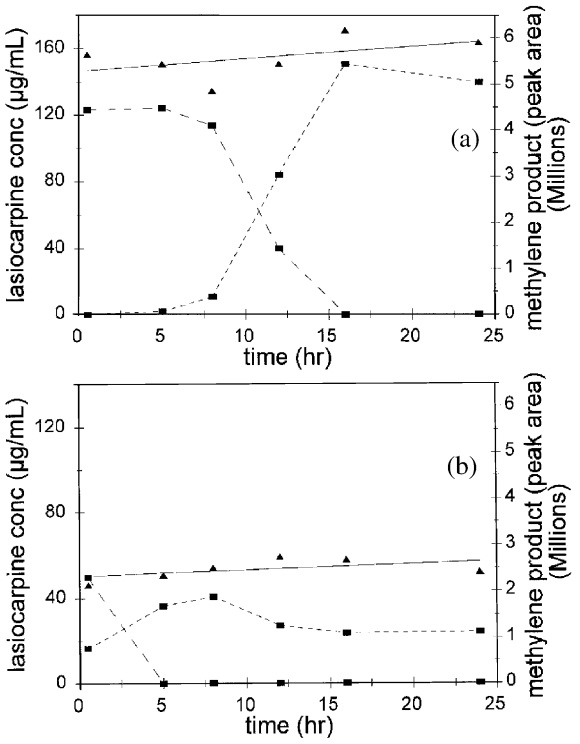

Fig. 7. GC/MS analysis results from cultures growing on lasiocarpine: (a) *P. heliotrinreducens*; and (b) L4M2. Lasiocarpine concentration results from one sterile culture (▲) and the linear regression (——) and one viable culture (– –■– –). Methylene product peak area results from the same viable culture (----■----).

throughout the 24-h period; the slopes were −0.99 and −0.27 μg/ml h in the cultures of *P. heliotrinreducens* and L4M2, respectively. No 1-methylene compounds were detected at any time in the sterile controls.

Fig. 7 shows results from the cultures grown on lasiocarpine. The lasiocarpine concentration represents the total of all four compounds present in the initial material (Fig. 3). The *P. heliotrinreducens* completely metabolized the lasiocarpine within 16 h, with production of both 7α-angelyl-1-methylene-8α-pyrrolizidine and 7α-tiglyl-1-methylene-8α-pyrrolizidine reaching a plateau in 16 h. A lag time of approximately 5 h was observed in both the metabolism of the lasiocarpine and in the production of the 1-methylene products. The L4M2 culture completely metabolized the lasiocarpine within 5 h, with production of both 7α-angelyl-1-methylene-8α-pyrrolizidine and 7α-tiglyl-1-methylene-8α-pyrrolizidine reaching a maximum in 8 h and then leveling off. In the sterile controls for both cultures, the lasiocarpine concentration remained relatively constant throughout the 24-h period; the slopes were 0.74 and 0.28 μg/ml h in the cultures of *P. heliotrinreducens* and L4M2, respectively. No 1-methylene compounds were detected at any time in the sterile controls.

Fig. 8 shows the results from the *P. heliotrinreducens* and L4M2 cultures grown on tansy pyrrolizidine alkaloids. The tansy pyrrolizidine alkaloid concentration represents the total of all seven compounds present in the initial material (Fig. 2). The cultures of *P. heliotrinreducens* did not metab-

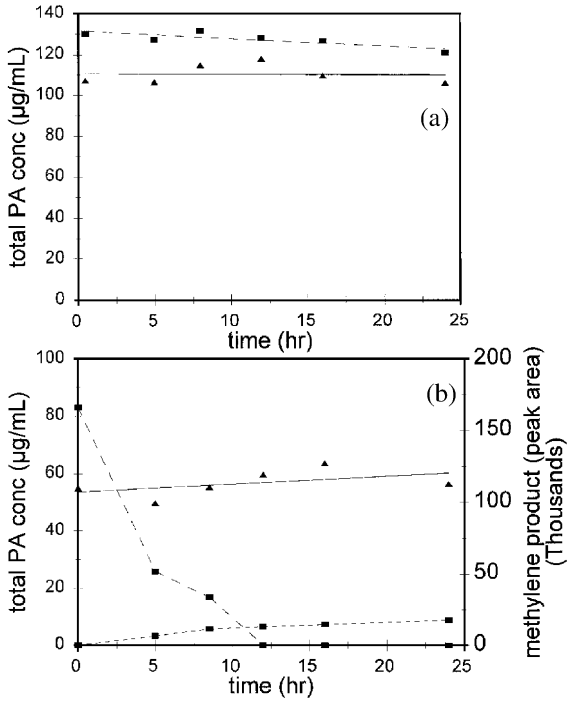

Fig. 8. GC/MS analysis results from cultures growing on tansy pyrrolizidine alkaloids. (a) *P. heliotrinreducens*. Total pyrrolizidine alkaloid concentration results from one viable culture (■) and one sterile culture (▲) and the linear regressions (– – – –, ——). (b) L4M2. Total pyrrolizidine alkaloid concentration results from one sterile culture (▲) and the linear regression (——) and one viable culture (– – ■ – –). Methylene product peak area results from the same viable culture (----■----).

olize the tansy pyrrolizidine alkaloids within the 24-h period; the slopes were −0.38 and −0.04 μg/ml h 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 [15].

With L4M2, the tansy pyrrolizidine alkaloids were completely metabolized within 12 h, with production of a small amount of 7β-hydroxy-1-methylene-8α-pyrrolizidine increasing throughout the 24-h period (Fig. 8b). The mass spectra for the 7α- and 7β-hydroxy-1-methylene-8α-pyrrolizidine compounds are essentially identical (Fig. 4a). All of the pyrrolizidine alkaloids in the mixture isolated from tansy have β-stereochemistry at C7 (Fig. 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 constant throughout the 24-h period (slope = 0.27 μg/ml h) and no 1-methylene compounds were detected at any time.

Fig. 9 shows 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-h period; the slopes were 0.56 and −0.33 μg/ml h 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 h. 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 h, with production of 7β-hydroxy-1-methylene-8α-pyrrolizidine (Fig. 9b). Due to the large degree of scatter in the results of the product peak area, it is impossible to determine from these data if the amount of product is stable or is changing. However, these data were 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 these two compounds

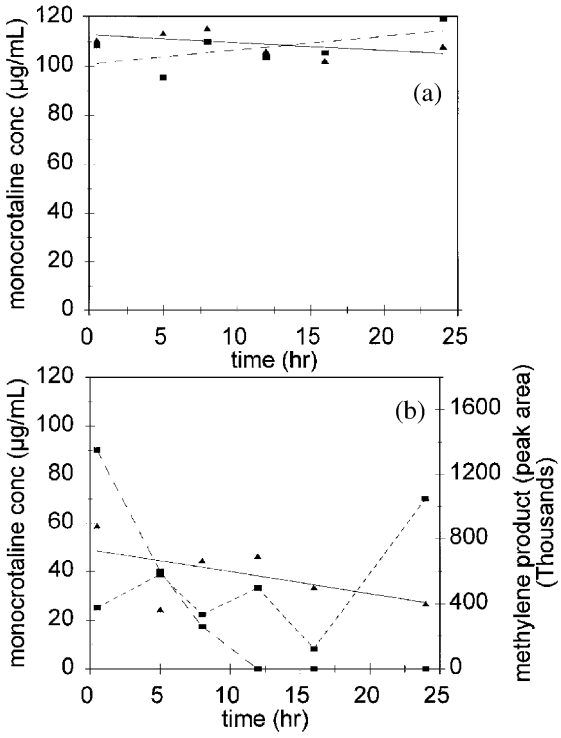

Fig. 9. GC/MS analysis results from cultures growing on monocrotaline. (a) *P. heliotrinreducens*. Monocrotaline concentration results from one viable culture (■) and one sterile culture (▲) and the linear regressions (– – – –, ——). (b) L4M2. Monocrotaline concentration results from one sterile culture (▲) and the linear regression (——) and one viable culture (– – ■ – –). Methylene product peak area results from the same viable culture (----■----).

are 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) [21]. In the sterile controls for L4M2, the concentration of monocrotaline remained relatively constant throughout the 24-h period (slope −0.91 μg/ml h) and no 1-methylene compounds were detected at any time.

4. Conclusions

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 L4M2 mixed culture (Fig. 1). The macrocyclic pyrrolizidine alkaloids isolated from tansy ragwort were not metabolized by *P. heliotrinreducens*; these same pyrrolizidine alkaloids were rapidly metabolized by L4M2 with the production of very low levels of the corresponding 1-methylene compound. The macrocyclic pyrrolizidine alkaloid monocrotaline was inconsistently metabolized by *P. heliotrinreducens* (either slowly or not at all); L4M2 rapidly metabolized monocrotaline, with production of the corresponding 1-methylene compound.

The L4M2 mixed culture 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 diester 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 diester 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 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-h period monitored, its concentration appeared 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 h is due to further metabolism or random error in the assay. This same trend was observed in duplicate cultures.

Experimental data indicate 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-fold lower 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 increased throughout the 24-h period, or if it reached a maximum at 12 h 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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