Identification of Trifluralin Metabolites from Rumen Microbial Cultures. Effect of Trifluralin on Bacteria and Protozoa

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Bacteroides ruminicola subsp. *brevis* (GA-33) and *Lachnospira multiparus* (D-32) degraded ^{14}C-trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) to α,α,α-trifluoro-N_4,N_4-dipropyltoluene-3,4,5-triamine, α,α,α-trifluoro-5-nitro-N_4-propyltoluene-3,4-diamine, α,α,α-trifluoro-N_4,N_4-dipropyl-5-nitrotoluene-3,4-diamine, α,α,α-trifluoro-N_4-propyltoluene-3,4,5-triamine, α,α,α-trifluoro-2,6-dinitro-*N*-propyl-*p*-toluidine, and polar products. Metabolites were identified by spectral comparison with authentic compounds. No loss of the trifluoromethyl group or cleavage of the ring of trifluralin was evident. Rumen bacteria were etiological agents found to degrade trifluralin in rumen fluid. However, of 12 characterized rumen bacterial strains, 10 did not degrade trifluralin. Based on endogenous gas evolved, volatile fatty acid production, viable population counts, and microscopic observations, rumen ciliated protozoa and bacteria tolerated relatively high concentrations of trifluralin.

Trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) was reported by Alder *et al.* (1960) to be a preemergent soil-incorporated herbicide. It is registered and marketed for use on alfalfa and other trifluralin-tolerant crops. A residue tolerance of 0.05 ppm for forage legumes was established under the Federal Food, Drug, Cosmetic Act of 1968. The possibility of ruminants ingesting the herbicide prompted Golab *et al.* (1969) to investigate the fate of trifluralin in a lactating cow and goats. Rumen fluid was found to degrade trifluralin to products that constituted the ultimate degradation of trifluralin in ruminants. These findings prompted an investigation to determine etiological agent(s) associated with rumen ingesta capable of degrading trifluralin. Effects of the herbicide on ruminal bacteria and protozoa were also considered.

MATERIALS AND METHODS

Labeled and Synthesized Compounds. Carbon-14 trifluralin preparations incubated with rumen microbes included ^{14}C-side chain-labeled trifluralin (propyl-1-^{14}C; specific activity, 3.08 μCi per mg) and a mixture of ^{14}C-uniformly ring-labeled and ^{14}C-trifluoromethyl-labeled trifluralin in a ratio of 1:5.7 (ring-trifluoromethyl-labeled; specific activity, 13.6 μCi per mg) (Golab *et al.*, 1969). These preparations were diluted in acetone to approximately 1 μCi per ml and stored in darkness at $-20°$ C.

General reaction procedures used for synthesis of trifluralin-related compounds (shown in Table I) were essentially those of Marshall *et al.* (1966).

Rumen Microorganisms. Rumen bacterial strains (*Bacteroides amylophilus*, H-18; *B. ruminicola* subsp. *brevis*, GA-33; *B. succinogenes*, S-85; *Butyrivibrio fibrisolvens*, 49; *Eubacterium ruminantium*, B_1C23; *E. limosum*, L-34; *Lachnospira multiparus*, D-32; *Peptostreptococcus elsdenii*, B-159; *Ruminicoccus flavefaciens*, C-94; *Streptococcus bovis*, FD-10; *Succinimonas amylolytica*, $B_2$4: and *Succinivibrio dextrinosolvens*, 24) were obtained from Marvin P. Bryant's collection, as reported by Fulghum *et al.* (1968). The strains were maintained on rumen fluid–trypticase yeast extract medium, RFTY (Fulghum *et al.*, 1968). Ruminal ciliates were obtained from calves faunated with *Isotricha prostoma* and *I. intestinalis*, and/or *Entodinium* sp. Ciliate-free rumen fluid was obtained from calves reared free of rumen ciliates. Manometric analysis and ciliate tracer studies were performed according to a previous report (Williams *et al.*, 1963).

Apparatus. Gas chromatographic separations were accomplished with a Barber-Colman Series 5000 gas chromatograph equipped with a ^{14}C-radioactive monitor and effluent splitters to facilitate simultaneous flame ionization detection and trapping with glass capillary tubes. Infrared spectra were taken by the micro-KBr technique with a Perkin-Elmer 337 grating infrared spectrophotometer equipped with a 4× beam condenser. Mass spectra were obtained with a Varian M-66 mass spectrometer using a solid sample inlet system. A Nuclear-Chicago liquid scintillation spectrometer was used for quantitation of radioactivity. Radioactive $^{14}CO_2$ was monitored as previously described (Williams, 1967). A Microferm fermenter (New Brunswick Scientific Co., N.J.) was used for continuous culture experiments. Thin-layer chromatograms were monitored for ^{14}C-radioactivity by a Packard 2 π scanner.

Culture Procedures. Trifluralin-^{14}C (either 2.0 μCi of side chain or ring-trifluoromethyl-labeled) was asceptically dispensed into sterile 300-ml round-bottomed flasks. A stream of nitrogen was used to evaporate the acetone so that ^{14}C-trifluralin was deposited as a thin film. The flask was purged with CO_2 and inoculated with 1.0 ml of an 18-hr-old bacterial culture (or mixture of rumen bacteria or rumen fluid) and 49.0 ml of sterile RFTY medium (or casein hydrolysate medium or basal medium, as shown in Table II). The flask was sealed with a neoprene stopper and incubated at 39° C in darkness. At 24 hr, the flask was opened and the contents were diluted with 50 ml of sterile medium. The flask was then stoppered and reincubated. This procedure was again repeated at 48 hr to give a total medium volume of 150 ml. After 72 hr, the flask contents were solvent-extracted and analyzed for ^{14}C products. Control flasks were prepared as above, except without bacterial or rumen fluid inocula. In experiments where trifluralin concentrations were varied, unlabeled trifluralin was added to the flasks and treated as above. In time-rate experiments, 3.0 ml of inoculated medium prepared as above was removed at intervals of 4 hr during incubation. These samples were solvent-extracted and chromatographed on one-dimensional tlc plates.

In continuous culture experiments, 30 g of rumen ingesta

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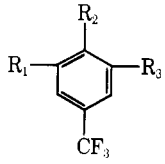
Table I. Trifluralin-Related Synthesized Compounds



Compd no.	Name	R_1	R_2	R_3
1	α,α,α-Trifluoro-2,6-dinitro-*N*-propyl-*p*-toluidine	NO_2	NHC_3H_7	NO_2
2	α,α,α-Trifluoro-2,6-dinitro-*p*-toluidine	NO_2	NH_2	NO_2
3	α,α,α-Trifluoro-5-nitro-N_4-propyltoluene-3,4-diamine	NO_2	NHC_3H_7	NH_2
4	α,α,α-Trifluoro-N_4,N_4-dipropyltoluene-3,4,5-triamine	NH_2	$N(C_3H_7)_2$	NH_2
5	α,α,α-Trifluorotoluene-3,4,5-triamine	NH_2	NH_2	NH_2
6	α,α,α-Trifluoro-N_4-propyltoluene-3,4,5-triamine	NH_2	$-NC_3H_7$ | H	NH_2
7	α,α,α-Trifluoro-5-nitrotoluene-3,4-diamine	NO_2	NH_2	NH_2
8	4-(Dipropylamino)-3,5-dinitrobenzoic acid[a]	NO_2	$N(C_3H_7)_2$	NO_2
9	α,α,α-Trifluoro-3,5-dinitrotoluene	NO_2	H	NO_2
10	α,α,α-Trifluoro-3,5-diaminotoluene	NH_2	H	NH_2
11	α,α,α-Trifluoro-N_4,N_4-dipropyl-5-nitrotoluene-3,4-diamine	NO_2	$N(C_3H_7)_2$	NH_2
12	α,α,α-Trifluoro-N_4-propyltoluene-3,4-diamine	H	NHC_3H_7	NH_2
13	α,α,α-Trifluoro-5-nitrotoluene-3-amine	NO_2	H	NH_2
Trifluralin	α,α,α-Trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine	NO_2	$N(C_3H_7)_2$	NO_2

[a] Carboxyl group substituted for a trifluoromethyl group.

was asceptically dispensed in a sterile 2.0-l. fermentation flask containing 300 ml of RFTY or basal medium (Table II) and ^{14}C-trifluralin (8.0 μCi of ^{14}C-carbon and 1.08 g of trifluralin). The fermenter was set at 39° C for 96 hr with a medium flow rate of 25 ml per hr. The medium was continuously bubbled with sterile 5% CO_2–95% N_2 and quantitatively monitored for $^{14}CO_2$ (Williams, 1967). Redox potential of the medium was > -123 mV (indigo carmine, E_0', pH 6.85). After 96 hr, the flask contents and medium effluent were freeze-dried, solvent extracted, and analyzed for ^{14}C-degradation products. The numerical criterion and *in vitro* cultural–rumen compatibility criterion described by Bryant (1959) were used as a basis that rumen microorganisms were cultured. (*B. fibrisolvens*, 49 under the above continuous culture procedures with RFTY medium, was maintained at an absorbancy reading of approximately 0.4 at 600 mμ wavelength as read in 13-mm cuvettes.)

Table II. Casein Hydrolysate Medium[a,b]

Component and percentage (w/v) in final medium

K_2HPO_4, 0.045	Propionic acid, 0.0002
KH_2PO_4, 0.045	Isobutyric acid, 0.001
$(NH_4)_2SO_4$, 0.09	Butyric acid, 0.0002
NaCl, 0.09	DL-α-Methyl-*n*-butyric acid, 0.001
$MgSO_4$, 0.009	Thiamine hydrochloride, 0.0004
$CaCl_2$, 0.009	Calcium-D-pantothenate, 0.0004
$CoCl_2$, 0.001	Nicotinamide, 0.0004
Hemin, 0.0003	Riboflavin, 0.0004
Yeast extract, 0.1	Pyridoxal hydrochloride, 0.0004
Pectin, 0.5	*p*-Aminobenzoic acid, 0.0002
Glucose, 0.05	Biotin, 0.0001
Cellobiose, 0.05	Folic acid, 0.0001
Soluble starch, 0.05	Vitamin B_{12}, 0.004
Resazurin, 0.0001	DL-Thiotic acid, 0.0001
Casein hydrolysate, 0.2	Na_2S, 0.05
Na_2CO_3, 0.4	
Cysteine hydrochloride, 0.05	
Acetic acid, 0.001	
Formic acid, 0.0002	

[a] Medium prepared with demineralized water under CO_2, pH 6.85; medium when prepared under 5% CO_2–95% N_2 used 0.02% (w/v) Na_2CO_3. [b] Basal medium consisted of all the above components [plus 0.002% (w/v) polyoxyethylene sorbitan monooleate] except hemin, yeast extract, carbohydrates, casein hydrolysate, and the fatty acids.

Gauze-strained rumen fluid (360 ml) was centrifuged at 13,000 × *g* for 20 min. The resulting cellular fraction was diluted to 90 ml with basal medium (Table II) and used as an inoculum for tubes containing different concentrations of trifluralin, as shown in Table VI. The basal medium tubes contained 2.0 ml of the inoculum, and the other set of tubes contained 0.1 ml of the cell fraction and 1.9 ml of RFTY medium. All preparations were incubated 96 hr at 39° C and analyzed for volatile fatty acid content.

Effect of trifluralin on rumen bacterial strains was determined as follows. Bacterial strains (No. D-32, H-18, $B_2$4, 49, B-159, FD-10, GA-33, C-94, L-34, 24, S-85, and B_1C23) were inoculated in casein hydrolysate medium (Table II) containing trifluralin at 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 5.0 mg/5.0 ml in 13-mm cuvettes. Mean absorbancy readings at 600 mμ wavelength were then taken of the inoculated preparations at 4, 8, 16, and 24 hr at 39° C. Lower absorbancy readings of inoculated medium containing no trifluralin when compared to inoculated medum with trifluralin were indicative of inhibition of growth of a given strain.

Extraction and Counting Procedures. Trifluralin and degradation products were liquid–liquid extracted from microbial media (usually 150 ml) with glass-distilled chloroform (6×, 500 ml each time). Water was removed from the extracts by the addition of $MgSO_4$. The extracts were filtered through Whatman No. 5 filter paper and evaporated to near dryness under nitrogen (Figure 1). All extractions were performed under a 40-W incandescent light to avoid photodecomposition (Marshall *et al.*, 1966). Nonchloroform-extractable materials were freeze-dried, extracted with methanol, and combusted as previously described (Williams *et al.*, 1968).

All counting of radioactivity was done with a liquid scintillation spectrometer. Samples were dissolved in dioxane and

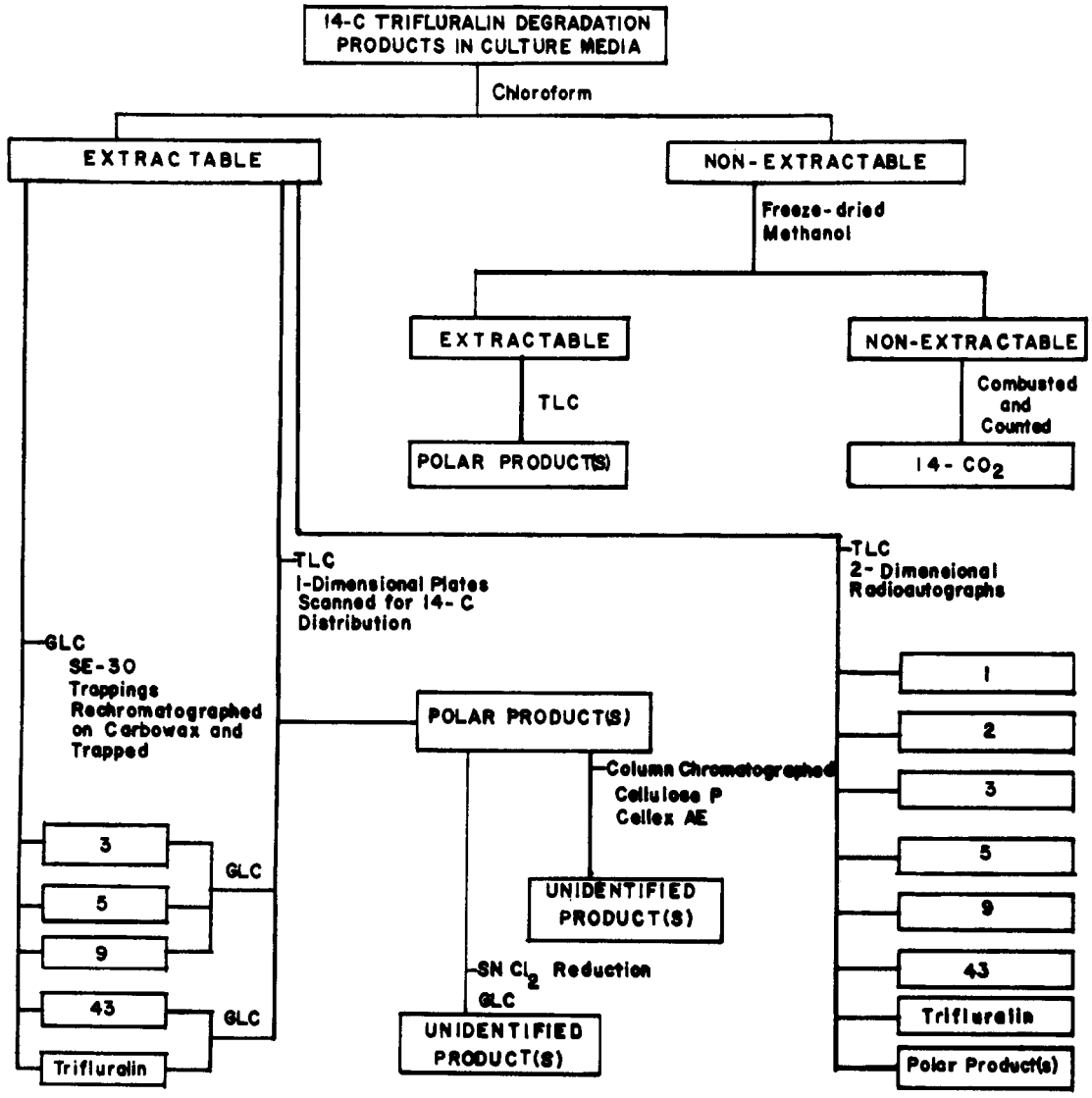


Figure 1. Extraction and chromatographic procedures for isolation and identification of trifluralin degradation products. Numbers in the chart were assigned according to the numbers and chemical structures listed in Table I

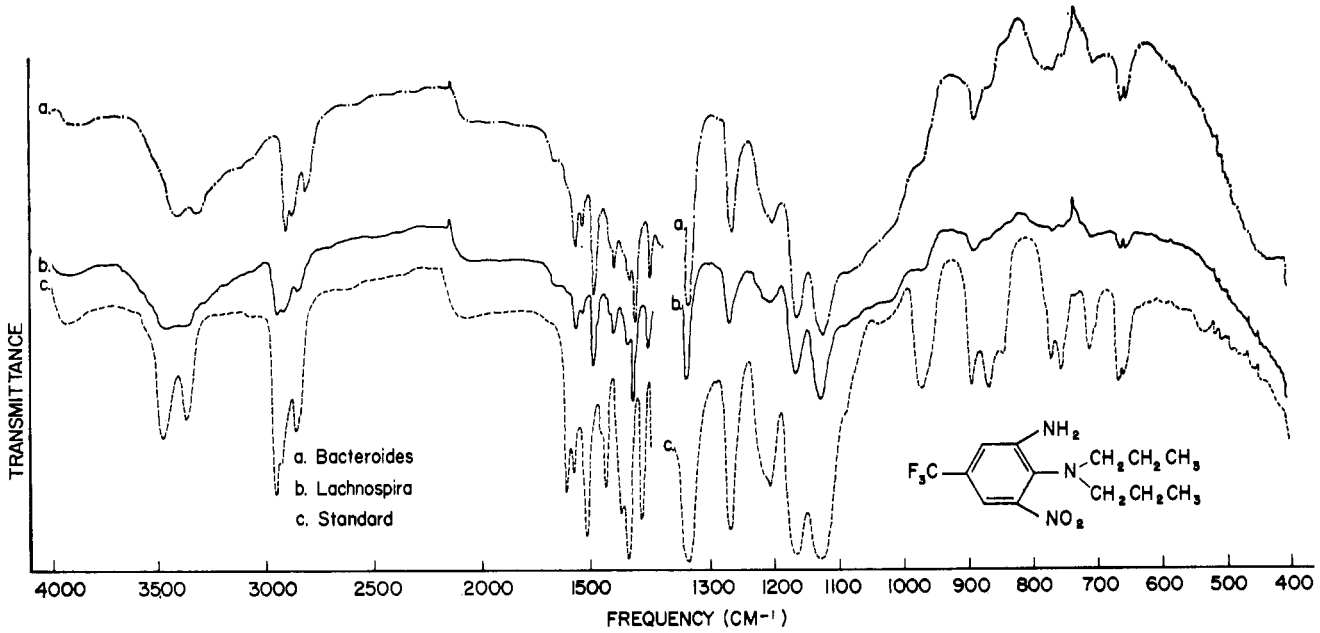


Figure 2. Infrared spectra of α,α,α-trifluoro-N_4,N_4-dipropyl-5-nitrotoluene-3,4-diamine. Experimental: (a) Bacteroides = *Bacteroides ruminicola* subsp. *brevis*, GA-33; and (b) Lachnospira = *Lachnospira multiparus*, D-32; Standard (c) = authentic sample

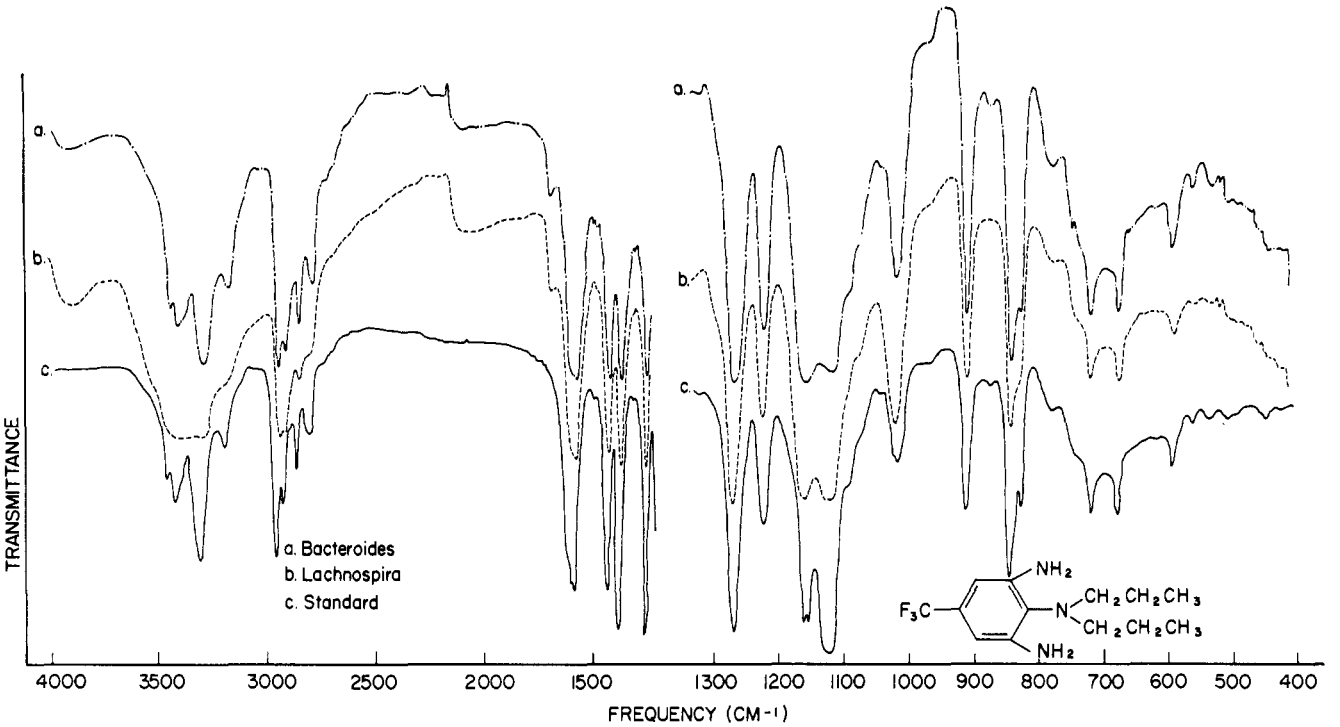


Figure 3. Infrared spectra of α,α,α-trifluoro-N_4,N_4-dipropyltoluene-3,4,5-triamine. Experimental: (a) Bacteroides = *Bacteroides ruminicola* subsp. *brevis*, GA-33; (b) Lachnospira = *Lachnospira multiparus*, D-32; Standard (c) = authentic sample

toluene scintillation solution and counted. Counting efficiencies were determined by internal standardization with toluene-*l*-^{14}C or by channels ratio standardization. Counting times ranged from 2 to 30 min, depending on the level of radioactivity in the samples.

Chromatographic Procedures. Glass plates (5- × 20- × 0.35-cm; 20- × 20- × 0.35-cm) coated with a 250-μ thick layer of silica gel HF-254 (Brinkmann, Lot 51251) was used for tlc (Figure 1). Plates were developed with benzene–ethylene dichloride (1:1) and *n*-hexane:methanol (97:3) according to Golab (1965). Other plates were run in diisopropylketone–formic acid–water (40:15:2) or isopropyl alcohol. One-dimensional plates were scanned for ^{14}C distribution. Recorded peaks of carbon-14 were measured for peak areas and compared to scans of known ^{14}C radioactivity applied to tlc plates. Two-dimensional plates were used for radioautography.

Volatile fatty acids were gas chromatographed and quantitated as previously described (Williams *et al.*, 1968). Trifluralin samples were gas chromatographed on two columns; (I) 2% SE-30 on Chromosorb W (60/80 mesh, 6-ft × 4-mm i.d., isothermal at 150° C) and (II) 2% Carbowax 20M on Chromosorb W (60/80 mesh, 6-ft × 4-mm i.d., 180° C for 12 min, then programmed to 245° C at 5° C/min). Helium (55 ml/min) was used as a carrier gas. Injection port and detector temperatures were set at 300° C. Trappings from these columns were accomplished by the use of capillary tubings fitted to an effluent–splitter port. The trapped products collected from column I were rechromatographed on column II (Figure 1). The products were then trapped in capillaries from column II and eluted onto KBr with solvent. The KBr was dried and the sample was analyzed by infrared, mass, and, in some cases, nuclear magnetic resonance spectrometry. The products were identified by spectral comparison with authentic compounds (Table I).

The ^{14}C-trifluralin degradation products, designated as polar products (Golab *et al.*, 1969), were stripped from tlc plates, solvent eluted, and chromatographed (about 30,000 dpm) on 25 g of cellulose P (1.8 × 40 cm; H^+ form, Bio-Rad Lab., Richmond, Calif.) by eluting in sequence with 72 ml of H_2O, 5.0 ml of 1 *N* NH_4OH, and 97.0 ml of H_2O. The effluent from the column was collected in 6.0-ml fractions at a rate of 0.5 ml/min. Fractions 7 through 14 and fraction 15 were concentrated and separately chromatographed on 25 g of

Table III. Degradation of ^{14}C-Ring Trifluoromethyl-Labeled Trifluralin by Rumen Microorganisms

Microorganism	Incubation time, hr	Percent of ^{14}C distributed on silica gel tlc plates[a] R_f 0–0.13[b]	R_f 0.29–0.55[c]	R_f 0.68–0.93[d]
GA-33[e]	0			100.0
	4	5.5	0.9	93.6
	8	18.6	15.9	65.5
	12	22.8	26.5	50.7
	16	40.8	32.5	26.7
D-32[e]	0			100.0
	4	8.7	1.3	90.0
	8	10.9	25.5	63.6
	12	27.2	36.2	36.6
	16	33.0	44.2	22.8
Bacterial mixture[e]	24	30.0	20.0	50.0
Ciliate-free rumen fluid	48	56.3	30.3	13.4
Entodinium sp.[f]	48	45.9	35.9	18.2
Isotricha sp.[f]	48	43.6	41.1	15.3

[a] Percent ^{14}C was based on the three zones of activity as 100%. Values given are taken as representative of the total ^{14}C (40 μg/ml) added to the cultures. Silica gel (250 μ); solvent, benzene–ethylene dichloride (1:1). Based on two-dimensional tlc and glc analysis, the ^{14}C activity was distributed as follows. [b] As polar products. [c] Mainly as α,α,α-trifluoro-N_4,N_4-dipropyltoluene-3,4,5-triamine with α,α,α-trifluoro-5-nitro-N_4-propyltoluene-3,4-diamine. [d] Trifluralin and α,α,α-trifluoro-N_4,N_4-dipropyl-5-nitrotoluene-3,4-diamine. [e] Incubated in RFTY medium. [f] Incubated in rumen fluid; Entodinia (584,000/ml), Isotrichs (10,230/ml).

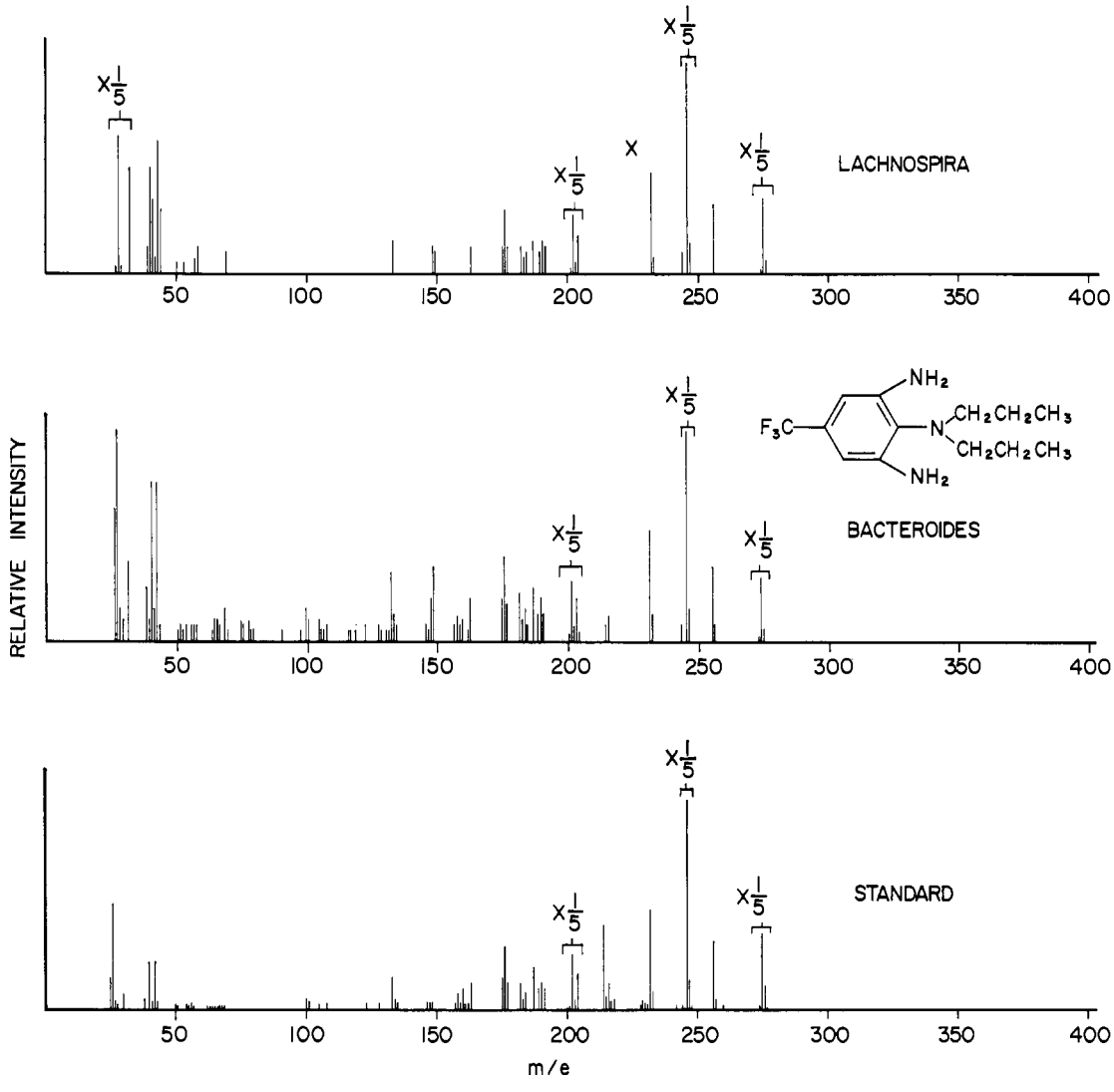


Figure 4. Mass spectra of α,α,α-trifluoro-N_4,N_4-dipropyltoluene-3,4,5-triamine. Experimental: Bacteroides = *Bacteroides ruminicola* subsp. *brevis*, GA-33; Lachnospira = *Lachnospira multiparus*, D-32; Standard = authentic sample

Cellex-AE (free NH_2 form, Bio-Rad Lab.) eluted with 90 ml of H_2O, 5.0 ml of 1 *N* NH_4OH, and then 85.0 ml of H_2O. Six-milliliter fractions were collected at a rate of 0.5 ml/min. The polar products were also extracted with methanol and treated to the esterification and acylation procedures of Gehrke and Stalling (1967).

Hydrolysis and Reduction Procedures. The ^{14}C-trifluralin polar products (about 20,000 dpm) were dissolved in chloroform or methanol in ampoules. The solvent was removed with a stream of nitrogen, and the products were mixed with either 4.0 ml of 6.0 *N* HCl or 4.0 ml of 6.0 *N* NaOH. The ampoules were then sealed with a torch and heated at 100° C for 72 hr. The samples were neutralized, extracted with diethyl ether (3×, 20, ml each time), and the extracts chromatographed by tlc and glc.

Reduction of ^{14}C-trifluralin polar products was accomplished by reacting the products with 100 mg $SnCl_2$ at 80° to 100° C for 20 min in a solution prepared by mixing 1 to 3 ml of methanol and 15 ml of 11.6 *N* HCl. The mixture was constantly stirred with a magnetic stirrer. A condenser and Dry Ice trap were used to trap any volatile ^{14}C-products produced during the reaction. The reaction was stopped by cooling the preparation to room temperature and neutralizing it with 2.5 *N* NaOH. The material was then extracted with methylene dichloride and analyzed by glc. Sublimation of the polar products was also investigated by using temperatures of 130 to 275° C.

RESULTS AND DISCUSSION

Recovery of Radioactivity. In all tracer experiments, 88 to 93% of the original ^{14}C radioactivity was recovered by chloroform extractions of rumen microbial media. Remaining ^{14}C in the aqueous phase (3.0 to 9.0%) could be accounted for by freeze-drying and extracting with methanol, and/or by combusting the samples to $^{14}CO_2$. The recovery values for all experiments indicated that ^{14}C-ring-trifluoromethyl-labeled trifluralin was not degraded to $^{14}CO_2$ nor $^{14}CH_4$. Rumen bacteria and ciliates degraded ^{14}C-side chain-labeled trifluralin to $^{14}CO_2$ by less than 0.4% in 72 hr. In these experiments, about 1% ^{14}C-trifluralin was volatilized and effectively trapped in magnesium perchlorate cartridges prior to the $^{14}CO_2$ traps (Williams, 1967). Recovery rates of carbon-14 from side chain-labeled trifluralin indicated that $^{14}CH_4$ was probably not produced.

^{14}C Distribution in Cultures. Experiments with washed cell suspensions of GA-33 and D-32 indicated the cells could take up, immediately upon mixing, about 50% of the trifluralin (1.8 μg/ml) dissolved in basal medium (Table II).

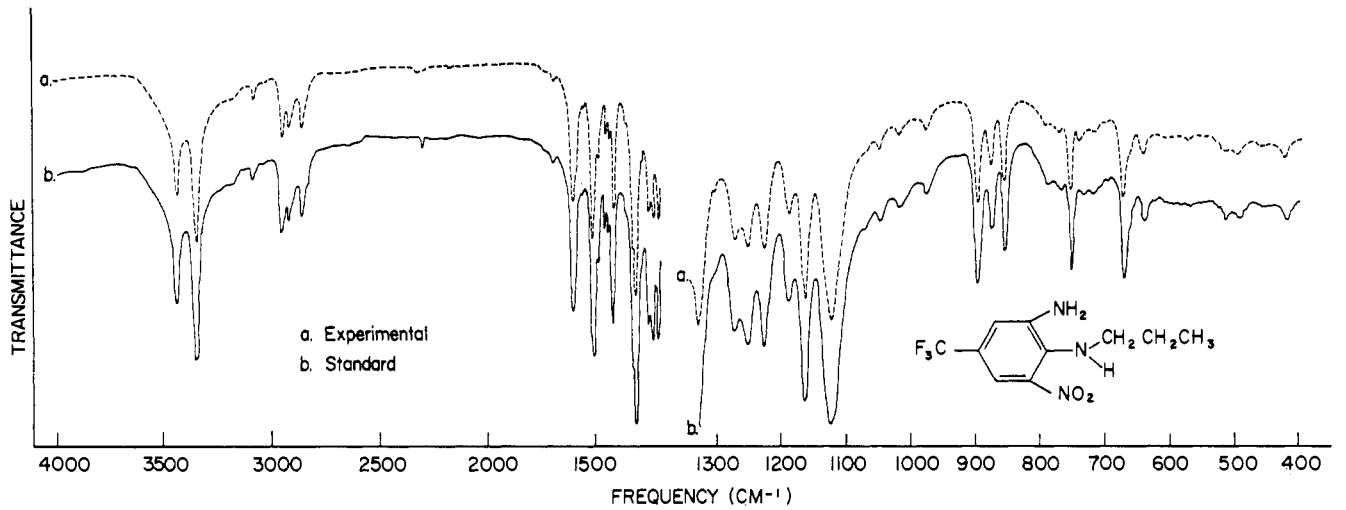


Figure 5. Infrared spectra of α,α,α-trifluoro-5-nitro-N_4-propyltoluene-3,4-diamine. (a) Experimental = *Bacteroides ruminicola* subsp. *brevis*, GA-33; (b) Standard = authentic sample

These strains, along with mixed bacteria, when grown in the presence of ^{14}C-ring-trifluoromethyl-labeled trifluralin, showed a progressive degradation of trifluralin to intermediate and polar products (Table III). Similar patterns of ^{14}C distribution were observed with ciliate-free rumen fluid or isotrichs and entodinia in rumen fluid incubated with ^{14}C-ring-trifluoromethyl-labeled trifluralin for 48 hr (Table III). Bacterial strains No. 49, B$_1$C23, B-159, L-34, C-94, FD-10, B$_2$4, 24, H-18, and S-85 did not degrade ^{14}C-trifluralin in 16 hr at 39° C. In other experiments where trifluralin concentrations were increased to 1000 μg/ml, there was a marked decrease in conversion rates by rumen bacteria (about 2% conversion in 72 hr at the 1000-μg level).

Identification of Trifluralin Metabolites. Both strains D-32 and GA-33 reduced one of the nitro groups of trifluralin to yield α,α,α-trifluoro-N_4,N_4-dipropyl-5-nitrotoluene-3,4-diamine (Figure 2; compound **11**, Table I). The second nitro group of trifluralin was also reduced by D-32 and GA-33. Comparison of infrared (Figure 3) and mass (Figure 4) spectra with an authentic sample identified it as α,α,α-trifluoro-N_4,N_4-dipropyltoluene-3,4,5-triamine (compound **4**, Table I). Compounds **11** and **4** (Table I) were consistently the predominant trifluralin metabolites found for strains D-32 and GA-33 and mixed rumen bacterial preparations.

Strain GA-33 degraded trifluralin by dealkylation and reduction to a compound identified as α,α,α-trifluoro-5-nitro-N_4-propyltoluene-3,4-diamine (Figure 5; compound **3**, Table I). Strain GA-33 also metabolized the parent compound to α,α,α-trifluoro-N_4-propyltoluene-3,4,5-triamine (Figure 6; compound **6**, Table I). Compounds **3** and **6** (Table I) were also found in D-32 preparations. Compound **1** (Table I) was detected by two-dimensional tlc radioautographs of D-32 and GA-33 and by infrared spectra of continuous culture preparations.

Characteristics of Trifluralin Polar Products. Polar products ran as a composite on tlc at R_f 0.74 (solvent, isopropyl alcohol) and R_f 0.60 (solvent, diisopropylketone–formic acid–water) and could be eluted from cellulose P columns in two fractions: fraction A (tubes 7 through 8) and fraction B (tubes 15 through 16). Total recovery from the column was 87%. On cellulose AE columns, polar product ^{14}C was eluted as two fractions with 63% of the carbon-14 recoverable. These experiments suggested that the polar fraction contained more than one compound.

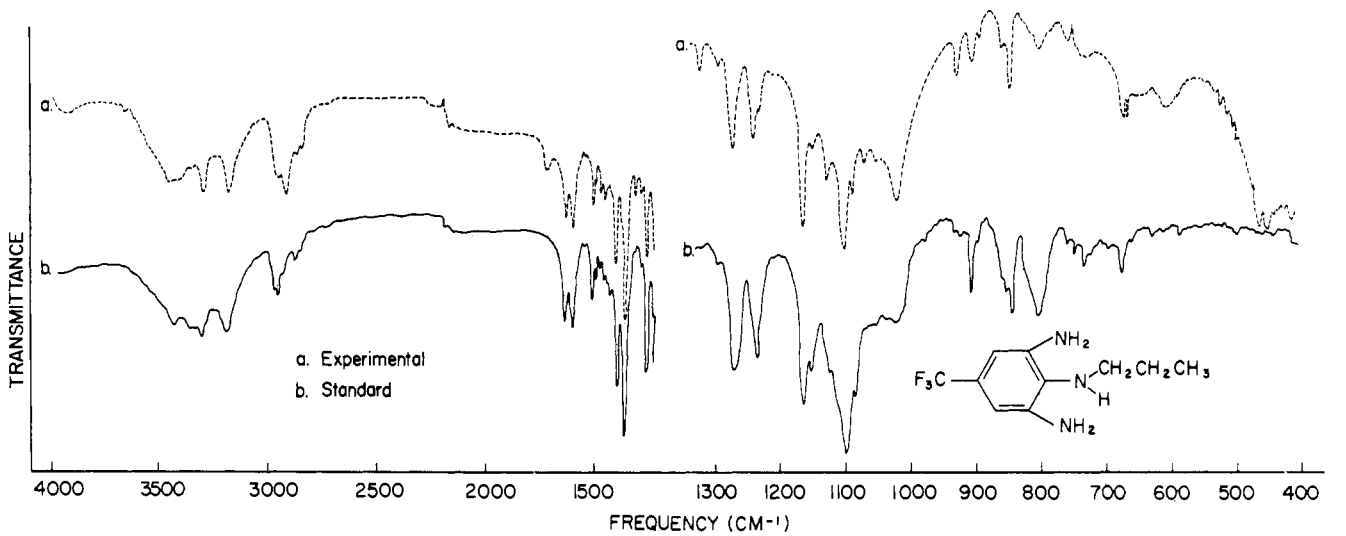


Figure 6. Infrared spectra of α,α,α-trifluoro-N_4-propyltoluene-3,4,5-triamine. (a) Experimental = *Bacteroides ruminicola* subsp. *brevis*, GA-33; (b) Standard = authentic sample

Table IV. Effect of Trifluralin on *Isotricha* sp. and Rumen Bacterial Gas Production

Trifluralin (mg/2.2 ml)	No. of observations		Gas evolved in μl per 80 min	
	isotrichs	Bacteria	per 10^3 isotrichs	per 10 mg bacteria
0	10	4	26.0	67.7
5	8	4	36.2	67.8
10	4	4	39.2	68.0

Table V. Effect of Trifluralin or Washed Bacterial Suspensions[a]

Trifluralin (mg/5.0 ml)	Viable population counts (−× 10^7/ml) at 72 hr incubation		
	Strain GA-33	Strain D-32	Mixture of bacteria
0	2.30	18.1	7.65
0.25	2.21	15.3	7.54
0.50	0.08	10.4	7.72
5.00	0.07	0.4	7.33

[a] Cell suspensions were exposed 2.5 hr to the trifluralin levels shown in the table. Viable population counts were then determined in RFTY medium containing the indicated levels of trifluralin.

Table VI. Effect of Trifluralin on Volatile Fatty Acid Production by a Rumen Ingesta Cellular Fraction Incubated with and without Trifluralin in RFTY Medium for 96 Hr at 39° C

Trifluralin (molarity)	Mean volatile fatty acid concentrations (μM/ml)[b]				
	Acetic	Propionic	Butyric	Valeric	Total
0[a]	44.60	8.20	3.60	0.80	57.20
0	73.80	21.30	13.00	12.10	120.20
8.8×10^{-5}	73.20	21.10	13.10	11.10	118.50
17.7×10^{-5}	72.30	21.30	13.60	11.50	118.70
3.6×10^{-4}	67.90	19.50	13.10	11.00	111.50
8.8×10^{-4}	67.20	21.40	16.10	11.90	116.60
17.7×10^{-4}	53.70[c]	16.60	12.10	10.50	92.90
36.0×10^{-4}	54.00[c]	16.80	11.80	9.80	92.40
7.2×10^{-3}	52.40[c]	17.90	12.60	9.80	92.70
17.7×10^{-3}	56.00[c]	17.90	13.00	7.80	94.70
36.0×10^{-3}	49.90[c]	16.80	12.10	8.80	87.60

[a] Zero time control was not incubated. [b] Values are given as means of triplicate tubes analyzed by glc in duplicate. [c] Based on analysis of variance, the values showed a significant ($P < 0.05$) decrease in acetic acid when compared to lower trifluralin concentrations or controls.

The polar products were resistant to acid and base hydrolysis, and acylation–esterification but were susceptible to reduction as reported by Golab *et al.* (1969). The reduced polar product (73%) could be sublimed at 200° C when held for 20 min. Ir and mass spectra of this material indicated the presence of a trifluoromethyl group and a propylamino group attached to an aromatic ring. No identification of the compound was made. None of the compounds in Table I were detected in reduced or nonreduced polar products. It is unlikely that these products were azo compounds since they are apparently degraded in rumen fluid (Katz *et al.*, 1969).

Effect of Trifluralin on Microbes. Manometric data indicated that endogenous gas production of *Isotricha* sp. and rumen bacteria was not suppressed at trifluralin concentrations of 0, 5, and 10 mg/2.2 ml of cell suspension (Table IV). With *Isotricha* sp., an increase in gas evolved was noted. However, this increase in gas evolved was not attributable to the protozoa utilizing trifluralin as a carbon source. *Isotricha* sp. and *Entodinium simplex* incubated for 80 min at 39° C with trifluralin (10 mg/2.2 ml) showed no cellular distortion or impairment of motility.

Strain GA-33 showed inhibition to trifluralin concentrations of 0.3 mg/5.0 ml and higher. Other strains (No. D-32, H-18, $B_2$4, 49, B-159, FD-10, C-94, L-34, 24, S-85, and B_1C-23) showed some inhibition at the 5-mg level but essentially no inhibition between 0- and 0.5-mg trifluralin levels. When mixed bacterial cell suspensions from rumen digesta were incubated with trifluralin, no reduction in viable populations was noted (Table V). Both GA-33 and D-32 showed suppression of growth at the 0.5- and 5.0-levels.

Effect of trifluralin on rumen microbial volatile fatty acid production is shown in Table VI. Rumen microbial activity in basal medium (Table II) indicated no effect due to different concentrations of trifluralin. When the trifluralin concentration was 17.7×10^{-4} *M* or higher in an enrichment medium, there was a significant ($P < 0.05$) decrease in acetic acid production. No significant effect on total volatile fatty acid production was observed at any of the trifluralin levels.

Rumen bacteria were found to be etiological agents in rumen ingesta capable of degrading trifluralin by dealkylation and reduction to intermediate products, which presumably were converted to polar products (Golab *et al.*, 1969). High concentrations of trifluralin had relatively little effect on rumen microbial populations, microbial endogenous gas evolved, and volatile fatty acids produced.

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