Environmental Assessment

PRODUCIL● (efrotomycin) Medicated Feed Premix
for Swine

1. Date: June 23, 1986

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4. Description of the proposed action:

The applicant has filed a New Animal Drug Application to permit the medication of swine feed with the antibiotic efrotomycin at a range of from 2 to 16 parts per million in order to increase the rate of weight gain and improve feed efficiency of growing/finishing swine.

The antibiotic substance efrotomycin will be produced at the Merck Cherokee Plant located at Danville, Pennsylvania, or at the Merck Stonewall Plant located in Elkton, Virginia, and pre-blended into an efrotomycin magnesium alginate granule with 20% efrotomycin A activity which will be further incorporated into a carrier system, composed of limestone, corncob flour, and mineral oil to provide a commercial medicated feed premix (type A article) containing 3.2% efrotomycin A, at a Merck feed premix blending facility at St. Louis, Missouri.

The medicated feed premix will be sold to feed additive distributors, feed manufacturers, and livestock raisers compounding rations for swine. These are, in general, more frequently located in rural areas of the midwest and southeastern United States.

The efrotomycin medicated feed is fed continuously from weaning to market weight to swine and the antibiotic and its metabolites are found in the swine urine and feces. These are spread as fertilizer on the agricultural land in the area where the swine are raised.

5. Identification of chemical substances that are the subject of the proposed action:

Efrotomycin is an antibiotic with a gram positive spectrum of antibacterial activity produced by the fungus Streptomyces lactamdurans, as a clean, tan free-flowing powder. Efrotomycin consists primarily of efrotomycin A_1 with smaller amounts of the related compounds: Efrotomycin Az (z isomer of A_1 which is the E form) and efrotomycin B's (ring closed compounds formed by Michael addition). The structures are shown in Figure 1. It is structurally closely related to the antibiotics goldinomycin and mocimycin. The CAS registry number is 56592-32-6.

The chemical names of the A_1 and B components are as follows:

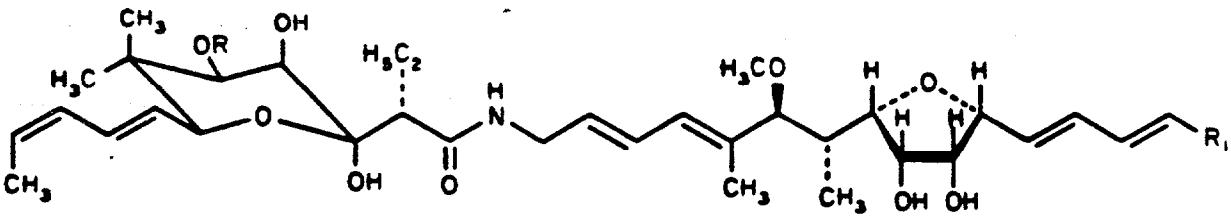
Efrotomycin A_1:

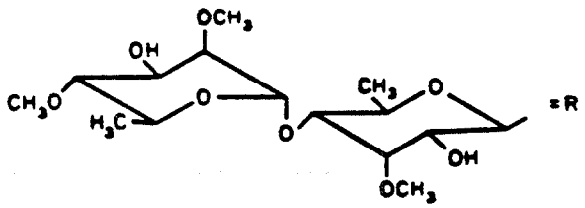
31-O-[6-deoxy-4-O-(6-deoxy-2,4-di-O-methylhexopyranosyl)-3-O-methylhexopyranosyl]-1-methylmocimycin

Efrotomycin B:

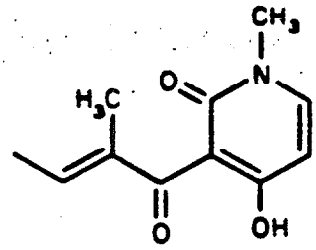
4-deoxy-31-O-[6-deoxy-4-O-(6-deoxy-2,4-di-O-methyl-α-L-manno-pyranosyl)-3-O-methyl-β-D-allopyranosyl]-4,9-epoxy-8,9-di-hydro-1-methylmocimycin

The structural formulae are as follows:



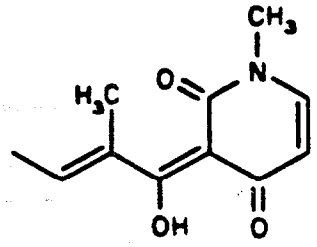


Efrotomycin $A_1, R_1 =$

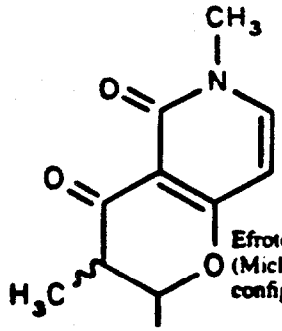


Efrotomycins A_1 and A_2 are tautomers

Efrotomycin $A_2, R_1 =$



Efrotomycin $B, R_1 =$



Efrotomycin B = ring-closed products (Michael adduct) with differing stereo configuration

Efrotomycin A_1 = at least 65%

Efrotomycin B = no greater than 30%

Total A_1 + B content = no less than 80%

Characterization:

The known efrotomycin components account for 85-90% of the material on a dry basis. Typically the product contains about 70-80% A_1, 5-15% of the B components, and 1-5% of the Az isomer. The remaining 10-15% consists of many components each present in small amounts. Because of their multiplicity and low levels, isolation of individual components is not practical. However, the data from several experimental observations suggest that they are related compounds that are precursors or degradation products.

Examination of 14 peaks in the chromatogram of a relatively impure sample using diode array ultraviolet scanning to obtain the spectra of each peak showed that all peaks had the typical maxima at ~230 nm and most also had maxima of various intensity at ~325 nm; both are characteristic of efrotomycin. The Az isomer has an additional maximum at ~280 nm and is one of the most different spectrums observed.

An additional observation that suggests that the impurities are closely related to efrotomycin is that in the preparation of radioactive efrotomycin by adding radioactive precursors to the fermentation and isolating the product by essentially the

normal process, the material isolated contains the radioactivity in the sample pattern; namely that the known efrotomycin compounds contained ~85% of the radioactivity and the other ~15% was spread across the chromatogram very similarly to the impurities in the normal product. The fact that the impurities are radioactive suggests that they arise from the metabolic activity of the microorganism that is involved in the formation of efrotomycin, or are degradation products formed during fermentation or isolation.

One compound that has been identified is the aglycone (goldinomycin) which was observed to be a minor component by NMR in one sample. It was also noted that all of the peaks in the very complex NMR spectrum were accountable for as efrotomycin related; which is consistent with the ultraviolet scan experiment.

In summary, the known efrotomycin components typically comprise about 85% of the product (specification minimum 80 area %) with the rest being a multiplicity of components which appear to be closely related to the main components. The isolation processes used are reasonably selective and consistent in that various modifications have all produced material with similar chromatographic profiles.

Chemistry:

Efrotomycin exhibits an ill defined decomposition point at about 110°C. It is miscible (forms gels) with most polar organic solvents and is relatively insoluble in non-polar solvents. Solubility in water is a function of pH, being 0.1 mg/ml at pH 4, 1 mg/ml at pH 7, and 10 mg/ml at pH 9.5.

Efrotomycin is optically active with a specific rotation $[\alpha]_D^{25°}$ of about -72° (C=1, methanol). A highly purified sample of the A_1 component had a specific rotation of -92° under the same conditions. The ultraviolet spectrum in methanolic phosphate buffer (pH 7.5) is characterized by maxima at ~326 and ~233 nm with A1%1cm values of ~275 and ~545 respectively. Purified A_1-isomer (95 area % pure by liquid chromatography) had an A1%1cm value of 350 at 325 nm and 632 at 220 nm with a shoulder value of 595 at 233 nm. A similarly purified sample of the B components had an A1%1cm value of 85 at 322 nm.

The solid is reasonably stable, losing about 5% a year at room temperature. Solution stability is a function of light, temperature and pH. Solutions are rapidly degraded at pH 12 and pH 2.5 and more slowly when exposed to light or heat. The reversible isomerization between the A_1 and B components is

affected by pH. Cyclization by a Michael reaction occurs at acidic pH and may be reversed at high pH. The rates are slow with only 4% B formed from A_1 at pH 6 in 25 days at 30°C, but essentially all of a sample of B has been converted to A_1 after 25 days at 30°C at pH 9. No change is observed in A_1 at pH 8 or in B at pH 5 under these conditions.

Antibacterial Activity:

Efrotomycin is a narrow spectrum antibiotic.(1) Among the genera tested for susceptibility in vitro it is most active against isolates of Moraxella, Pasteurella, Yersinia, Haemophilus Clostridium, Streptococcus and Corynebacterium. The drug is as active by oral administration as by the subcutaneous route. Blood levels rise rapidly to high concentrations, after oral dosing. Two peaks occur which may indicate biliary excretion and re-adsorption. Urinary excretion is minimal. The high blood concentrations may explain, in part, the in vivo activity against pathogens such as Bordetella bronchiseptica which are relatively insensitive in vitro.

Toxicology:

The following table (Table I) summarizes the toxicity testing completed with efrotomycin and provides a summary to findings of those tests.

MK-0621: Summary Table

5/21/85

Type of Study	Species	Sex	Duration	Route	Dosage Levels (mg/kg/day)	Findings
Ames test					2-20 mcg/plate	Negative
V-79 Chinese hamster cell point mutation assay					0.04-0.4 mM	Negative
UDS assay in rat hepatocytes					0.02-0.13 mM	Negative
Acute	Mouse	F	14 days	oral		LD_{50} > 20,000 mg/kg
Acute	Mouse	M&F	14 days	i.p.		LD_{50} approx. 900 mg/kg (both sexes)
Acute	Rat	M&F	14 days	oral		LD_{50} > 7500 mg/kg (both sexes)
Micronucleus assay	Mouse	M&F	2 days	oral	60,180,540	Negative
Teratogenicity	Mouse	F	Days 6-15 of gestation	oral	0,20,200,2000	No adverse effects at doses up to 2000 mg/kg/day.
Teratogenicity	Rat	F	Days 6-15 of gestation	oral	0,20,200,2000	Reduced fetal weights at highest dosage. No other significant effects.
Oral reproduction (*in utero* exposure phase of 14-week toxicity study)	Rat	M&F	8 weeks	oral	0,20,200,2000	Gestation period increased at highest dosage. No other adverse effects.
Subchronic (*in utero* exposure)	Rat	M&F	14 weeks	oral	0,20,200,2000	Salivation at 200 & 2000 mg/kg/day. Increased kidney and liver wgts. at high dose only. No effects observed at 20 mg/kg/day.

MK-0621: Summary Table (cont'd) 5/21/85

Type of Study	Species	Sex	Duration	Route	Dosage Levels (mg/kg/day)	Findings
Subchronic	Dog	M&F	14 weeks	oral	0,20,200,2000	Emesis and salivation in high dose group only. Increased alkaline phosphatase in all treated groups. Microscopic evidence of kidney damage only in high dose group.
Subchronic	Dog	M&F	14 weeks	oral	0,1,2.5, 5 & 10	Kidney wgts. relative to body wgts. increased in high dose group only. No other significant effects.
Primary dermal & ocular irritation	Rabbit	M&F	14 days	dermal and ocular	500 mg dermal 100 mg ocular	Single dermal application did not result in irritation. Chemosis and ocular discharge found immediately following ocular instillation. After 72 hrs. all eyes normal.
Dietary range-finding	Rat	M&F	14 weeks	oral	0.5, 1.0, 2.0 and 5% in diet	Nephrotoxicity in both sexes at 2.0 and 5.0% concentrations. NEL 1.0% in the diet.
Dietary range-finding	Mouse	M&F	14 weeks	oral	"	Nephrotoxicity in both sexes at 2.0 and 5.0% concentrations. NEL 1.0% in the diet.

6. Introduction of substances into the environment

a) Through manufacturing

1) Identity of chemicals expected to enter the environment

Organic and non-organic chemicals will be emitted into the atmosphere as a result of manufacturing operations. The chemicals expected to enter the atmosphere includes sulfuric acid (CAS #7664-93-9), heptane (CAS #142-82-5), isopropyl alcohol (CAS #67-63-0), t-butylamine (CAS #75-64-9), n-butyl alcohol (CAS #71-36-3), methyl isobutyl ketone (CAS #108-10-1), and n-hexane (CAS #110-53-3).

Three liquid streams will be generated from the isolation of fermentation whole broth. The three streams will contain water, methyl isobutyl ketone, soybean oil (MI* 5287), lecithin (MK 5287), lactic acid (CAS #598-82-3), boric acid (CAS #10043-35-3), ammonium molybdate (CAS #12027-67-7), zinc sulfate (CAS #7733-02-0), calcium chloride (CAS #10043-52-4), cupric chloride (CAS #7447-39-4), manganese sulfate (CAS #7785-87-7), and ferrous sulfate (CAS #7720-78-7).

Dust will be emitted into the atmosphere during the formulation process. A waste water stream generated

*MI Merck Index, 9th Edition

during formulation will contain alginic acid (CAS #9005-32-7), magnesium hydroxide (CAS #1309-42-8), mineral oil (MI 6972), limestone (MI 5330), corncobs, and traces of efrotomycin.

2) Identity of the location of emission expected to enter the environment and controls to contain and treat waste

Bulk manufacturing will occur in Elkton, Virginia, and Danville, Pennsylvania, where air emissions of volatile organic emissions and effluent from a biological treatment plant will enter the environment. Formulation of a 20% granule will take place at Elkton, Virginia. All other formulation operations will take place in St. Louis, Missouri. Volatile organic chemicals, at Elkton and Danville, will be controlled by equipment, such as surface condensers and wet scrubbers, where applicable. The three liquid streams generated from the whole broth isolation will be treated in a wastewater treatment facility at each manufacturing site. Treatment consists of equalization, neutralization, bio-oxidation, secondary settling, sludge dewatering, and incineration. Effluent from the Elkton facility will be discharged into the south fork of the Shenandoah

River. Effluent from the Danville facility will be discharged into the north branch of the Susquehanna River. Dust from the formulation steps in St. Louis will be controlled by exhaust hoods and a series of filter bags. Dust from granule formulation at Elkton will be controlled by bag filters. Wastewater from equipment washings at St. Louis will be discharged to the Metropolitan St. Louis Sewer District.

3) Identity of any applicable federal, state, and local emission requirements (including occupational exposure limits)

Discharge to the Shenandoah River from the Elkton facility treatment plant is regulated by NPDES Permit Number VA0002178, which is administered by the Virginia State Water Control Board. Non-hazardous solid waste (trash, paper, plastic, etc.), generated during the manufacturing process, is burned in an on-site incinerator. The trash incinerator is subject to Commonwealth of Virginia Regulations for the Control and Abatement of Air Pollution. The trash incinerator operates under Permit Number 20524, which is administered by the Virginia Air Pollution Control Board. Air emissions of volatile organic chemicals

are subject to the Virginia Regulations for the Control and Abatement of Air Pollution, Section III and Appendix M. Air emissions are subject to Permit Number 20524, which is administered by the Virginia Air Pollution Control Board.

Discharge to the Susquehanna River from the Danville facility treatment plant will be subject to and in compliance with the Rules and Regulations of the Pennsylvania Department of Environmental Resources (Title 25, Part 1, Subpart C, Article II - Water Resources) and NPDES Permit Number PA 0008419 which is administered by the Pennsylvania Department of Environmental Resources. Air emissions of volatile organic compounds (VOC) will be subject to and in compliance with the Rules and Regulations of the Pennsylvania Department of Environmental Resources (Title 25, Part 1, Subpart C, Article III -- Air Resources). Non-hazardous solid waste (trash, paper, etc.) will be disposed of off-site to a licensed sanitary landfill and will be in compliance to the Rules and Regulations of the Pennsylvania Department of Environmental Resources (Title 25, Part 1, Subpart C, Article VI -- Solid Waste Management).

The control of dust is subject to Title 10, Code of Missouri State Regulations, Chapter 5 -- Air Quality Standards and Air Pollution Control Regulations for the St. Louis Metropolitan Area. The discharge of effluent to the sewer is subject to Title 10, Code of Missouri State Regulations, Chapter 7 -- Water Quality Discharge to the sewer will also be subject to the Metropolitan St. Louis Sewer District Ordinance Number 2289.

Occupational exposure limits for some chemicals are established by the American Conference of Governmental Industrial Hygienists (ACGIH). The Threshold Limit Values (TLVs) of the chemicals used to manufacture efrotomycin includes sulfuric acid (1 mg/m^3, TWA); sodium hydroxide (2 mg/m^3, Ceiling); heptane (1600 mg/m^3, TWA); manganese sulfate (Ceiling of 5 mg/m^3); ferrous sulfate (TWA of 1 mg/m^3 as Fe); N-butanol (150 mg/m^3, Ceiling); isopropyl alcohol (980 mg/m^3, TWA); n-hexane (180 mg/m^3, TWA); and methyl isobutyl ketone (205 mg/m^3, TWA). The TLV for t-butylamine has not been established, but the ACGIH recommends a ceiling value of 15 mg/m^3. An

exposure control limit of 5 mg/m^3, TWA, is recommended by ACGIH for nuisance dust.

4) Determination of compliance with current emission requirements and the effect the contemplated action will have upon compliance with current emission requirement

The Stonewall Plant (Elkton, Virginia), the Cherokee Plant (Danville, Pennsylvania), and the St. Louis Plant (St. Louis, Missouri) are currently in compliance with the regulations which address the control and disposition of air emissions, aqueous waste, and non-hazardous solid waste. The production of efrotomycin will not affect compliance with existing environmental regulations at any of the facilities mentioned above.

b) Through use

The projected use of efrotomycin in swine involves inclusion of the drug in the feed at levels of 2 to 16 ppm daily from starter to finisher hogs, approximately 7 to 100 kg body weight and roughly 4 to 24 weeks of age. The animals may be contained in a pasture, a large commercial feedlot, a slotted-floored house or a solid concrete-

floored facility. The dirt lot or pasture and the solid concrete-floored facility comprise the majority of the swine operations and represent the extremes in dilution of the solid wastes for use as fertilizer.[2] However, since nitrogen content of fertilizer is often used as the basis for determining application rates,[3a] the total amount of drug per acre introduced through fertilization with swine excreta should be comparable regardless of the dilution of the manure.

The daily intake of drug will depend upon the level of efrotomycin in the feed and upon the feed intake of the animal. Swine dosed at the maximum level, 16 ppm in the feed, would receive an average of 36.8 mg of drug per day, based upon an average of 2.3 kg of feed consumed by a 45 kg hog[2] about the average weight of hogs to be treated. Hogs would spend about 24 weeks in the feedlot, from starter or feeder weight (7-25 kg) to market weight (100 kg).[2] The average hog would receive 6.2 g of efrotomycin while in the feedlot.

16 ppm (μg/g) x 2.3 kg feed/day/average = 36.8 mg efrotomycin/day

36.8 mg/hog/day x 24 weeks x 7 days/week = 6.2 g/hog

At high feedlot densities, 250 hogs/acre,[2] there would be 250 hogs/acre x 6.2 g efrotomycin/hog = 1.55 kg drug/acre in the manure in the feedlot.

Since the average raw waste production in the swine feedlot is about 3.5 kg/hog/day,[2] the average concentration of drug and metabolites in manure would be 36.8 mg/day/3.5 kg waste/day = 10.5 ppm. This average corresponds well with the drug-residue levels observed in the excreta of two barrows fed ^{14}C-labeled efrotomycin at 16 ppm for seven days. The labeled drug was 98.8% pure A_1-isomer. Excreta were collected pre-dose, daily for 7 days on drug and for 2 days off drug. Approximately 60% of the dose was recovered from one animal, and about 77% was recovered from the other during the 9 days of collection. The urine from each barrow contained only about 2% of the dose, with residue levels of 0.3 to 0.5 ppm in the urine from days 2 through 7. The remainder of the drug was excreted in the feces, where residue levels ranged from about 7 to 17 ppm from days 3 on drug through 2 days off drug, with an average residue level for those days of about 11 ppm.

Because nitrogen is often the nutrient that limits plant growth and because nitrogen poses the greatest threat to ground water contamination, it is often used as the basis for determining application rates.(3a) The United States Department of Agriculture recommends the use of no more than 12 tons of fresh animal manure per acre.(4)

Indiana's Cooperative Extension Service recommends application rates of 225 pounds of nitrogen per acre, while Extension Services and Experiment Stations in North Carolina, Iowa, and Maine all recommend application rates based upon soil testing and nitrogen content of the waste.(3a) The Wisconsin Manure Management Plan recommends applying manure at rates which minimize the nitrate-nitrogen accumulation in the soil by using application rates based upon crop removal of nitrogen.(3b) The amount of nitrogen this plan estimates that can be applied without accumulating nitrates is 250 pounds per acre. For swine, with 10 pounds nitrogen per ton of manure,(4) this would be 25 tons of manure per acre. If 25 percent of the nitrogen is lost prior to application, the maximum rate could be 33 tons per acre. Single applications on a land disposal

basis might be two times the annual rate, or 50 tons per acre (500 pounds nitrogen per acre).(3b)

In a non-random survey of dairy farmers, 12% exceeded 300 pounds nitrogen per acre and 7% exceeded 400 pounds per acre.(3c) Application of approximately 570 lbs N/acre using swine manure injection into the soil has been reported.(3d) This heavy application was followed by only 80 lbs N/acre in the form of anhydrous ammonia for two years. In a liquid swine manure management system in Texas, settled solids from the liquid manure storage pits were collected, hauled to the field, and injected 10 inches below the soil surface.(3e) Nitrogen loading rates of 600 lbs N/acre/year were used for coastal Bermuda grass, annual hay, oats, and hybrid sudangrass.

Thus, application of 50 to 250 pounds nitrogen (5 to 25 tons of swine manure) per acre is recommended, while 570 to 600 pounds nitrogen (57 to 60 tons of manure) per acre appears to be the highest application rate used for swine wastes. Using swine feces containing 17 ppm efrotomycin and metabolites (the highest level seen in the feces of the two barrows dosed at 16 ppm in the feed), fertilizing

at 60 tons per acre and plowing or injecting 6 inches into the ground would lead to a drug plus metabolite level of 1.0 ppm in the soil.

$$\frac{17 \text{ mg residue}}{\text{kg feces}} \times \frac{1 \text{ kg}}{2.2 \text{ lb}} \times \frac{2000 \text{ lb}}{1 \text{ ton (U.S.)}} \times \frac{1 \text{ g}}{1000 \text{ mg}} = \frac{15.45 \text{ g drug-equivalent}}{\text{ton feces}}$$

$$\frac{15.45 \text{ g residue}}{\text{ton feces}} \times \frac{60 \text{ ton feces}}{\text{acre}} \times \frac{1 \text{ acre}}{43{,}560 \text{ ft}^2} \times \frac{1000 \text{ mg}}{\text{g}} = \frac{21.3 \text{ mg drug-equival}}{\text{ft}^2}$$

$$1 \text{ ft}^2 \times 6\text{" deep} \times \frac{144 \text{ in}^2}{\text{ft}^2} \times \frac{16.4 \text{ cm}^3}{\text{in}^3} \times \frac{1.5 \text{ g}}{\text{cm}^3 \text{ soil}} = \frac{21{,}254 \text{ g soil}}{\text{in 1 ft}^2 \times 6\text{" deep volume}}$$

$$\frac{21.3 \text{ mg drug equivalent/ft}^2}{21{,}254 \text{ g soil}} \times \frac{1000 \text{ µg}}{\text{mg}} = 1.0 \text{ µg/g (ppm) drug-equivalent}$$

Thus, the worst-case usage of 60 tons per acre of swine feces which contain the highest expected level of residue leads to a soil concentration of efrotomycin and metabolites of 1 ppm. Using recommended fertilization rates of 5 to 25 tons of swine manure per acre and the expected average level of 11 ppm of residue in manure from animals dosed at 16 ppm efrotomycin in the feed leads to drug-residue concentrations of 54 to 270 ppb in the soil.

The efrotomycin levels in ground water in contact with fertilized fields can be calculated using the efrotomycin levels in the soil and the Freundlich equation. The Freundlich equation states that the soil concentration equals a constant times the solution concentration raised to a power and has the form:

$$(x/m) = kc^{1/n}$$

where x/m is the soil concentration in micrograms per gram, c is the solution concentration in micrograms per gram, and k and n are constants. When n = 1, the Freundlich K equals the distribution coefficient (Kd) between soil and water.

The binding of efrotomycin to five soil types is discussed in Section 7.b) Sorption/Desorption. Efrotomycin is poorly bound to sand with about 17% sorbed. The binding to four other soil types did not correlate to soil texture, pH or organic matter content. Freundlich constants for desorption ranged from 13 to 514 for k and 0.981 to 1.3 for n.

If swine manure containing the "worst-case" level of 17 ppm of drug-residue is applied to sand at 60 tons per acre, resulting in a drug concentration in soil of 1 ppm, then roughly 17% of that would be bound leading to a solution concentration of 0.83 x 1 ppm = 0.8 ppm. This would be the worst-case level of efrotomycin in ground water. For the other soils, since n≈1, then the solution concentrations would be approximately the soil concentrations divided by the Freundlich k. Soil concentrations of 1 ppm would result in solution concentrations in the range of 0.002 to 0.08 ppm (1/514 to 1/13, respectively) in the loam, silt loam, sandy loam and clay loam soils tested.

Application of highest recommended fertilization rate of 25 tons per acre of swine manure containing the expected average level of 11 ppm of residue would result in a concentration of 0.2 ppm (0.83 x 0.270 ppm) ground water in contact with sand and a range of approximately .0005 to 0.02 ppm (0.270/514 to 0.270/13, respectively) in water in contact with the other four soils tested.

Excreta from two barrows fed for seven days with ^{14}C-labeled efrotomycin A_1 at 16 ppm were examined to deter-

mine the percent of parent drug and for the presence of isomers and metabolites. The urine from each barrow contained only about 2% of the doses, while 60% and 77% of doses were recovered in the feces through two days off drug. The A_1-isomer accounted for about 60% of the drug-residue in the feces, and the B-isomers accounted for about 20% of the residue. Polar metabolites were not extracted by the work-up procedure. In the urines, the A_1-isomer accounted for only 35% of the residue and B-isomers only about 5%. At least two polar compounds were observed by chromatography. Overall then, there was not much metabolism of the drug and it will be mostly efrotomycin A_1- and the B-isomers which will be introduced into the environment through the feeding of commercial efrotomycin to swine.

7. Fate of emitted substances in the environment

The following laboratory tests were conducted to predict the fate of efrotomycin which may enter the environment.

a) Aqueous stability

The stability of efrotomycin A_1-isomer was determined at 25°C in the dark in buffered solutions at pH 5, 7, and 9 and also in methanol. The stability of samples at pH 7

and in methanol was also studied under ambient laboratory temperature and lighting. All glassware used in the experiment was sterilized by autoclaving to exclude microbial contamination.

Aliquots of ^{14}C-labeled efrotomycin A_1 in methanol, 0.1 ml, 10409 disintegrations per minute (DPM), and 0.418 micrograms, were added to each of 40 sterilized centrifuge tubes. The methanol was evaporated to dryness under nitrogen that was passed through a 0.2 micron filter to sterilize it.

All buffer concentrations were 0.05 M. All buffers were sterilized by filtering through 0.20 micron filters. Five milliliters of the appropriate, sterile filtered buffer or methanol was added to each centrifuge tube. The efrotomycin A_1 was dissolved by vortex mixing for 1 minute. Samples for the dark study were individually wrapped in aluminum foil to exclude light and placed in a reciprocating water bath set at 25°C. Samples for ambient laboratory conditions were placed in a test tube rack on a lab bench at Merck Sharp & Dohme Research Laboratories, Rahway, NJ. Zero time for all samples was 4:35 p.m. EDT on August 15, 1984.

Time and temperature were recorded at each assay time. Benchtop samples were exposed to approximately 10 hours of fluorescent lighting and 14 hours of dark per day. Incident light was not recorded.

One sample at each pH and in methanol was assayed at 0 days, 4:35 p.m. EDT on August 15, 1984. One sample under each condition, pH 5, pH 7 (light and dark), pH 9, and methanol (light and dark) was assayed at 1, 2, 5, 7, 13, and 29 days. A total of 40 samples was used.

At assay times, 909.7 micrograms of unlabeled efrotomycin was added as cold carrier and mixed by vortexing. The volume of 0.05 molar monobasic potassium phosphate needed to adjust pH 9 samples to between pH 6 and 7 was determined by adding 0.5 ml aliquots of the KH_2PO_4 buffer to 5.0 ml of control pH 9 buffer and measuring the pH. Two ml of KH_2PO_4 buffer was found to yield a pH of 6.9 and was the volume added to the pH 9 samples in the experiments to improve the extraction recovery. One to 1.5 milliliters of HPLC grade ethyl acetate was used to extract each aqueous sample. Extractions were done in duplicate. Samples were mixed by a combination of

vortexing and shaking for 1 minute. Ethyl acetate layers were removed and duplicates were pooled, and their volumes recorded. One-tenth milliliter of each was taken for scintillation counting to determine extraction recovery.

The recovery of radioactivity decreased as the experiment proceeded, indicating that there was less non-polar (extractable) material with time. About 90% of the radioactivity was extractable at 0 days for the pH 5 and 7 samples and about 60% was extractable by 29 days. For the pH 9 samples, extractability decreased from 78% at day 0 to 69% at day 29.

The remainder of the organic layer was evaporated to dryness under nitrogen at 30°C. The methanolic samples were evaporated to dryness under nitrogen at 30°C immediately after the cold carrier was added. The residues from all samples were reconstituted with 0.2 ml of 70:30; methanol:water. High Performance Liquid Chromatography (HPLC) was used to isolate the efrotomycin A_1-isomer for ultraviolet (UV) absorbance analysis. Five 1.0 ml fractions were collected from the HPLC eluate for each sample at the expected elution time for

efrotomycin A_1. The UV absorbances of the fractions were measured from 500 nm to 195 nm. The optical densities at 233 nm and 320 nm were recorded for the two fractions in each sample which contained the greatest UV absorbance. Six-tenths ml aliquots of the two fractions from each sample that had the greatest UV absorbance were taken for scintillation counting and placed into plastic mini vials.

The specific activity (S.A.) of each fraction was calculated using the equation:

$$\text{Specific Activity} = \frac{\text{Radioactivity (DPM/ml)}}{\text{Concentration (mg/ml)}}$$

where the radioactivity was determined by LSC, and concentration in mg/ml is by UV absorbance. The Theoretical Specific Activity is the expected specific activity of the test sample plus cold carrier, and is calculated by the equation:

$$\text{Theoretical Specific Activity} = \frac{\text{Radioactivity}}{\text{Total mass efrotomycin } A_1}$$

where radioactivity was determined by scintillation counting. The total mass of efrotomycin A_1 was the amount of cold carrier added plus the mass contributed by the labeled material, which was negligible compared to the mass of the cold carrier added.

The percent (reverse isotope dilution assay percent, RIDA %) of radioactivity represented by undegraded efrotomycin A_1 was determined by:

$$\text{RIDA \%} = \frac{\text{Specific Activity} \times 100}{\text{Theoretical Specific Activity}}$$

For efrotomycin A_1 at pH 5, 7, and 9 in the dark and pH 7 in the light, the RIDA % of efrotomycin A_1 decreased from approximately 100% at day 0 to 35%, 64%, 75%, and 43%, respectively, by day 29. In methanol, efrotomycin degraded from 111% at day 0 to 89% of the radioactivity in the sample in the dark by day 29 and to 82% of the radioactivity in the sample exposed to laboratory light.

Regression analysis was performed on the data from each group by a least squares regression of the logarithm of RIDA % vs. time to obtain the degradation rate constants and half-lives. The half-lives for hydrolysis in aqueous solution in the dark at pH 5, 7, and 9 were determined to be 21.6 days, 49.2 days, and 89.3 days, respectively. Hydrolysis was slower in methanol in the dark, with a half-life of 106.5 days. The rate of hydrolysis was faster for samples exposed to laboratory light, where the half-life was 26.0 days in an aqueous solution at pH 7. A

similar increase in the hydrolysis rate was observed for a methanolic solution exposed to laboratory light, where the half-life was 87.4 days.

The half-life at pH 9 was greater than 4 times the half-life at pH 5. These findings are consistent with the reported pKa of 6 for the phenolic proton of the A_1-isomer. Ionization of this functionality has an observable effect on its rate of hydrolysis.

The 29-day samples were re-chromatographed on the HPLC system and radioactivity and absorbance versus time were compared to characterize the hydrolysis products of efrotomycin A_1. The profile from pH 5, day 29, showed the greatest accumulation of radioactivity under peaks other than the parent compound, where 43.0% eluted radioactivity was A_1-isomer, 15.4% represented B-isomers, 9.5% was more polar (solvent front) and 14.0% was less polar (methanol wash) than the major isomers. Small amounts of radioactivity were seen in the 29-day profiles of the other samples, but no significant accumulation was observed. No further characterization of the efrotomycin hydrolysis products was done.

In the environment, samples of efrotomycin in ground water, for example, may be expected to completely degrade (i.e., 4 half-lives = 93.75% degraded) from 2.9 months at pH 5 to 11.9 months at pH 9. This incorporates the reasonable pH range for most soils.

b) Sorption/Desorption

The sorption and desorption characteristics of efrotomycin A_1-isomer with five soil types (silt loam, loam, sand, sandy loam, clay loam) of varying physical properties have been determined. A preliminary test was run to assure the applicability of the analytical method, a screening test was performed to provide a semi-quantitative measure of sorption and desorption of the test chemical, and an advanced test provided quantitative measurements.

Carbon-14-labeled efrotomycin A_1 stock solution in methanol was 0.206 mg/ml and 2.35 µCi/ml (11.41 µCi/mg). The position of the radiolabel is carbon 7.

All chemicals and solvents were reagent or HPLC grade. In-house deionized water was further filtered through activated carbon and mixed-ion exchange resin in a commercial ultrapurification system.

All solutions equilibrated with soil were aqueous solutions of calcium chloride. A 0.01 M calcium chloride solution was used to best approximate the ionic environment of water in soil. This solution was prepared by adding 0.444 g of anhydrous calcium chloride to 400 ml of deionized distilled water that had been boiled for 10 minutes to remove carbon dioxide. The pH of this solution was measured and found to be 6.70.

Liquid samples were radioassayed by mixing 1 to 3 ml of sample with 18 ml of scintillation cocktail. Radioactivity in each aliquot was measured for 10 minutes by scintillation counting. All soil samples were dried and analyzed by combustion in a sample oxidizer. Blank and control oxidizer standards were assayed to determine the oxidizer efficiency. All samples were combusted for 40 seconds. Trapped $^{14}CO_2$ and scintillation cocktail were delivered by the oxidizer to scintillation vials. Due to the small amount of oxidizable (organic) material in soil, a small hard pellet of non-organic, non-oxidizable material was recovered from the oxidizer. Each of these was placed in a scintillation vial with 18.0 ml of scintillation cocktail and counted. In all samples, the radioactivity of the pellets was at background level.

Preliminary test

Ten ml of the $CaCl_2$ solution was added to 2.0 grams of Riverside, California, loam soil (See Table II for soil characteristics) in four separate centrifuge tubes. These samples were equilibrated by rotating for 16 hours at room temperature in the dark. Following equilibration these samples were centrifuged. One ml aliquots of each supernate were taken for scintillation counting. Five ml aliquots were removed from each tube and placed into clean centrifuge tubes. To the blank, 0.07 ml methanol was added, and to each of the two samples (one tube broke on centrifugation) was added 0.02 ml of the test chemical solution and 0.05 ml of a 9.36 mg/ml solution of unlabeled efrotomycin as "cold carrier" to yield 20250 DPM/ml, 92.3 µg/ml. A 1.0 ml aliquot of the blank and each sample was taken for scintillation counting. Recovery in the spiked aqueous phases was 91.2% and 89.9%, respectively, for the two samples. In the preliminary test, no attempts were made to account for the remainder of the radioactivity.

The blank and the two samples were each extracted twice with equal volumes of ethyl acetate. Duplicate extracts

were pooled, the volumes were recorded and 0.1 ml of each was taken for scintillation counting. Ethyl acetate extraction recoveries were 100.6% and 99.7% while the amounts remaining in the aqueous phase were 0.9% and 1.1% for the two samples, respectively. The total recovery of the two samples was 101.5% and 100.8%. The pH values for $CaCl_2$ solution after equilibration with each of the five soils used in this experiment are included in Table II.

The remainder of each extract was evaporated to dryness under nitrogen and the residues were dissolved in 0.2 ml of 70% methanol/water as was a control solution. High Performance Liquid Chromatography (HPLC) was used to isolate efrotomycin A_1-isomer for ultraviolet (UV) absorbance analysis and liquid scintillation counting (LSC) for specific activity determination. The specific activity of the isolated efrotomycin A_1 was determined in 1.0 minute collections of the effluent from the HPLC system. The concentrations of efrotomycin A_1 in each of two fractions corresponding to the elution time of the efrotomycin peak were determined by dividing the UV absorbance value at 320 nm by the A1%1cm (323.4 AU) and multiplying by 10,000 µg/ml (i.e., 1% w/v = 1 g/100 ml = 10,000 µg/ml).

TABLE II

PROPERTIES OF TEST SOILS FOR SORPTION/DESORPTION

Soil Source	Three Bridges, NJ	Riverside, California	Lakeland, Florida	Lufkin, Texas	Newton, Iowa
pH	7.5	6.7	7.5	7.5	5
Organic Matter (%)	2.1	2.5	0.1	1.1	4.6
C.E.C. (meq/100 g)	12.5	12.3	1.5	8.7	24.29
Bulk Density (g/cc)	1.12	1.23	1.73	1.42	(~1.5)
Water Retention (% @ 1/3 atm)	30.3	17.8	1.2	15	-----
Mech. Analysis (%)					
Sand	11.6	49.6	91.6	57.6	26
Silt	61.6	35.6	5.6	27.6	46
Clay	26.8	14.8	2.8	14.8	28
Soil Texture	Silt Loam	Loam	Sand	Sandy Loam	Clay Loam
pH of $CaCl_2$ Solution After Soil Equil.	6.65	6.09	6.7	6.76	5.5

The observed specific activity of the isolated efrotomycin A_1 was determined by dividing the concentration in the effluent fractions by the radioactivity per milliliter. Dividing the observed specific activity by the theoretical specific activity (spiked radioactivity divided by the mass of cold carrier added) and multiplying by 100 gives the percent of the radioactivity in the sample which is efrotomycin A_1 (Reverse Isotope Dilution Assay % or RIDA%). The mean RIDA% for the spiked samples (n=2) was 101.6%, indicating all the radioactivity in the samples was efrotomycin A_1.

The preliminary test demonstrated the applicability of the analytical methods.

Screening test

Screening tests were performed on five different soils of various composition and characteristics (Table II). The screening test for each soil was made up of one blank and one control, on which triplicate analyses were performed, and three replicate samples with test chemical only, each analyzed once.

A working solution of the test compound was made by adding 0.059 ml of the test chemical stock solution to 3.65 mg of unlabeled efrotomycin A_1-isomer in 1 ml methanol. This mixture was diluted to 150 ml with $CaCl_2$ solution to yield 24.38 µg/ml, 2016.3 DPM/ml of efrotomycin (specific activity = 82.70 DPM/µg). Soil samples were made by adding 5.0 ml of this working solution to 1.0 g of soil and stoppering in a 15 ml centrifuge tube. Blanks were made by adding 5.0 ml of $CaCl_2$ solution to 1.0 g of soil in stoppered centrifuge tubes. Controls were made by adding 5.0 ml of the working solution to empty centrifuge tubes and stoppering them. Equilibrations were performed over 16 hours with all samples protected from light.

Each soil mixture was centrifuged and aliquots of the supernatant liquid layers (1.0 ml) were assayed for the test chemical by scintillation counting.

Triplicate 1.0 ml aliquots of the working solution averaged 2105.35 DPM. Each sample for the sorption process contained 5 ml x 2105.35 DPM/ml = 10526.75 DPM. The amount of radioactivity bound to soil following equilibration was determined by subtracting the total DPM

in solution from the initial total DPM. Amount bound in micrograms was determined by dividing DPM bound by specific activity (82.70 DPM/µg). The percent bound to soil was calculated by dividing µg bound by total µg added. Any soil that bound more than 25% of the test chemical was considered to readily sorb the test chemical, and desorption processes were attempted on these soils. Only Lakeland, Florida sand, did not readily sorb efrotomycin (mean sorption % = 16.94%). Desorptions were not carried out with this soil.

A total of 4.0 ml of supernate was removed from each blank, sample and control, and the supernate not analyzed was discarded. This was replaced with 4.0 ml of $CaCl_2$ solution. All samples were again stoppered and equilibrated for 16 hours in the dark. This was the first desorption step. Aliquots from each tube were taken and analyzed identically to the adsorption procedure above. The desorption step was repeated.

For any soil to which efrotomycin was readily sorbed (greater than 25%) and from which it was not readily desorbed (less than 75% of the fraction sorbed), the

performance of the advanced test was indicated. This was the case for four soils, Three Bridges, New Jersey, slit loam (63.44% sorbed, 27.95% desorbed), Riverside, California, loam (61.20% sorbed, 29.97% desorbed), Lufkin, Texas, sandy loam (91.03% sorbed, 7.57% desorbed), and Newton, Iowa, clay loam (98.22% sorbed, 1.76% desorbed).

Advanced test

The advanced test was carried out in two parts, a soil kinetics test to determine the time necessary to reach equilibration and the determination of binding parameters.

1. Soil kinetics test

The working solution for the soil kinetics test was prepared by dissolving 3.72 mg of unlabeled efrotomycin in 1.0 ml methanol. Two-tenths ml of labeled test chemical solution and 149 ml of $CaCl_2$ solution were added. The final observed concentrations were 7038.5 DPM/ml and 25.04 µg/ml (specific activity = 281.1 DPM/µg). The soil kinetics test was performed on the four soils indicated from the screening test that demonstrated significant binding without subsequent desorption.

One gram of each soil was placed into six centrifuge tubes. To one sample of each soil, 5.0 ml of $CaCl_2$ solution was added as a blank. To the other samples, 5.0 ml of the working solution was added. A control of 5.0 ml working standard was added to an empty centrifuge tube as a control. All samples were placed on a rotary mixer and covered with foil to exclude light.

At each sampling time, one tube from each soil was taken off of the mixer for analysis. Triplicate 1.0 ml aliquots of each supernate were taken after centrifugation and counted. The blank for each soil and the control sample (no soil) were removed at 1.18 hours, with the first sample for each soil. A single 1.0 ml aliquot from each blank was counted while triplicate 1.0 ml aliquots were taken from the control sample for counting. Triplicate, 1.0 ml aliquots from each soil type were taken at 1.18, 2, 4, 7, and 24 hours.

Free µg amounts per ml were calculated by dividing free DPM values by the specific activity (281.1

DPM/µg). Micrograms bound were determined by subtracting the number of µg free per ml solution from the number of µg added per ml of working solution (25.04 µg). Percent bound was calculated by dividing µg bound by µg added (25.04 µg) and multiplying by 100. From plots of percent efrotomycin bound to soil versus time for all four soil types, it was determined that equilibration was essentially complete after 16 hours. Sixteen hours was therefore used for sorption and desorption during the isotherm determination. Three Bridges silt loam equilibrated at 78.6% bound, Riverside loam at 75.3% bound, Lufkin sandy loam at 96.2% bound, and Iowa clay loam at 99.0% bound, all in the range of binding observed in the screening test for each soil. The control sample analyzed at 1.18 hours had a mean percent binding of 1.1% (6963.3 DPM/ml in solution, after sorption of 7038.5 DPM/ml added, or 98.9% free).

2. Isotherm determination

Four working solutions were used in this phase of the experiment and were made as follows:

Working Solution W: 0.05 ml of the labeled test chemical solution was added to 0.032 ml of an unlabeled efrotomycin solution, 0.1935 mg/ml in methanol. This solution was evaporated to dryness under a stream of nitrogen at 30°C, re-dissolved with 0.534 ml methanol and diluted with 80.0 ml of $CaCl_2$ solution to yield 3205.1 DPM/ml and 0.205 µg/ml (specific activity = 15634.6 DPM/µg).

Working Solution X: Identical to working solution W, except 0.390 ml of unlabeled efrotomycin solution, 0.1935 mg/ml in methanol was used. The concentrations were 3205.1 DPM/ml and 1.06 µg/ml (specific activity = 3023.7 DPM/µg).

Working Solution Y: Identical to working solution W, except 2.18 ml of unlabeled efrotomycin solution, 0.1935 mg/ml in methanol was used. The concentrations were 3205.1 DPM/ml and 5.37 µg/ml (specific activity = 596.85 DPM/µg).

Working Solution Z: Identical to working solution W, except 11.14 ml of unlabeled efrotomycin solution,

0.1935 mg/ml in methanol was used. The concentrations were 3205.1 DPM/ml and 26.9 µg/ml (specific activity = 119.15 DPM/µg).

The adsorption isotherm experiment was performed using triplicate samples of each soil for each concentration, a blank for each soil and a control for each concentration. One and one-half grams of the appropriate soil was added to each sample tube and each blank tube. Seven and one-half ml of the appropriate working solution was added to each sample and the controls, and 7.5 ml of $CaCl_2$ solution was added to each blank. All blanks, samples and controls were covered with foil and were equilibrated for 16 hours on the rotary mixer. Following equilibration, all samples and blanks were centrifuged and supernatants were aliquotted for scintillation counting. Triplicate 1.0 ml aliquots were taken from the blanks and the controls and single 3.0 ml aliquots were taken from each sample for counting. Three ml from each blank, sample and control was discarded.

The remaining solution/soil was vortexed and stirred to make a uniform slurry. Paper combustion cups were

weighed, and between 0.2-0.4 gram of slurry was weighed out from each sample and blank tubes. Each combustion cup was allowed to stand overnight and re-weighed to determine the amount of solution in each sample by difference, and the amount of soil when solution weight was subtracted.

Meanwhile, 6.0 ml of $CaCl_2$ solution was added to each blank, sample and control to replace the total volume removed. All tubes were stoppered and allowed to mix for 16 hours in the dark for the first desorption step. Following equilibration, supernate and slurry samples were removed as described in the adsorption step. The desorption step was repeated.

Solution weight from slurry was calculated by subtracting the dry weight from the wet weight. Density of the solution was assumed to be 1.0 g/ml. Therefore, the solution weight and volume were used interchangeably. Soil weight from the slurry was calculated by subtracting the tare weight from the dry weight. Radioactivity per gram soil (DPM/g) was determined by subtracting (solution volume x DPM/ml solution) from net DPM in soil, all divided by the soil weight from the slurry. DPM/g

soil was converted to microgram/gram soil by dividing by specific activity for the aqueous solution used. Similarly, DPM/ml solution was converted to microgram/ml solution by dividing by specific activity. The adsorption distribution constant, K_d, was determined by dividing the concentration on the soil (in microgram/gram soil) by the concentration in solution (in microgram/ml). This parameter gives an estimate for each soil of the amount of test chemical bound to soil when the solution concentration in equilibrium with the soil is 1.0 microgram/ml. The distribution constant extrapolated to 100% organic matter content of the soil, K_{om}, was calculated by dividing K_d by the percent organic matter and multiplying by 100. The distribution constant extrapolated to 100% organic carbon content, K_{oc}, was determined by multiplying K_{om} by 1.724.[5] The mean and standard deviations were calculated for K_{om} and K_{oc} for each soil.

The mean sorption distribution coefficients (K_d) varied considerably for the four soils. For the Three Bridges silt loam, K_d was 17.79 (±7.60). The K_d of Riverside loam was 8.34 (±1.56), for Lufkin sandy

loam, K_d was 51.02 (±12.09) and for Iowa clay loam, K_d was 293.96 (±67.62). This variation in distribution coefficient did not seem to correlate with any single parameter in Table I. K_d did not correlate with organic matter concentration as demonstrated by the large range of K_{om} and K_{oc} values. If organic carbon were a single source of test chemical binding, then K_{om} and K_{oc} would be expected to be the same for all soils.

The Freundlich binding parameters were determined from least squares linear regression analysis of the data to the equation:

$$\log (x/m) = \log (k) + 1/n \log (c)$$

which is derived from the Freundlich binding equation:

$$(x/m) = kc^{1/n}$$

In both equations, x is the amount of adsorbate per mass, m, of adsorbent (soil). C is the solution concentration at equilibrium and k and n are constants. When n = 1, the Freundlich constant k equals the distribution constant

K_d. Since the log (x/m) equation is in the form of a linear equation, the slope of the line is equal to 1/n, or n equals 1/slope. The intercept is equal to log (k), or k equals ten to the (intercept) power. Table III contains a summary of the isotherm constant k (Freundlich constant), n, K_d, K_{om}, and K_{oc} for each soil. Reasonable agreement was observed between Freundlich (k) values and K_d values, with the greatest divergences in their values where n was farthest away from unity. There was also no correlation between Freundlich constant (k) values and any of the test soil parameters in Table II.

Mass balance, or accountability of the amount of material recovered throughout the experiment was determined using the data from the sorption portion of the advanced test. Amounts in solution and in soil were calculated by multiplying the solution volume or soil mass by the concentrations of solution or soil per unit measurement. The total amount (µg) was the sum of the solution and soil amounts. The theoretical amount per sample was equal to 7.5 ml times the concentration of each test chemical solution, and % accountability was the total divided by the theoretical total times 100. Examination of the data indicated there was a trend toward lower accountability at the higher concentrations. The reason for this is not known.

TABLE III

SUMMARY OF SOIL BINDING PARAMETERS FOR EFROTOMYCIN A_1

PARAMETER	SOILS			
	THREE BRIDGES, NEW JERSEY	RIVERSIDE, CALIFORNIA	LUFKIN, TEXAS	NEWTON, IOWA
Sorption				
Freundlich (k)	13.39	7.98	46.43	335.80
n	1.27	1.10	1.03	0.955
K_d	17.79	8.34	51.02	293.96
K_{om}	846.90	333.80	4638.50	6390.30
K_{oc}	1460.10	575.50	7996.80	11017.00
Desorption				
Freundlich (k)	24.02	12.52	86.16	513.90
n	1.30	1.03	1.04	0.981

The sorption to and desorption from soil of efrotomycin does not appear to be related to any single parameter, or simple combination of parameters for each soil presented in Table II. Although the soils with the lowest and highest % organic matter (sand and clay loam) demonstrated the least and most binding, respectively, the soil with the second most amount of binding had the second lowest % organic matter. The same is true for the cationic exchange capacity parameter. Also, the soil with the lowest pH exhibits the most binding, as expected from the ionization of efrotomycin above pH 6, but the other soils with similar pH values to each other exhibit a wide variety of binding characteristics.

It was discovered early in the experiments that at the lower concentrations (<25 ppm) a significant amount of efrotomycin binding to the glass tubes occurred. Since the preliminary and screening tests, and the advanced kinetics test were performed at or greater than 25 ppm, binding of the test chemical to glass was minimal (less than 5%), and no efforts were made to change the methodology to account for this binding. The isotherm experiments, however, required concentrations as much as

125 times lower than these experiments (~0.02 ppm). It was found necessary, therefore, to physically measure the amount of bound material on the soil rather than to assume that the remainder of the test chemical no longer in solution was all bound to soil. This was accomplished by oxidizer combustions of the soil samples. This binding to glass appears for the most part to be reversible, as evidenced by the amount of control sample that was desorbed in the isotherm determination tests.

The binding of efrotomycin to soil seems to be not completely reversible. A sample of each soil following the screening test was extracted with duplicate volumes of methanol, and these methanolic washes were pooled and counted. Only 16 to 20% of the amount of test chemical still bound after two desorptions was extracted off of the soil by methanol. Similar results were obtained when the soils were extracted with ethyl acetate, which is a very good solvent for efrotomycin in the experimental pH range.

HPLC radioactivity profiles from the preliminary test demonstrated that the test chemical was not degraded by

assay methodology, since the great majority of the radioactivity eluted under the UV peak for efrotomycin A_1.

The mobility of a compound in soil is commonly related to the K_{oc} for that compound. By this method, efrotomycin is highly immobile in the Newton, IA and Lufkin, TX soils. Although the K_{oc}'s of efrotomycin in the Three Bridges, NJ and Riverside, CA soils are considerably lower than the other two soils studied, the values are still an order of magnitude higher than values associated with mobile compounds.(6) The mobility of efrotomycin in most soils is expected to be low due to the high amount of binding.

c) Octanol-water partition coefficient

The distribution coefficients for the major isomer of efrotomycin (A_1) were determined in pre-saturated 1-octanol and aqueous buffers of pH 5, 7 and 9 at 25° in the dark at 1 and 24 hr.

Chemicals and solvents were certified, reagent or HPLC grade. In-house deionized water was further filtered through activated carbon and mixed-ion exchange resin in a

commercial ultrapurification system. To pre-saturate the solvents, one hundred milliliters of deionized water was added to one hundred milliliters of 1-octanol, one hundred milliliters of deionized water was added to one hundred milliliters of ethyl acetate, and one hundred milliliters of 1-octanol was added to two hundred milliliters each of pH 5, pH 7 and pH 9 buffers. The reagents were mixed in separatory funnels by shaking, and the layers were separated after standing and settling.

The efrotomycin A_1 stock solution was prepared by diluting 0.4 ml of efrotomycin, 2.31 µCi/ml (206 µg/ml), to 10 ml with methanol. Duplicate one ml aliquots of the stock solution were taken and combined with 15 ml of scintillation cocktail for scintillation counting for 20 minutes.

One ml aliquots of the efrotomycin A_1 stock solution were pipetted into seven, 15 ml test tubes and evaporated to dryness with nitrogen. Ten ml of pre-saturated deionized water was added to tube #1. The tube was wrapped in foil and left to stand at room temperature for one week.

To tubes #2 through #7, 5 ml of pre-saturated 1-octanol plus 5 ml of a pre-saturated buffer solution were added. To two additional tubes, #8 and #9, 5 ml of pre-saturated 1-octanol and potassium phosphate buffer pH 7 was added. These tubes were control 1 hr and 24 hr samples.

Tubes #2-9 were agitated with a vortex mixer for about 3 minutes each. Tubes #2, 4, 6 and 8 were placed in a 25° shaker water bath for 1 hour. Tubes #3, 5, 7 and 9 were placed in a 25° shaker water bath for 24 hours.

After the equilibration periods, the tubes were centrifuged for 5 minutes to separate the layers. The octanol layers were drawn off. The remaining buffer layer was re-centrifuged to separate any residual 1-octanol. One ml aliquots were taken from both the 1-octanol and aqueous buffer layers and combined with 15 ml of cocktail for scintillation counting for 10 minutes. The control tubes #8 and #9 were handled by the same procedures.

Duplicate 1 ml aliquots were taken from the efrotomycin A_1 stock solution to determine the decompositions per minute (DPMs) in the starting material. The average net

DPMs was 229,577. One ml aliquots were taken from control tubes #8 and #9, and used as backgrounds for scintillation counting. One ml aliquots were taken in duplicate from tubes #1 through #7. All aliquots were combined with 15 ml of cocktail and counted for 10 minutes in a scintillation counter.

The DPMs for the duplicate 1 ml aliquots of 1-octanol and buffer layers were averaged. The distribution coefficients (K_D) were calculated by dividing the average DPMs/ml in the 1-octanol layers by the average DPMs/ml in the buffer layers. The total radioactivity recovered for each equilibration was determined by multiplying the average DPM/ml values by 5 ml, then summing the DPMs in the 1-octanol and buffer layers. The percent radioactivity recovered was calculated by dividing the total radioactivity recovered by the starting radioactivity and multiplying by 100.

The octanol/water distribution coefficients for efrotomycin at pH 5, 7 and 9 were 133, 57 and 0.037 at 1 hour and 111, 60 and 0.029 at 24 hours, respectively. The agreement in the values at 1 and 24 hours at each pH

indicates that equilibration was complete by 1 hour. The values reflect the ionization of the phenolic proton above pH 6. Quantitative recovery was realized for all samples.

The remaining aqueous layers were extracted twice with two, 3 ml portions of pre-saturated ethyl acetate. The extracts were evaporated under nitrogen and reconstituted in 100 µl HPLC run solvent. A 20 µl portion of each was taken for scintillation counting and a 20 µl portion of each was chromatographed. The eluates were collected for scintillation counting. Because of the low radioactivity in the pH 5 and 7 aqueous layers, insufficient radioactivity was extracted to verify the purity of the A_1-isomer by HPLC. Ethyl acetate was also not successful in extracting sufficient radioactivity from the pH 9 aqueous layers, because of the ionization of the A_1-isomer at that pH. However, based upon the consistency of K_D values at 1 and 24 hr, and from aqueous stability data for the A_1-isomer, no degradation of the A_1-isomer should have occurred and the K_D values should be valid.

The low values of these octanol/water distribution coefficients, 122 at pH 5, 59 at pH 7, and 0.033 at pH 9, (means of 1- and 24-hour values), indicate efrotomycin is not expected to bioconcentrate in aquatic nor terrestrial organisms.

d) Photodegradation

The stability of efrotomycin A_1-isomer was determined in direct sunlight under sterile conditions in buffered solutions at pH 5, 7 and 9 in Rahway, NJ on June 19, 1984.

All chemicals and solvents were reagent or HPLC grade. In-house deionized water was further filtered through activated carbon and mixed-ion exchange resin in a commercial ultrapurification system.

All buffer concentrations were 0.05 M. All buffers were sterilized by filtering through 0.20 micron filers. The carbon-14 labeled efrotomycin A_1 stock solution was 0.206 mg/ml methanol and 2.35 microCurie/ml.

All glassware used in the experiment was sterilized by autoclaving to exclude microbial contamination. Aliquots

of efrotomycin A_1 stock solution, 0.4 ml each, were added to three 10 ml sterilized volumetric flasks, and evaporated to dryness under a stream of nitrogen which was passed through a 0.20 micron filter. To each flask, 10.0 ml of pH 5, 7 or 9 buffer was added and the efrotomycin dissolved. Duplicate 1.0 ml aliquots of each were taken for scintillation counting. Seven, 1.0 ml aliquots of each solution were aseptically transferred to sterilized centrifuge tubes and stoppered.

Two samples at each pH were assayed at 0 time, and one sample at each pH was securely wrapped in foil to exclude light. The remaining samples were placed in direct sunlight at 3:38 p.m. June 19, 1984 in the afternoon in Rahway, NJ (42 degrees N. latitude). One sample at each pH was taken for assay at 0.25, 0.50, 0.75, and 1.0 hr. The foil wrapped sample was also taken for assay at 1.0 hr. Rather than determine each sample temperature, a -20 to 107°C thermometer was used to measure the temperature at each sampling time in a stoppered tube containing 1.0 ml distilled water. The amount of incident solar radiation was measured at each time.

At assay times, the temperature and solar radiation were recorded. One-tenth ml of unlabeled efrotomycin, 3.29 mg/ml in methanol (cold carrier) was added to each sample at assay time, and the samples were mixed. Each sample was extracted twice with equal volumes of ethyl acetate. The two extracts of each sample were pooled, the volume recorded, and 0.1 ml aliquots were taken for scintillation counting to determine the extraction recovery. The remainder of each extract was evaporated to dryness under nitrogen and the residues were dissolved in 0.1 ml of 70% methanol/water. High Performance Liquid Chromatography (HPLC) was used to isolate the efrotomycin A_1-isomer for ultraviolet (UV) absorbance analysis and Liquid Scintillation Counting (LSC) for specific activity determination.

It was observed that only 7-19% of the radioactivity was extracted from the pH 9 samples. These samples were re-extracted with duplicate ethyl acetate volumes after the addition of 2.0 ml of the pH 5.0 buffer. The ethyl acetate from each sample was pooled with the previous extracts, and 0.1 ml was taken for LSC. The remainder of the extract was evaporated under nitrogen and analyzed by HPLC.

Five, 1.0 ml fractions were collected from the HPLC eluate for each sample at the expected elution time for efrotomycin A_1. The UV absorbances of the fractions were measured from 500 nm to 195 nm, and the optical densities at 233 nm and 320 nm were recorded for the two fractions in each sample which contained the greatest UV absorbance. The optical density at 320 nm was used to calculate the solution concentration for RIDA determinations.

Six-tenths ml aliquots of the two fractions from each sample that had the greatest UV absorbance were taken for scintillation counting and placed in plastic mini-vials with 5.0 ml scintillation cocktail.

The extractability of radioactivity from the aqueous layer into ethyl acetate decreased with time at pH 5, indicating that there was less non-polar (extractable) material. At pH 7 the decrease in radioactivity was less pronounced. At pH 9 the recovery was very low, due to the deprotonation of the phenolic group at the C_4 position in the A_1-isomer at this pH, making the molecule much more polar. The pH of the aqueous phase of these samples

was adjusted to approximately 6.9 with two volumes (2 ml) of the pH 5 buffer and re-extracted with two volumes of ethyl acetate as before. The total recovery data for both extraction steps at pH 9 ranged from 59-83%. The recovery of radioactivity from the adjusted pH 9 samples was lower throughout the experiment than for the other samples. This was not investigated, but may be due to slow protonation when the pH of a solution of efrotomycin is decreased. It was also noted that when the ethyl acetate extracts of the neutralized pH 9 samples were evaporated, the solid material that resulted was more intensely yellow than the other samples. Upon reconstitution with 70% methanol/water, the color disappeared within a few seconds, and the solutions resembled the other samples. Neither the color intensity nor the lower recover had any effect on the reverse isotope dilution assay percent (RIDA%) since changes should have affected labeled and unlabeled species in the same manner once the cold carrier was added.

RIDA percents were determined by:

RIDA % = (S.A./Theor. S.A.) x 100.

The specific activity (S.A.) of each fraction was calculated using:

$$\text{Specific Activity} = \frac{\text{Radioactivity (DPM/ml)}}{\text{Concentration } (\mu g/ml)}$$

where the radioactivity was determined by LSC, and concentration in µg/ml was determined by UV absorbance. Theoretical specific activity (Theor. S.A.) was the expected specific activity of the test sample plus cold carrier, and was calculated by:

$$\text{Theor. S.A.} = \frac{\text{Radioactivity Added}}{\text{Total mass efrotomycin } A_1}$$

where the radioactivity was determined by scintillation counting.

The total mass of efrotomycin A_1 was the amount of cold carrier added plus the mass contributed by the labeled material (i.e.: radioactivity added divided by the labeled stock solution specific activity). Ideally, the contribution to the total mass of efrotomycin by the radioactive species present is negligible. The contribution in this experiment was approximately 8 micrograms, while the added unlabeled species was 329 micrograms, or the labeled contribution was 2.4% of the total mass. Since decomposition was less than 10 to 20%

for most sample points in this experiment, 337 micrograms was taken to be the total mass of efrotomycin in the calculations. For samples at pH 5 and 7, where decomposition progressed significantly, the mass of efrotomycin approached 329 micrograms, but this small correction was not included in calculations.

Regression analysis was performed on the data from each pH by a least squares regression of the logarithm of RIDA % versus time. The half-life ($t_{1/2}$) of decomposition in sunlight at pH 5 was 0.25 hr. At pH 7, the half-life was 1.54 hr and at pH 9, 2.52 hr. The rate constants for photolysis of efrotomycin at pH 5, 7 and 9 are 2.81 hr^{-1}, 0.451 hr^{-1} and 0.275 hr^{-1}, respectively. Samples maintained outside in the dark showed little or no loss of test chemical.

The nature of the photolysis products was not determined, however, an HPLC radioactivity profile at pH 5 at 1.0 hr showed that the majority of the radioactivity eluted at about 20 minutes. This retention time did not correspond to any previously identified efrotomycin isomers. Peaks due to efrotomycin A_1 and the two major B-isomers eluted

at 10.18 minutes, 26.15 minutes, and 28.67 minutes, respectively. The photolysis products have been determined to be less toxic than efrotomycin A_1 by an assay using Daphnia magna. (See Section 8a, Toxicity of Efrotomycin A_1-Isomer and its photoirradiation products to Daphnia Magna

The greater stability of efrotomycin at pH 7 and 9 is consistent with a reported pk_a of 6 for the phenolic proton of the A_1-isomer. Ionization of this functionality has an observable effect on its rate of sunlight photolysis. Despite the relative stabilities of the higher pH samples, essentially complete sunlight decomposition (93.75%) would be expected after ten hours (4 half-lifes at pH 9) of sunlight exposure. Efrotomycin would not be expected to readily transfer into nor accumulate in the aquatic environment as the intact parent compound, based on its rapid photolysis.

e) Biodegradation

The biodegradation potential of efrotomycin in soils was investigated by the carbon dioxide evolution method.

The amounts of ^{14}C-carbon dioxide (CO_2) and ^{14}C volatiles released upon biodegradation of carbon-14 labeled efrotomycin A_1 and ^{14}C-glucose in soils, incubated aerobically in the dark, were determined for a period of 98 days. The test system was a 250 ml Erlenmeyer flask, containing 50.0 g dry weight of soil, attached to a series of glass scintillation vials. Effluent air from each flask was vacuum purged through vials containing appropriate scintillation cocktail for trapping the radiolabeled degradation products. Quantitation of trapped radioactivity was performed by liquid scintillation counting.

A total of 20 incubation flasks were used to test the biodegradation of ^{14}C-efrotomycin A_1 (20.1 ppm), ^{14}C-efrotomycin A_1 (20.1 ppm with 100 mg glucose), and ^{14}C-glucose (25.2 ppm with 100 mg glucose), each in two soil types in triplicate. A single incubation flask, containing untreated soil, was used for each soil type to measure background radioactivity independent of test or reference chemicals. The use of a glucose amendment provided the soil microbial population with optimal nutrient supply for a viable culture.

A solution of ^{14}C-efrotomycin A_1 in water, a total of 60.0 ml containing 30.1 mg ^{14}C-efrotomycin A_1 with a specific activity of 0.944 µCi/mg in three bottles of 20.0 ml each, and a solution of uniformly labeled ^{14}C-glucose in water, 44.0 ml containing 55.44 mg ^{14}C-glucose with a specific activity of 0.78 µCi/mg, were prepared. The test chemicals were stored in the freezer until used for the experiments. The efrotomycin was labeled via fermentation with a single carbon-14 label at position 7. The glucose was purchased.

Soils for this study were a sandy loam soil (Freehold, New Jersey), 1.2 kg, and a clay loam soil (Watchung, New Jersey), 1.5 kg. Soil characteristics were analyzed by Rutgers University Soils and Crops Department. The sandy loam soil had a pH of 6.1, an organic matter percent of 0.72, a field capacity of 42.13%, a cation exchange capacity of 7.2 meq/100 g and a texture of 62% sand, 18% silt and 20% clay. The clay loam soil had a pH of 5.0, an organic matter percent of 1.44, a field capacity of 75.91%, a cation exchange capacity of 17.4 meq/100 g and a texture of 32% sand, 40% silt and 28% clay.

The field capacity for the soils was determined by submerging a portion of soil in water, draining off the excess water, and drying the soil. Weights of soil prior to and after drying were used to calculate the percent field capacity on a dry weight basis.

The dose of test or reference compounds was dissolved in sufficient distilled deionized water to moisturize the soils to 73.6% (sandy loam) or 80.4% (clay loam) of field capacity.

The amount of solution, containing water and test compound (1.0 mg ^{14}C-efrotomycin A_1) or reference compound (1.26 mg ^{14}C-glucose with 100 mg glucose) required to re-moisturize 50 g portions of soil was placed in the bottom of 250 ml Erlenmeyer flasks. Three incubation flasks for each soil type contained ^{14}C-efrotomycin A_1, (1.0 mg), ^{14}C-efrotomycin, (1.0 mg with a 100 mg glucose amendment), or ^{14}C-glucose, (1.26 mg with 100 mg glucose). The sandy loam or clay loam soil, 50.0 g, (dry weight) was then added to each flask and gently shaken to ensure the even distribution of the solution in the soil. Each flask was then covered with aluminum foil. Prior to connecting the outlet port to the trapping system and

sealing the inlet port, an initial weight of the test vessel was recorded. A single incubation flask containing untreated soil, re-moisturized with distilled deionized water, served as a control for each soil type.

Triplicate 0.1 ml aliquots of the ^{14}C-efrotomycin A_1 and ^{14}C-glucose solutions were quantitated by liquid scintillation counting and served as a dose check.

The test system consisted of a 250 ml glass Erlenmeyer flask with a screw cap fitted with inlet and outlet ports for air exchange connected to a series of traps to capture evolved radioactivity. Each flask was covered with aluminum foil to maintain the soil in the dark.

Effluent air from each flask was passed through the vials which served as traps. The first vial was a backflow trap. The second and third vials contained a universal scintillation cocktail and served to trap any volatile test chemical or volatile test chemical biodegradation products other than carbon dioxide. The fourth vial was another backflow trap. The fifth and sixth vials contained a carbon dioxide trapping scintillation

cocktail. The radioactivity in all the trapping vials with scintillation fluid was counted in order to account for all radioactivity originally added to each flask. For the reference chemical and the untreated soil samples, the same series of 6 vials was used.

Each incubation flask was flushed daily by vacuum purging. The traps were replaced and counted by liquid scintillation counting daily for the first 8 days and at 2-10 day intervals thereafter. Flasks were re-weighed periodically to detect moisture loss from the soil. Moisture was replenished as necessary with distilled deionized water. The test system was maintained at 22-26°C. At the end of the study, soil from each flask was combusted in triplicate to assay for radioactivity.

At each sampling interval, the radioactivity evolved from the soil was measured by liquid scintillation counting of the trap and its entire contents. The disintegrations per minute (DPM) of radioactivity in each trap from a treated test vessel was corrected for background by subtracting the average of the DPM found in the two analogous traps for the untreated soil of the same type. A cumulative total DPM was determined for each treated test vessel by

adding the corrected DPM from the two volatiles or CO_2 traps to the cumulative totals for the vessel from the previous interval. Results for the three replicate test vessels representing one soil type and one chemical treatment type were averaged at each interval to obtain the mean cumulative CO_2 or volatiles evolved to that date. Both the mean and standard deviations for the cumulative data were determined at 98 days. Each mean cumulative DPM value for CO_2 and volatiles was calculated as a percent of dose by dividing the cumulative DPM by the DPM originally put into that vessel.

Radioactivity mass balance was calculated for each incubated soil sample by adding the cumulative volatile DPM, the cumulative CO_2 DPM, and the activity remaining in the soil. Triplicate portions of the soil were combusted in an oxidizer and the resulting $^{14}CO_2$ was trapped for scintillation counting.

Scintillation counting was performed for 2 or 5 minutes each sample. Efficiency was computed for each sample using external channel ratio values and curve fitting to previously established quench sample data or factory standard quench sample data.

The mean cumulative $^{14}CO_2$ evolved in soils dosed with ^{14}C-efrotomycin A_1 was 6.3 and 19.3% for sandy loam and clay loam soils, respectively, based on the initial addition of 2,271,835 DPM. A moderate amount of degradation occurred, and an estimate of time required for 50% degradation of ^{14}C-efrotomycin A_1 could not be made. Material balance at the end of the experiment ranged from 65.3 to 82.6% (mean of 71.2%) for sandy loam and from 68.6 to 78.1% (mean of 74.7%) of the total ^{14}C-efrotomycin applied.

The mean cumulative CO_2 evolved in soils dosed with ^{14}C-efrotomycin A_1 with 100 mg glucose was 5.9 and 23.9% for sandy loam and clay loam soils, respectively, based on the initial addition of 2,271,835 DPM. A minimal amount of degradation occurred, and an estimate of time required for 50% degradation of ^{14}C-efrotomycin A_1 could not be made. Material balance at the end of the experiment ranged from 80.2 to 89.6% (mean of 83.7%) for sandy loam and from 81.2 to 98.2% (mean of 89.8%) of the total ^{14}C-efrotomycin A_1 applied.

The mean cumulative $^{14}CO_2$ evolved in soils dosed with ^{14}C-glucose with 100 mg glucose was 45.4 and 41.3% for sandy loam and clay loam soils, respectively, based on the initial addition of 2,287,511 DPM. A significant portion of the evolved $^{14}CO_2$ occurred in 7 days with an apparent plateau reached in both soil types by 10 days. Individual replicates of the sandy loam soil evolved $^{14}CO_2$ at 36.6, 37.4, and 62.1% of the initial dose after 98 days. Individual replicates of the clay loam soil evolved $^{14}CO_2$ at 38.2, 42.2, and 43.5% of the initial dose after 98 days. These levels of evolved $^{14}CO_2$ indicate a viable microbial population in the soils. Material balance at the end of the experiment ranged from 52.1 to 86.6% (mean of 63.7%) for sandy loam and from 65.0 to 71.5% (mean of 68.8%) for clay loam of the total ^{14}C-glucose applied.

Based on the results obtained during the study, efrotomycin was found to degrade moderately in sandy loam soil (5.9% and 6.3%), and in clay loam soil (23.9% and 19.3%), unamended and amended with glucose respectively, during the 98-day period under laboratory conditions. No estimate of the time required for 50% degradation of efrotomycin could be made.

f) Vapor pressure

The vapor or sublimation pressure of efrotomycin has been determined by the Knudsen-effusion method. The measurements were carried out over the temperature range from about 150 to 170°C. The pressure ranged from 4×10^{-3} to 2×10^{-2} torr. The data were fitted to the integrated form of the Clausius-Clapeyron equation, assuming a constant heat of vaporization, to determine the vapor pressure at 25°C.

The pressure (P) of a gas (vapor) which is effusing through an orifice of an otherwise closed container can be calculated from the Knudsen-effusion expression as follows:

$$P = \frac{W}{t} \cdot \frac{1}{A} \cdot \sqrt{\frac{2\pi RT}{M}}$$

where W/t is the rate of weight loss (g/sec), A is the area of the orifice (cm^2), R is the gas constant (8.31×10^7 erges/°K-mole), M is the molecular weight and T is the temperature (°K).

A sample of efrotomycin A_1, approximately 15 mg, was hermetically sealed in a DuPont DSC sample cup. An orifice was made in the lid of the cup with a pointed needle. A photomicrograph of the sealed cup was obtained under incident light at 75X magnification. The sample cup

was then placed in an Omnitherm TGA module which was connected to a DuPont 1090 Thermal analyzer. The weight of the sample and cup were determined according to the manufacturer's suggested procedure. The TGA module was evacuated. The temperature was increased to about 130°C and the weight loss of the sample measured. Once the predetermined amount of solvent had been lost, the temperature was increased to about 160°C and the weight loss of the sample measured for a 10-minute period. The data were recorded and saved on the DuPont system and later transferred to a microcomputer for analysis. The temperature of the system was decreased to about 155°C and the system equilibrated at that temperature. The weight loss was measured for 10 minutes as before. The temperature was then increased to about 165°C and the weight loss measured for the same period. The temperature was increased once again to about 170°C for an additional weight loss measurement. The temperature of the system was then decreased to 154/155°C for the final weight loss measurement.

Similar experimental manipulations were carried out for benzoic acid. The temperature ranged from 40 to 60°C and only three temperatures were employed.

The Knudsen-effusion method has been used to measure the sublimation (vapor) pressure of solids with relatively low sublimation pressure. The application of the Knudsen-effusion method to efrotomycin requires overcoming some minor difficulties related to residual solvent adhering to the efrotomycin; efrotomycin retains residual solvents as determined by gas chromatography (GC) and titration (for t-butylamine). The GC determined solvents are lost below 100°C during a thermogravimetric (TG) weight loss determination. The t-butylamine is not as readily removed. The amine is apparently lost at about 130°C as measured by TG analysis. The temperature at which the amine is lost is dependent on the TG condition (vacuum or N_2 flow). Once the amine is removed, efrotomycin decomposes between 175 and 180°C. If the solvents are not removed, efrotomycin decomposes at lower temperatures (heating in a closed DSC cup results in decomposition at about 100°C). The solvent must be removed before the sublimation pressure measurements are made. Conveniently measured weight losses for efrotomycin are attainable in the 150 to 170°C range.

The data from the five isothermal weight loss measurements for efrotomycin were transferred to a microcomputer. A least-square analysis of the weight loss time data yielded the rate of weight loss as the slope of the least square fitted straight line for each temperature. The least square correlation coefficients were greater than 90% for each line and averaged 96% for the five lines. The average temperatures over each 10-minute weight loss measurement interval were calculated. These data pairs (W/t, T) were used to calculate the pressure from the Knudsen-effusion expression.

The weight loss-temperature data for benzoic acid were treated in a similar manner.

The log of the sublimation pressure (in torr) for efrotomycin versus the reciprocal of the absolute temperature data were fitted by least square analysis to an integrated (straight line) form of the Clausius-Clapeyron equation.

$$\log P = A - {}^{B}/_{T}$$

where B is the slope and is related to the heat of sublimation as follows:

$$\Delta H_s = 2.303\ R \cdot B$$

and A is the intercept and is related to the entropy of sublimation as follows:

$$\Delta S_s = 2.303\ R \cdot (A-2.881)$$

The values of A and B for efrotomycin from the least squares analysis were found to be 19.99 and 9954, respectively. These correspond to a ΔH_s of 45.6 kcal/mole and ΔS_s of 78.3 cal/°K-mole. The correlation coefficient for the least square analysis of the log P versus 1/T data was 95%. The 95% correlation and the technique of increasing and decreasing the temperature during the weight loss data collection indicates that the system was at equilibrium during the sublimation pressure measurements.

The extrapolated vapor pressure at 25°C was found to be 4 X 10^{-14} torr, that is:

$$\log P = 19.99 - (9954/298°K) = -13.41$$

$$P = 4 \times 10^{-14} \text{ torr.}$$

The data for benzoic acid was reduced in a like manner, and the calculated heat of sublimation was 19.8 kcal/mole which is 9% lower than the literature value of 21.8

kcal/mole. The agreement between the measured and literature values for benzoic acid indicates the accuracy and precision of the method.

g. Environmental fate

Based upon the laboratory experiments, the fate of efrotomycin in the environment can be predicted.

Because of its low volatility, efrotomycin will not significantly partition into the atmosphere. Degradation processes in the atmosphere are therefore not proposed.

Based upon the low values of K_{OW} at pH 5, 7, and 9, and its ionization above pH 6, efrotomycin would be expected to leach out of swine feces and into the freshwater, estuarine and marine exosystems. Efrotomycin, while having good stability in solution, would be extremely susceptible to photodegradation in that environment studies demonstrate a maximum half-life of ~2 1/2 hours under laboratory conditions.

Because of the low K_{OW} values, efrotomycin would not be expected to bioaccumulate in aquatic organisms. Efrotomy-

cin would be sorbed out of water by most soils. Degradation of efrotomycin by soil bacteria would occur, though slowly. It may not be readily available for biodegradation because of its strong binding to soil.

8. Environmental effects of released substances

The following laboratory studies were conducted to determine the potential effects of efrotomycin released into the environment.

a) Toxicity of efrotomycin A_1 isomer and its photoirradiation products to Daphnia magna

The toxicities of efrotomycin A_1 and its photoirradiation products were determined using Daphnia magna. The photoirradiated efrotomycin was prepared by dissolving a sample of efrotomycin enriched in the A_1-isomer in well water at 96 ppm. The solution was exposed to sunlight for a cumulative total of 57 hours, reducing the amount of the A_1-isomer to about 3% as determined by HPLC.

The efrotomycin used in this study had a weight percent of 82.7% of the A_1-isomer and HPLC area percents of 88.8% A_1, 1.2% B, and 2.9% A_2 isomers.

Photoproducts were prepared by dissolving 202.4 mg of efrotomycin in 2.0 liter well water. This sample had a pH of 8.2 and the well water had a pH of 8.1, both within the seasonal variation reported for the well water so no pH adjustments were made to the solution. The concentration of the efrotomycin solution, determined by its UV absorbance, was 95.9 ppm in efrotomycin.

The solution for photoirradiation in a 2.0 liter Erlenmeyer flask was covered with a watch glass and set outdoors at the Merck site in Rahway, NJ at 9:55 a.m. on August 23, 1985. The weather on August 23, 1985 was clear, with temperatures rising from mid-60's (°F) to low 80's by afternoon.

A zero-time sample of about 0.1 ml was removed before setting the sample outside. Twenty-five microliters of the zero-time sample was chromatographed on a reversed-phase HPLC system using 70% methanol and 30% 0.15% phosphate buffer, pH 6 at 1 ml/min. All samples were chromatographed using these conditions except the percent of methanol was decreased to 69% on warm days to try to keep the retention time of the A_1-isomer at 9 to

10 minutes. The A_1-isomer eluted at 9.4 minutes and only two small peaks eluted ahead of it. The injection solvent peak produced a characteristic disturbance at the column void volume at about three minutes and a 100% methanol wash of the column appeared on the chromatograph between 35 and 45 minutes.

A sample was taken at 1:45 p.m. (3 hr. 45 min. exposure) and 25 µl was chromatographed. There had been little decomposition of the A_1-isomer, presumably because of the high concentration of the material and due to the high pH, where efrotomycin is most stable. The sample was left out for a total of 7 hr. 25 min., then refrigerated. Exposure was continued on sunny days with periodic chromatographs to check the extent of photodegradation. The sample was refrigerated overnight and on cloudy days. On September 4, 1985, an aliquot taken at 56 hr. 34 min. indicated the A_1-isomer was almost totally converted to photoproducts, so the sample was brought indoors after 57 hours cumulative exposure.

A second injection of the 56 hr. 34 min. aliquot was made. Injecting 50 µl instead of 25 and using a

four-fold greater UV detection sensitivity, the A_1-isomer was observable eluting at 11 to 13 minutes. The retention time was verified using undegraded efrotomycin A_1. To determine the amount of A_1-isomer left, the chromatogram was photocopied and divided into two regions, the absorbances from the A_1-isomer and from all other materials, excluding the solvent peak absorbance. The regions were then cut out and weighed. A_1 component was estimated to be about 3% of the total by this method. A photocopy of the chromatogram was also divided into regions by dropping perpendiculars at the start and finish of the A_1-isomer UV peak, again excluding any solvent peak contributions. By this method, it was estimated that about 38% of the material was more polar than the A_1-isomer, about 13% was A_1 and material which eluted in the region of the A_1 peak, and about 50% was less polar material. There were no single photodegradation products, but rather a mixture of compounds both more polar and less polar than the starting compound.

The acute toxicity of the starting efrotomycin and of the photodegradation mixture to Daphnia magna were deter-

mined. The procedures for static bioassay, as described in Methods of Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians[7] and Standard Methods for Examination of Water and Wastewater[8], were used in this experiment. The adult Daphnia were fed algae (Selenastrum capricornutum) at least every three days prior to testing and supplemented with a suspension of Tetramin®/cerophyl. The daphnids were identified to species using the taxonomic key presented by Pennak[9].

The static Daphnia bioassay was conducted in 250-ml glass beakers containing 200 ml of aged well water with acceptable chemical characteristics. These vessels were kept at 20 (±2.0) °C in a temperature controlled area. The lighting was maintained at 50-70 foot candles on a 16-hour daylight photoperiod, with 30 minute simulated dawn and dusk periods.

For efrotomycin A_1, an initial range-finding experiment was conducted using 10 Daphnia each in exposure concentrations of .01, 0.1, 1.0, and 10 mg/l. A fifth preliminary test concentration of 100 mg/l was added to more accurately determine the range for definitive concentrations. Test concentrations were prepared based

on total compound. -Deionized water was used in the preparation of all working stock solutions. The results of the preliminary test were that efrotomycin at 48 hours caused no deaths or abnormal behavior at 0.1 to 10 ppm, but caused 30% mortality at 100 ppm.

From this information, five concentrations in duplicate of the test compound with 10 Daphnia (first instar less than 24-hours old) per beaker were selected for the definitive bioassay. These concentrations were a geometric series ranging from 15 to 250 mg/l and included a control. All concentrations were observed once every 24 hours for mortality and abnormal effects such as surfacing, clumping of the daphnids together and daphnids lying on the bottom of test chambers. The results of the 48-hour definitive test were that efrotomycin caused no mortalities or abnormal behavior at 15 or 31 ppm, caused abnormal behavior (surfacing) in 5% of the daphnids at 62 ppm, caused 35% mortality at 125 ppm and caused 70% mortality at 250 ppm. Abnormal behavior was noted in one of the surviving daphnids at 125 ppm (out of 13 survivors) and in 5 out of 6 survivors at 250 ppm.

The 24- and 48-hour LC_{50} values and corresponding 95-percent confidence limits were determined by an LC_{50} computer program developed by Stephen, et al.[10] This program calculated the LC_{50} statistic and its 95-percent confidence limits using the binomial, moving average angle and probit methods because no one method is appropriate for all possible sets of data. The method of calculation selected was that which gave the narrowest confidence limits for each separate analysis.

The 24- and 48-hour LC_{50} values for efrotomycin A_1 were >250 and 180 ppm respectively. Ninety-five percent confidence limits could not be calculated for the 24-hour data. The LC_{50} and 95% confidence limits (140-230 ppm) for the 48-hour data were calculated by the probit method. All results were based on the nominal concentrations of 15, 31, 62, 125, and 250 mg/l. The no-effect concentration, based on the lack of mortality and abnormal effects, was 31 mg/l after 48 hours. The abnormal effects of mortality, surfacing and erratic movement were observed in the 62, 125, and 250 mg/l test concentrations. A yellow color increasing with increasing concentrations was observed in all test concentrations.

The dissolved oxygen concentrations ranged between 8.1 and 8.6 mg/l. These values represented 88 and 93 percent saturation at 20°C, respectively, and were considered adequate for testing.[7] The pH values of the treated chambers were consistent with the control and ranged from 8.6 to 8.7.

For photoirradiated efrotomycin A_1, an initial range-finding experiment was conducted using 10 Daphnia each in exposure concentrations of 0.959, 9.59, and 95.9 mg/l. Test concentrations were prepared based on total compound. Aged well water was used as dilution water for all test concentrations. The results of the preliminary test were a lack of mortalities or abnormal behavior at the tested concentrations at both 24 and 48 hours.

From this information, and because 96 ppm was the concentration of the undiluted photoirradiated solution, 5 concentrations in duplicate of the test compound with 10 Daphnia (first instar less than 24-hours old) per beaker were selected for the definitive bioassay. These concentrations were a geometric series ranging from 6 to 96 mg/l and included a control. All concentrations were

observed once every 24 hours for mortality and abnormal effects such as surfacing, clumping of the daphnids together, and daphnids lying on the bottom of test chambers.

The 24- and 48-hour LC_{50} values and corresponding 95-percent confidence limits were determined by an LC_{50} computer program in the same manner as for efrotomycin A_1. However, in the definitive test, there were no mortalities or abnormal behavior at any test concentration at 24 and 48 hours. Therefore, the 24- and 48-hour LC_{50} values for photoirradiated efrotomycin were both >96 mg/l. Ninety-five percent confidence limits could not be calculated. All results were based on the nominal concentrations of 6, 12, 24, 48 and 96 mg/l. The no-effect concentration, based on the lack of mortality and abnormal effects, was 96 mg/l after 48 hours.

The dissolved oxygen concentrations ranged between 8.4 and 8.6 mg/l. These values represented 91 and 93 percent saturation at 20°C, respectively, and were considered adequate for testing[7]. The pH values of the treated chambers were consistent with the control and ranged from 8.5 to 8.6.

The 48-hour LC_{50} (95% confidence interval) for efrotomycin A_1 was 180 ppm (140 to 230 ppm) and the 48 hour no-observable-effect concentration (NOEC) was 31 ppm. There were no mortalities or sublethal effects in the diluted or undiluted solution of the photoirradiated efrotomycin, so the 48-hour NOEC was 96 ppm (the highest level tested). Comparison of the NOEC values indicated photoirradiation of efrotomycin led to a less toxic solution, relative to the starting material.

b) Evaluation of the effects of efrotomycin on the germination of four plant species

Seed germination tests were conducted in petri dishes in a regulated temperature cabinet. The effects of efrotomycin were measured by comparing the germination times of the treated seeds with the untreated seeds of each test species.

This study was conducted on four plant species: Pinto bean - Phaseolus vulgaris L., Rye grass - Lolium perenne L., Cucumber - Cucumis sativus L., and Corn - Zea mays L. None of the seeds had been treated with fungicides or insecticides.

The seed germination studies were conducted in an environmental chamber equipped with automatic day/night light and temperature controls.

The efrotomycin used was 74.9 wt.% A_1-isomer. Chromatographic analysis (HPLC) indicated the material was 80.84 area % A_1-isomer and 16.34 B-isomers, based upon materials absorbing UV light near 230 nm.

A preliminary (range-finding) seed germination test was conducted under both a 16-hour light/8-hour dark cycle as well as under total darkness because of suspected light sensitivity of the product. A single replicate was conducted under each light condition at 100, 10, 1, 0.1, and 0 ppm of the product.

Seeds were examined and obviously non-uniform and cracked seeds were discarded. An excess of each seed type was disinfected by rinsing in deionized water, soaking for 10 minutes in a 10% Clorox solution, re-rinsing, and finally soaking for 1 hour in deionized water. The beakers containing the drained seeds were covered to minimize moisture loss until the seeds were placed in the petri

dishes. The soaked seeds were spread on wet paper toweling and indiscriminately chosen to fill each petri dish previously prepared with two filter papers. Each dish was filled with 50 seeds and labeled as to seed type, test concentration, and replicate number. The dishes were marked on the edge with a Sharpie● pen.

The product was formulated by weighing 30 mg of efrotomycin and transferring it to a 300 ml Erlenmeyer flask containing 100 ml of deionized water. The product was brought to 300 ml and stirred magnetically on a stirplate. The product did not go into solution at this test concentration.

Acetone:deionized water (1:99) was tried as a mixed solvent system since considerable experience with it in plant treatment has shown no ill effects. Efrotomycin dissolved readily and the formulations were thereafter made with acetone:deionized water (1:99).

Twenty milligrams of efrotomycin was weighed, dissolved in 2 ml of acetone in a test tube, transferred to a 250 ml Erlenmeyer flask containing 100 ml of deionized water, and

brought to 200 ml with deionized water. The 100-ppm solution was sonicated for 10 minutes to ensure dispersion. Serial dilutions were made from the 100-ppm solution for the 10-, 1-, and 0.1-ppm solutions.

The treatments were accomplished by pipetting 6 ml of the test products in each petri dish. Six milliliters of acetone:deionized water (1:99) was used for the controls. The petri dishes containing treated and untreated (acetone control) seeds were placed in the incubator without randomization. The temperature was set at 30°C. Temperatures and humidities were recorded using a high/low thermometer and a humidity gauge. The 16-hour light/8-hour dark exposure group was placed on the top shelf. The dark-only exposure group was placed on the bottom shelf. The dark exposure was accomplished by covering the dishes on the lower shelf with panels of transite covered with black polyethylene sheeting.

Seed germination was recorded daily until test concentrations could be established for the definitive tests. Mold was a factor in terminating observations in some instances. The criterion for germination was a

sprout of at least 1/8 inch in length. The germinated seeds were removed when recorded.

The cumulative number of seeds germination and cumulative percent germination for the data from the preliminary test were calculated and analyzed statistically using the dependent t-test.

The dependent t-test is used when pairs of objects in two different groups are matched on the basis of one variable. In this case, seeds receiving the same treatment at each point in time were compared to see how they differed under the two light conditions. The number of measures in each group must equal to perform this test. Data entry was terminated when no values were available for one group.

The results of the dependent t-test showed no significant differences in the germination percents between the light/dark and dark only conditions except in the case of corn at 1 ppm and in the acetone control, in pinto bean at 100 and 0.1 ppm and in rye grass at 100 ppm. However, it was decided that insufficient data were available to come to a statistically valid conclusion.

The definitive tests were conducted at 100, 60, 36, and 21.6 ppm. The 100 ppm of efrotomycin was formulated with acetone:deionized water (1:99). The solution was sonicated for 10 minutes and serial dilutions were prepared. An acetone:deionized water (1:99) solution and a negative control (deionized water only) were included in the test series.

Since light appeared to have negligible effect on the preliminary test, the definitive seed germination tests were conducted in total darkness.

Seed disinfection was conducted in an identical manner as in the preliminary tests. The soaked seeds were divided into two equal parts and spread on wet paper toweling. Seeds were placed in each previously prepared petri dish containing two filter papers. Twenty-five indiscriminately chosen seeds from each of the two groups of seeds were placed in each petri dish for a total of 50 seeds/dish (replicate). The petri dishes were labeled with the seed type, test concentration, and replicate number.

The petri dishes containing the seeds were numbered in three series of 1-1 through 1-48. The treatments were assigned to the petri dishes using randomization tables. An incubator position diagram was prepared which consisted of 48 positions per shelf. There were 3 shelves and these positions were the same for each of the three shelves. A set of 48 random numbers was obtained for each shelf. The first random number representing one of the 48 treatment groups was placed in Position 1, the second in Position 2, etc., until all 48 numbers were used and all shelves were filled.

The treatment volume used was 6 ml for cucumbers and rye grass, and 8 ml for pinto beans and corn. The treatments were dispensed with a 10-ml graduated pipette.

The treated seeds were placed in the incubator using the described randomization table. The number of seeds germinated in each petri dish was recorded daily for corn, beans, and rye grass through 10 days or until 90% had germinated in the controls with the exception of rye grass which was terminated after 12 days. Cucumber germination was recorded at more frequent intervals due to the speed of germination of this seed type.

Temperatures and relative humidities were recorded daily as in the preliminary tests. The temperature was set at 30°C for the tests. The moisture level was maintained in the petri dishes by adding deionized water as needed during the germination observation.

Cumulative numbers of seeds germinated and cumulative percent seed germination were calculated.

The statistical methods used for the definitive data consisted of repeated measure analysis of variance (ANOVA) and Dunnett's many-on-one t-tests if the ANOVA was significant. In repeated-measure models, repeated measurements were made of the same variable (cumulative % seed growth) for each case at different times under different conditions (treatment levels). A statistical software program performed an ANOVA on repeated measure designs by distinguishing between a factor that classifies the cases into groups (treatment) and a factor for which each subject is measured at all levels (time). A calculation of significance was made for each, as well as the interaction between the two.

The number of measures in each group must be equal to perform the ANOVA tests. Data entry was terminated when no values were available for one group; except in the case of cucumber. Cucumber was analyzed only through 48 hours. This should not effect the conclusions from the data.

The two control groups (negative and acetone) were evaluated statistically to determine the effect of the cosolvent, acetone (1:99, v/v), on germination. The Chi-square test was used to test the differences in the frequencies of germination of seeds grown in the presence (acetone control) and the absence (negative control) of the cosolvent acetone. The frequency of germination of corn seeds exposed to acetone (1:99, v/v) was significantly different from that of the negative control (Chi-square equals 18.273, $p < 0.001$). The ANOVA was performed on the negative and acetone controls. The other seed types were not influenced by acetone. The two controls for rye grass, cucumber and pinto beans were combined to obtain more power in the tests.

Comparison of Percent Seed Germination by ANOVA

Seed Type	Control	Among Groups (Concentrations)*	Within Repeated Measures (Days)	Interaction Between Groups and Repeated Measures (Days)**
Corn	Negative†	1.75 (4,25) p < 0.171	981.8 (2.50) p < 0.001	3.99 (8,50) p < 0.004††
Corn	Acetone#	0.91 (4,25) p < 0.472	782.2 (2,50) p < 0.001	0.98 (8,50) p < 0.449††
Pinto Beans	Combined##	0.87 (4,31) p < 0.493	514.1 (3,93) p < 0.001	1.06 (12,93) p < 0.401††
Rye Grass	Combined##	0.49 (4,31) p < 0.745	2,196.4 (9,279) p < 0.001	1.08 (36,279) p < 0.380††
Cucumber	Combined##	0.95 (4,31) p < 0.448	1,814.0 (3,93) p < 0.001	1.96 (12,93) p < 0.072††

* The F statistic, corresponding degrees of freedom, and p, probability of a greater F.

** Interaction between groups (concentrations of efrotomycin) and repeated measures (days)

† Seeds treated with deionized water without acetone as cosolvent as controls.

†† Probability values (p) of a larger value of F obtained by BMDP-2V are Greenhouse-Geisser estimates.

Seeds treated with deionized water with acetone as cosolvent (99:1, v/v) as controls.

Negative and acetone controls combined.

The mean percent cumulative germination of the four seed types did not appear to be affected by the concentrations of efrotomycin. The group's (concentrations of efrotomycin) contributions to the ANOVA were not significant. The contributions of the within factors (repeated measures) were found to be significant at greater than the 5% level. This result was expected because seeds germinated in a time-dependent manner. The interactions of the treatment groups and the repeated measures were not significant for pinto beans, rye grass, and cucumbers. However, the interaction for the negative control on corn germination was significant ($F = 3.99$, $d.f. = 8, 50$, $p < 0.004$). This result may be expected given that the efrotomycin concentrations were prepared with acetone and deionized water (1:99, v/v). In addition, a significant interaction was not observed with the acetone control ($F = 0.98$, $d.f. = 8, 50$, $p < 0.449$).

The results for the analysis of corn seed growth versus negative control for Day 1 (the only day in which a significant difference was detected between treatment groups and the control) were further broken down according to Dunnett's t-test:

Group (Efrotomycin Concentration)		Percent Cumulative Germination Mean	Standard Deviation	Median	Comparisons Dunnett's t-test Groups	t	d.f.	p[a]
1	Control[b]	52.2	6.3	52.0	1 vs 2	1.34	25	<0.488
2	21.6 ppm	44.7	12.4	45.0	1 vs 3	2.52	25	<0.060
3	36.0 ppm	38.0	9.1	41.0	1 vs 4	3.12	25	<0.016
4	60.0 ppm	34.7	11.8	34.0	1 vs 5	3.17	25	<0.014
5	100.0 ppm	34.3	7.6	32.0				

a Two-tailed test of significance.
b Negative control

The mean cumulative percent germination of the negative control was not significantly different than the 21.6- ($t = 1.34$, d.f. = 25, $p < 0.488$) and the 36.0-ppm ($t = 2.52$, d.f. = 25, $p < 0.060$) treatments, but was significantly greater than the 60.0- ($t = 3.12$, d.f. = 25 $p < 0.016$) and the 100-ppm ($t = 3.17$, d.f. = 25, $p < .014$) treatments.

Corn was the only seed which indicated a statistically significant difference in the means at some interval between the two controls. Further exploratory analysis indicated that this difference was at Day 1. As a result, Dunnett's many-on-one t-test indicated that 60- and 100-ppm treatments showed statistically significant lower percent cumulative germination compared to the negative control. No other day or seed showed any significant

difference between the two controls. All seeds indicated significantly higher cumulative germination uniformly among all treated groups indicating no significant treatment effects, except that for corn against the negative control on Day 1.

No seeds showed a visibly adverse effect in terms of phytotoxicity. Some mold and rotting occurred, but was not considered product oriented because it occurred in controls as well as in treated seeds.

The mean percent cumulative germination of corn, pinto beans, cucumbers, and rye grass did not appear to be affected by 100, 60, 36, and 21.6 ppm of efrotomycin in distilled water containing 1% acetone by volume. The data for all seeds except corn were analyzed versus the pooled control data (negative and acetone controls). Efrotomycin had no significant effects on the germination of corn when compared to the acetone control. However, when the corn data was compared to the negative control (distilled water), there was a statistically significant lower percent cumulative germination for the 100- and 60-ppm treatment levels on Day 1 only.

c) Evaluation of the effects of efrotomycin on the growth of four plant species

Seedling growth studies were conducted in a glass greenhouse in flats containing white quartz sand. The effects of efrotomycin were measured by comparing the heights of seedlings receiving nutrient solution containing efrotomycin with the heights of seedlings receiving nutrient alone. The terminal root lengths, dry shoot and root weights of the efrotomycin-treated and untreated seedlings were also compared.

The efrotomycin used was 74.9 wt.% A_1-isomer. Chromatographic analysis (HPLC) indicated the material was 80.84 area % A_1-isomer and 16.34 B-isomers, based upon materials absorbing UV light near 230 nm.

This study was conducted on four plant species: Pinto bean - Phaseolus vulgaris L., Ryegrass - Lolium perenne L., Cucumber - Cucumis sativus L., and Corn - Zea mays L. None of the seeds had been treated with fungicides or insecticides. Seedling growth tests were started by germinating seeds in an incubator under a light/dark cycle. The germinated seeds were acclimatized to the

greenhouse lighting for 48 hours before treatment. The seedlings were maintained in the greenhouse throughout the treatment period. A humidifying unit was integrated into the heating system.

Preliminary Tests

Preliminary tests were conducted to establish the test concentrations, the sampling times, and the tests duration for conducting the definitive tests. Unreplicated tests of five plants per concentration were used in the preliminary studies.

Excess seeds were germinated between wet paper towels to allow an indiscriminate choice of uniform-sized seedlings. The towels were rolled in waxed paper and placed in a beaker with 5 cm of deionized water. The beakers were placed in an incubator which was set at 30°C on a 16-hour light/8-hour dark cycle.

The germinated seeds were removed from the incubator after 3 days. Twenty-five uniform seedlings were laid out on paper marked off with 25 squares. The seedlings were then chosen indiscriminately and planted one in each flat until

all were planted. Immediately after planting, 400 ml of nutrient solution was added to each flat.

Efrotomycin was formulated in 2% acetone/98% nutrient solution at the 400-ppm level. Serial dilutions were made with nutrient solution to obtain the 40-, 4-, and 0.4-ppm levels.

The 400-ppm level precipitated out 4 hours after treatment. The upper level was reformulated at 100 ppm using 1% acetone/99% nutrient solution.

The two levels of acetone (1% and 2%) in the preliminary test had no effect on the decision on the levels of efrotomycin used in the definitive test.

The treatment-nutrient solutions were added to the seedling flats and the flats were indiscriminately placed in the growth area without regard to treatment. Control flats received nutrient solution without the test product, but with the 1% acetone cosolvent. The solutions were

added to the flats twice each week. However, for the 100-ppm level, the treatments were added on the same day as the planting.

The treatment-nutrient or nutrient-only solutions were added to plastic containers larger than the seedling flats. The flats containing the seedlings were placed in the test solution containers to obtain a bottom-watering system. Twice per week the flats were removed from the treatment solution containers. The treatment containers were rinsed, the flats were returned to the treatment containers, and 250 ml of fresh treatment solutions were added. Deionized water was used for any additional water requirements. This procedure standardized the treatment level for all flats.

Seedling shoot lengths were recorded at 1- to 3-day intervals throughout the seedling growth period of 21 days.

The average shoot growth was added to each subsequent time to determine cumulative growth for each plant species. The percent cumulative change in elongation of the shoots was compared among days and concentrations.

The growth of corn seedlings was continuous over the growth period at concentrations from 40 to 0.4 ppm efrotomycin. The rate of shoot growth of corn plants grown in the presence of 100 ppm efrotomycin was much slower than control seedlings grown in the presence of the control solution of acetone:water (1:99, v/v). A similar pattern of growth was observed for ryegrass.

The patterns of growth observed for pinto beans and cucumbers reached plateaus in approximately 8 days. Pinto bean plants exposed to 100 ppm efrotomycin appeared to have a greater growth rate than controls or plants grown in the presence of lower efrotomycin concentrations. Growth of cucumber seedlings in the presence of 100 ppm efrotomycin was initially more rapid than the controls, but slowed over time.

The growth patterns of the four plant species exposed to four concentrations of efrotomycin and the acetone controls provided evidence for suitable ranges (0.4 to 80 ppm) of efrotomycin for the definitive tests. The highest concentration tested (100 ppm) appeared to retard the growth of corn, ryegrass, and cucumber seedlings. Growth

of pinto bean seedlings in the presence of 100 ppm efrotomycin was enhanced beyond the control growth.

Definitive Tests

Procedures for the definitive tests were the same as the preliminary tests, except for the number of replicates and the test concentrations. The definitive tests were conducted in five replicates. After reviewing the preliminary test results, the test concentrations of 80, 20, 4, and 0.4 ppm of efrotomycin were used. The definitive tests included a negative control in addition to the acetone control.

After the final shoot and root length measurements were noted, the seedlings were gently pulled from the sand and washed of all clinging particles. The roots were separated from the shoots by cutting the seedlings at ground level. The separate plant materials were pooled by flat, placed in a drying oven for 6 hours at 70°C, and weighed on a balance having a precision of ±0.1 mg to determine the dry weights of the shoots and roots.

The growth of seedlings of the four plant species treated with efrotomycin was evaluated by examining the increase in shoot length over a 21-day growth period, and by measuring the dry weights of shoots as well as the length and dry weights of roots at termination of the growth period. Control plants were grown in the presence and absence of the cosolvent, acetone. Acetone:water (1:99, v/v) was required to prevent precipitation of efrotomycin. Statistical comparisons of the effects of efrotomycin were compared to the acetone controls. This treatment was considered appropriate because acetone (as cosolvent) ensured the miscibility of efrotomycin in deionized water.

The average length of corn plants treated with efrotomycin increased with time. The average shoot length of plants treated with 80 and 20 ppm efrotomycin appeared to deviate from the control pattern of growth after 9 and 15 days post-treatment, respectively.

Analysis of variance (ANOVA) on repeated measures of shoot length at each interval over the 21-day growth period revealed a significant difference [F = 12.82, d.f. =

(36,864), $p < 0.001$] among the four concentrations and the acetone control. The interaction of the repeated measures (days post-treatment) of shoot length within replicates and among concentration was significant at better than the 5% level of F [F = 1.91, d.f. = (144,864), $p < 0.018$].

The Greenhouse-Geisser estimates of probability were chosen because of the observed deviations from sphericity. A breakdown of the individual effects of time and concentration revealed significant differences between the mean shoot length of the acetone control and that of the plants treated with 20.0 and 80.0 ppm efrotomycin after days 14 and 9, respectively. The shoot lengths of the corn plants treated at 4 and 0.4 ppm of efrotomycin were not significantly different than those of the acetone control group on any treatment dates.

The means of the shoot weights for the four concentrations of efrotomycin and the acetone control were significantly different [F = 5.011, d.f. = (4,20), $p < 0.006$] according to the one-way analysis of variance. A comparison of the mean shoot weights (mg/plant/replicate) for the acetone control and the four concentrations of efrotomycin

revealed significant differences only between mean growth of the acetone control and that of the plants treated with 80.0 ppm efrotomycin. Mean shoot weights for the control and the 0.4-, 4.0-, and 20.0-ppm treatments were not significantly different.

The observed differences in the growth of the corn shoots may be compared to the growth of the roots. The root lengths were treated statistically as root lengths (mm/replicate). The means of the corn root lengths for the acetone control and the four concentrations of efrotomycin were significantly different [$F = 17.703$, d.f. $= (4,20)$, $p < 0.001$]. The acetone control and the 0.4 and 4.0 ppm treatments were not significantly different.

The dry weights of corn roots were treated statistically as mg/plant/replicate because of the variable number of plants grown to term in each replicate. The mean dry weights of the corn roots for the acetone controls and the four efrotomycin concentrations were not significantly different [$F = 2.10$, d.f. $= (4,20)$, $p < 0.119$].

The growth of pinto bean shoots was rapid over the first four days but reached a plateau after Day 5. The cumulative percent change in pinto bean shoot length was nearly uniform at each concentration of efrotomycin after Day 2.

ANOVA on repeated measures of pinto bean shoot growth revealed a statistically significant [$F = 2.38$, d.f. = (36,900), $p < 0.023$] interaction between time (days post-treatment) and concentration of efrotomycin (ppm). However, the interaction between time, concentration, and replication [mean shoot length (mm/replicate)] was not statistically significant [$F = 1.24$, d.f. = (144,900), $p < 0.203$]. The difference in these two results may be explained in terms of the contribution of each factor [time (days), concentration, and replication]. The disparity may be due to a lack of precision in replication.

A comparison of the means of the dry shoot weights (mg/replicate) of the pinto beans from each test concentration and from the acetone control revealed a significant difference [$F = 6.528$, d.f. = (4,20), $p < 0.002$]. However, a breakdown of the means into groups

indicated no significant negative differences between the acetone control and any treatment group.

Significant negative differences were observed between the root lengths of the acetone control and the 0.4 ppm [t = -8.51, d.f. = (20), p < 0.001] and 4.0 ppm [t = -7.21, d.f. = (20), p < 0.001] treatments. The mean root length of the 80-ppm treatment was significantly different [t = 3.97, d.f. = (20), p < 0.003] than the acetone control.

The mean dry root weight of the acetone control was significantly greater than the 20.0- [t = 4.56, d.f. = (20), p < .001] and 80.0-ppm [t = 6.73, d.f. = (20), p < 0.001] treatments. The mean dry root weight of the 0.4 ppm treatment group was not significantly different [t = -1.69, d.f. = (20), p < 0.294] than the control, while the 4.0 ppm treatment was significantly greater [t = -4.49, d.f. = (20), p < 0.001].

The growth of cucumber shoots was rapid over the first six days and reached a plateau thereafter. The interaction between repeated measures (days) and concentration of efrotomycin (ppm) was significant [F = 2.08, d.f. =

(36,891), $p < 0.039$] by ANOVA on repeated measures. However, the interaction between repeated measures (days), concentration (ppm), and replication [shoot lengths (mm/replicate)] was not significant [$F = 1.32$, d.f. = (144,891), $p < 0.131$].

The t-test on repeated measures provided evidence of a lack of trend among replicates, days, and concentrations of efrotomycin. The differences among means of the shoot lengths may have occurred by chance.

The means of the cucumber dry shoot weights (mg/replicate) of the acetone control and four treatment concentrations of efrotomycin were tested statistically according to a one-way ANOVA. The means were significantly different [$F = 13.941$, d.f. = (4,20), $p < 0.001$]. However, the mean dry weight of the cucumber control was significantly less than the 4.0-ppm [$t = -2.79$, d.f. = (20), $p < 0.038$] treatment, yet significantly greater than the 80.0-ppm treatment [$t = 4.20$, d.f. = (20), $p < 0.002$].

Similar results were observed for mean root lengths. Although the means of the root lengths were significantly

different [F = 18.405, d.f. = (4,20), p < 0.001] among concentrations, the breakdown of the means revealed a significantly negative difference between the acetone control and the 0.4-ppm treatment [t = -3.15, d.f. = (20), p < 0.017] and a positive difference between the acetone control and the 80.0-ppm treatment [t = 5.03, d.f. = (20), p < 0.001]. The mean root length of the controls was significantly greater than the 80.0-ppm treatment but significantly less than the 0.4-ppm treatment.

The means of the ranks of the cucumber root dry weights (mg/replicate) were significantly different among concentrations [F = 41.238, d.f. = (4,20), p < 0.001]. The rank transformation was required because of the heterogeneity of the variances.

The mean root weights of the control were significantly greater than the 4.0-, 20.0-, and 80.0-ppm treatments.

The average length of ryegrass plants treated with efrotomycin appeared to increase proportionally with time. The number of plants surviving in each replicate was, however, variable. The treatments did not have a

significant effect on survivability ($z = 1.54$, $p < 0.124$) according to a test for trend between plants surviving to term and plants dying within replicates.

The average cumulative growth of surviving ryegrass plants indicated a substantial reduction in growth was observed for the 80.0-ppm treatment. The ANOVA on repeated measures (days) revealed a significant interaction between days post-treatment and concentration of efrotomycin [$F = 13.95$, d.f. = (36,648), $p < 0.001$]. However, as observed with the pinto bean growth tests, the interaction of days, concentration, and replication was not significant [$F = 0.96$, d.f. = (144, 648), $p < 0.531$). The differences between the mean shoot length of the controls and the treatments according to repeated-measure t-test were significant for the 20.0- and 80.0-ppm treatments after 16 and 5 days, respectively.

These results appear to be reflected in the measurements of dry shoot weights. Comparisons were made among the means of the dry shoot weights (mg/plant/replicate) of surviving plants. The means of the control and the treatments were significantly different [$F = 10.708$, d.f.

= (4,20), $p < 0.001$]. A breakdown of the means revealed a significant difference between the control and the 80.0-ppm treatment [$t = 5.04$, d.f. = (20), $p < 0.001$] according to Dunnett's t-test.

The means of the root lengths of the ryegrass plants treated with efrotomycin and the acetone control were significantly different [$F = 27.267$, d.f. = (4,20), $p < 0.001$]. Again, the mean root lengths of the control were significantly greater than the 20.0- [$t = 3.61$, d.f. = (20), $p < 0.006$] and 80.0-ppm [$t = 6.58$, d.f. = (20), $p < 0.001$] treatments.

The means of the dry root weights (mg/plant/replicate) of the ryegrass plants treated with efrotomycin and the acetone control were significantly different [$F = 3.247$, d.f. = (4,20), $p < 0.033$]. However, unlike the means of the root lengths, the breakdown of the means according to Dunnett's t-test (two-tailed $\alpha = 0.05$) did not reveal significant differences.

In summary, the growth pattern of the corn seedlings from the preliminary to the definitive tests appeared to indicate an effect level at concentrations greater than or equal to 20 ppm. The effects of efrotomycin at these concentrations were time dependent. The mean shoot lengths of corn plants grown in the presence of 20 and 80 ppm efrotomycin were significantly less than the controls. However, the weights of the shoots of corn plants treated with 20 ppm were not significantly different than the controls, while those of the 80 ppm-treatment were significantly less than the control. Only the mean length of the roots was influenced by efrotomycin at 20 and 80 ppm. The rate of growth of the plants in terms of shoot length and root length appeared to be adversely affected by efrotomycin. However, this effect was not reflected in root weights.

The effects of efrotomycin on the other seed types, pinto bean, cucumber, and ryegrass, were more difficult to interpret than the effects on corn. The growth of pinto bean shoots was not influenced by efrotomycin in a

dose-dependent manner. The mean weights of pinto bean shoots treated with efrotomycin were not significantly different than the controls. Root lengths and weights of plants treated with 80 ppm were significantly less than the controls. Unlike the corn plants, efrotomycin appeared to adversely affect growth of bean roots at 20 and 80 ppm.

In a similar manner to the growth of pinto beans, the growth of cucumber shoots was not influenced by efrotomycin in a dose-dependent manner. However, the dry weights of the roots were observed to be negatively influenced by efrotomycin as a function of concentration. This result was, however, not observed for root lengths.

A similar ambiguity was observed for the growth of ryegrass shoots and roots. Although plants grown in the presence of efrotomycin died before termination of the test, lethality could not be attributed to the concentrations of efrotomycin. As observed for the growth of the corn seedlings, the growth of ryegrass shoots was negatively influenced by efrotomycin at 20 and 80 ppm.

These results were reflected in the weights of the shoots as well as the root lengths but not the root weights. The growth rate of the ryegrass shoots, like those of the corn plants, was affected by efrotomycin at higher concentration, 20 and 80 ppm.

Except for cucumber dry root weights at 4 ppm, adverse effects were not observed in any species treated at 4 or 0.4 ppm with efrotomycin.

d) Earthworm (manure worm)

The toxicity of efrotomycin was determined to the earthworm species (Eisenia foetida, manure worm) in artificial soil. Two range-finding tests were performed along with a reference toxicant test. No definitive test was conducted.

While Eisenia foetida is not a common species in soil, it is common in sewage beds, particularly in trickling filters, where it is exposed to industrial chemicals. Eisenia foetida is extremely prolific, has a short life-span, and readily adapts to laboratory culture. It is for these reasons that Eisenia foetida was selected as the test organism. Worms were maintained in a 50:50 mixture of manure and peat at pH 7.0 with ionic conductivity of <6.0 mmho. Cow and horse manure were both added to the breeding boxes for the first range-finder. Cow manure only was added to the stock used for the second study. All worms used in the tests were mature with clitellum and were selected from two breeding boxes in the laboratory. Live worm weights were taken prior to the addition of worms to the test containers for both range-finding test and at test termination for the second

range-finding test. Soil was rinsed off the worms before weighing.

The test chemical, a brown powder, was stored in the freezer. It contained 74.9 wt % efrotomycin A_1.

The test media consisted of 70% sand, 10% sphagnum peat, and 20% kaolinite clay.

The doses for this study were calculated in terms of milligrams (mg) of test chemical per kilogram (kg) of artificial soil. Weights of test chemical used in both range-finding tests were corrected for percent active ingredient (80%).

The range-finding tests were conducted at concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg. A control was run with the second range-finding test, but not with the first range-finding test. For the first range-finding test, the proper amount of test material per kilogram of soil was weighed into a vial and mixed with 10 grams of fine ground

quartz sand for the 10, 100, and 1000 ppm levels. These 10 grams of dosed sand were then mixed with 490 grams of prepared artificial soil and roller mixed for 15 minutes. For the 1.0 ppm level, an appropriate amount of test compound was mixed with 20 grams silica sand and 10.0 grams of this stock was mixed with the 490 grams artificial soil. The remaining 10 grams of dosed sand was diluted with 90 grams of silica sand and 10 grams was then mixed with 490 grams artificial soil for the 0.1 ppm level. After dosing the artificial soil, moisture was supplied by 125 ml deionized water and 5 grams of cow manure and 5 grams of calcium carbonate were added to 1.75 liter glass test containers (95 l x 95 w x 190 h mm). Ten worms were added to the soil surface of each test container and each container was covered with perforated plastic film to reduce soil substrate evaporation. The test containers were maintained at 25.0 x ± 2.5°C in continuous light. Temperatures were monitored by direct reading from an NBS traceable calibrated thermometer inside the chamber. Mortality and observations were recorded on days 7, 14, and 28. Additional deionized water to maintain soil moisture and 5 g of cow manure were added to each test container at each mortality assessment.

For the second range-finding test the proper amount of test compound for the 0.1, 1.0, and 10 ppm concentrations was dissolved in 100 ml deionized water, mixed with 50 grams cow manure, and incorporated into 1.0 kg (dry weight) of artificial soil previously moistened with 250 ml deionized water. The 100 and 1000 ppm doses were incorporated into 10 grams of silica sand by roller mixing for 15 minutes. The dosed sand was then incorporated into the food (cow manure):water slurry (50 grams:100 ml) by mixing with a spatula, and then the food:water:test compound slurry was incorporated into 990 grams of artificial soil previously moistened with 250 ml deionized water. Ten worms were added to the soil surface of each test container and each container was covered with perforated plastic film to reduce soil substrate evaporation. All test containers were identified with project number and test compound concentration. The test containers were maintained in an incubator at 20 ±2°C in continuous light. Temperatures were monitored by direct reading from an NBS traceable calibrated thermometer inside the chamber. Mortality and observations were recorded on days 7, 14, 21, and 28. Additional deionized water to maintain soil moisture and 50 g of cow manure

were added to each test container at each mortality assessment.

Mortality was assessed on days 7, 14, and 28 by emptying the soil into a tray and sorting out the worms. Worms were classified as dead, moribund, soft and flaccid, soft, turgid, or normal on each mortality assessment day.

Insufficient mortality was observed on Day 28 of either range-finding test to calculate an LC_{50} with 95 percent confidence intervals.

In the first range-finding test, there were 10% mortalities at Day 7 and 14 at 0.1 and 10.0 ppm and no deaths at any other levels. At Day 28, there was 20% mortality at 0.1 ppm, 10% mortalities at 1, 10, and 100 ppm and no mortalities at 1000 ppm.

In the second range-finding test, there were no mortalities in the control, 0.1, 1, 10, 100, or 1000 ppm levels at 7, 14, and 21 days. At 28 days, there were 10% mortalities at 100 and 1000 ppm, but none at the other levels or controls. Sublethal effects were monitored in

the second study. All living worms were normal at all observation times, 7, 14, 21, and 28 days, for all levels and the controls.

Weights of test worms for Day 0 and 28 were measured for the second range-finding test. Mean weights for worms exposed to 0.1, 1.0, and 10 ppm efrotomycin for 28 days increased 8.9, 7.6, and 8.8 percent, respectively. A decrease in mean weight of 5.2, 12.4, and 4.8 percent was observed after 28 days for the 0, 100, and 1000 ppm dose levels, respectively.

Soil moisture data indicates that the moisture content remained relatively constant throughout the second test.

A reference toxicant test was conducted using copper sulfate ($CuSO_4$). Since water was the solvent for the reference toxicant, copper sulfate was dissolved in 100 ml of water and added to 50 grams of feed. The compound: water:feed mixture was added to 1.0 kg of artificial soil and mixed. Worms were added thereafter. The 28-day LC_{50} for E. foetida exposed to the reference toxicant, copper sulfate, is 657 ppm.

The results of the two range-finding tests with E. foetida showed that efrotomycin did not elicit a dose-related toxic response over the concentration range of 0.1 to 1000 ppm. No sublethal effects were seen over this range of concentrations. There also was no trend in worm weight gain or loss with concentration. Therefore, no definitive test was performed and no LC_{50} could be calculated.

e) Respiration

The effects of the test chemical efrotomycin on the microbial respiration of two well characterized soils were determined over a time period of 21 days using a laboratory model system. Treated and untreated control samples were prepared in respirometer vessels consisting of amber screw cap bottles sealed with rubber septa. The test chemical and characterized soils were incubated aerobically for 20 days. On a predetermined schedule, analysis of the head gas from the respirometers was performed to determine the CO_2 evolution by gas chromatography. Average rates of total CO_2 evolved were computed and presented both graphically and tabularly to determine the effects of the test material efrotomycin on soil respiration.

Two soil types were used in the study, a loamy sand and a silt loam. The soils were collected fresh from sites in Georgia and Arkansas, and were sieved through a 2 mm wire mesh. The sieved soils were characterized as follows:

Soil Analysis

PARAMETER	LOAMY SAND	SILT LOAM
Source	Georgia	Arkansas
Percent Sand	80.0%	23.8%
Percent Silt	10.6%	64.4%
Percent Clay	9.4%	11.8%
Percent Organic Matter	2.4%	1.2%
pH	6.0	5.5
Field Capacity	22.55%	49.92%
Bulk Density	0.6338	1.0232
CEC	9	12
Base Saturation	84.1%	53.6%
K-(potassium) Saturation %	3.3%	1.7%
Mg (magnesium) Saturation %	8.8%	16.1%
Exchangeable Sodium %	5.3%	1.2%

All calculations were performed on a dry weight basis.

Fifty grams (dry weight) of soil were weighed into 250 ml amber screw cap bottles with rubber septum tops. Triplicate samples were prepared for the four concentra

tions, untreated control, and sterile control samples of each soil type. Next, the moisture content of the loamy sand samples was adjusted to 44% of field capacity, prior to incubation by the addition of 0.2 ml distilled deionized water to each sample. The final adjustment to 62% field capacity was made at the time of dosing by the addition of 2 ml of the dosing solution. The silt loam samples were adjusted to 44% of field capacity by the addition of 3.73 ml of distilled deionized H_2O to each sample. The final adjustment to 64% field capacity was made at the time of dosing by the addition of 5 ml of the dosing solution. The field capacity adjustment was performed in this manner to ensure moisture for the microbial population.

Soil samples then incubated at 20°C ± 1 for a period of 3 days to stabilize microbial population and to establish uniform moisture content. Prior to the 3-day pretreatment period the sterile control samples were sterilized for 1 hour using 15 psi steam at 121°C. The sterile control soils were sterilized 2 additional times for 1 hour each using 15 psi at 121°C.

A representative sample from each soil type was used to estimate the fungal and bacterial population by plate count procedure. Soil samples extracted with phosphate buffer and serial dilutions of the extract in phosphate buffer were plated on prepared agars. The total colony forming units per gram (CFU/g) of soil for fungi, bacteria and actinomycetes were respectively 6.4×10^4, 1.3×10^5 and 9.9×10^4 for the loamy sand and respectively 1.2×10^5, 1.2×10^5 and 9.8×10^4 for the silt loam.

The test chemical efrotomycin was a brown, free flowing powder with an active ingredient (purity) of $\geq$80 percent. It was stored in a freezer when not in use.

Two attempts were made to prepare a 434 ppm (A.I.) stock solution of efrotomycin, both were unsuccessful because of insolubility of the test chemical. A third attempt was made using a 4% methanol and water solution. The test compound did dissolve, but gave opaque milky solution which cleared to a brown clear solution. Analysis of the final stock solution using UV Spectophotometry confirmed that the solution contained 477 ppm (A.I.) of efrotomycin.

Concentrations of 0.05, 0.2, 2.0, and 20.0 ppm were made from the appropriate volume of stock solution mixed with the appropriate amount of distilled deionized water in each test vessel containing 50 g (dry weight) of preincubated soil. Sterile controls and positive controls were prepared using distilled, deionized water only.

The test system was each dosed container of 50 g (dry weight) of soil. The test systems were identified with project number, dose concentration, replicate number, and soil type. The samples were capped with rubber septa immediately after the addition of each dose and flushed with CO_2 free air and placed in an environmental incubator at 20°C until analysis.

Methanol was used as a co-solvent in preparing the stock solution. The amount of methanol used was 10 ml in a volume of 250 ml or 4%. No solvent control was run because the actual amount of methanol in each dose was minimal and therefore would not affect the respiration of the microbial population.

The percent carbon dioxide in the head gas of the respirometer vessels was determined at 24 and 48 hours after soil treatment and subsequently at 48-hour intervals, thereafter, with the final analysis conducted at 21 days. Carbon dioxide levels in each respirometer vessel were determined by the drawing 250 ml of head gas using of 250 ml Pressure-Lok syringe and analyzing the sample by a gas chromatograph equipped with a thermal conductivity detector. The GC system supplied a good separation of CO_2 from other gasses in a reasonable amount of time (approximately 2.7 minutes). Each sample was injected and allowed a run time of 3.5 minutes.

At each sampling interval external standards of 1.0, 0.4, 0.2, and 0.1 $\%CO_2$ were used to calibrate the gas chromatograph. Standards were also analyzed throughout the run to assure operating conditions did not vary and to obtain a more representative calibration curve for the instrument on each day.

Some samples were rejected due to poor chromatographic conditions as described below:

a. Poor nitrogen recovery indicating a bad injection of nitrogen. Assuming that the amount in the headspace of the respirometer vessel remained constant then any sample with less than 80% of the maximum nitrogen peak area was rejected.

b. Poor integration: When integration proceeded below the printer baseline the sample was rejected because the tracing could not be visually inspected for interfering peaks.

c. Poor injection: Samples rejected due to apparent poor injection technique. The poor chromatography seen in these injections may have been due to instrument malfunction, but this could not be remedied at the time of anlysis.

d. Poor peak resolution: Peaks not completely resolved, therefore, peaks could not be differentiated from each other and consequently rejected. Integration included both peaks and consequently CO_2 value was erroneously high.

All respirometer vessels of a soil type were to be flushed with CO_2 free air when the positive control for that soil type were determined to have a CO_2 content in the head gas of 1.0 percent or greater upon analysis. Carbon dioxide free air was obtained by bubbling bottled compressed air through a 1 Normal solution of sodium hydroxide. The CO_2 free air was then dried by passing it through 1 foot of drying agent. Bottles were flushed by inserting two syringe needles through the septa with CO_2 free air passed through one syringe and exhausted through the other syringe needle. The CO_2 levels did not reach 1.0 percent in the positive controls for both soil types and consequently the soils were not flushed during the duration of the study.

For each day's analysis, a calibration curve of peak area versus percent CO_2 of external standards was prepared. These plots were found to be linear with correlation coefficients of 0.990 and greater.

The percent CO_2 in each sample was determined by linear regression using the calibration curve for the day the sample was analyzed and correlating the peak with percent

CO_2 in the sample. Then the mean and standard deviation for the triplicate samples in each concentration were computed to give the average percent CO_2 in each group of samples.

The results of the respiration of the two soil types were computed as the mean of replicates versus time for all treatment levels and controls.

Because of the data deletion due to unsatisfactory chromatographic conditions, for statistical analysis the data were grouped into three weekly intervals as follows: week 1 - days 1, 2, 4, 6; week 2 - days 8, 10, 12, 14; and week 3 - days 16, 18, 21. For each replicate the mean percent CO_2 was computed for each week. Appropriate statistical techniques for testing progressiveness of response with increasing doses of a drug were performed on the weekly mean percent CO_2 data in order to compare the time-response curve for each dose of efrotomycin with that of the control. The results of the analysis are summarized as follows:

Loamy Sand Soil - no inhibition of enhancement of CO_2 production was observed at 0.05, 0.2 and 20.0 ppm treatments. However, at 2.0 ppm dose CO_2 production was stastically significantly higher than the control.

Silt Loam Soil - Enhanced CO_2 production was observed at 0.2, 2.0 and 20.0 ppm of efrotomycin treatment.

For both soil types, the mean percent CO_2 for the sterile control group was statistically significantly greater than the untreated control at weeks 2 and 3. The reason for this unexpected phenomenon is not known.

f) Nitrification

The effects of efrotomycin on microbial nitrification in two characterized soils were determined over a 4 week exposure period using a laboratory model system.

Two soil types were used to determine the effects of efrotomycin on soil nitrification. Soils were preincubated for 3 days to establish microbial populations. Subsamples of each soil type were treated

with efrotomycin at 0.05, 0.20, 2.0 or 20.0 ppm of efrotomycin. Untreated samples both sterilized and non-sterile were dosed only with water and served as controls. All soil samples were dosed with ammonia on the same day as with efrotomycin or water ("day 0" treatment regimen), some again 15 days later ("day 14") and some both 15 and 27 days after efrotomycin additions ("day 28"). All samples were extracted with aqueous KCl 7-8 days after the last ammonia addition and the extract analyzed for ammonia, nitrate and nitrite concentrations. Average nitrification values were computed to determine the effects of efrotomycin on soil nitrification.

Two soil types were used in the study, a loam and a sandy loam. The soils were collected fresh from sites in Mississippi. Soils were taken at approximately 0-15 cm from the surface. Prior to use the soils were sieved through a 2 mm wire mesh. The soils were characterized as follows:

Soil Analysis

Texture	Sandy Loam	Loam
Source	Mississippi	Mississippi
Percent Sand	62.0	43.6
Percent Silt	30.4	47.6
Percent Clay	7.6	8.8
Percent Organic Matter	0.5	1.2
pH	7.5	7.5
Field Capacity		
(g water/100 g dry soil)	31.1	26.6
(g water/100 g wet soil)	23.7	21.0

The sandy loam soil contained 7.9% moisture (wet weight) and was adjusted to 63.1% of field capacity by the addition of 76.5 ml H_2O to 923.5 g of wet soil. The moisture content of the loam soil was 13.7% (wet weight), which was 65.2% of field capacity. It was used without moisture adjustment.

Next, twenty gram aliquots (wet weight) of soil were weighed into 250 ml amber screw cap bottles. Triplicate samples were prepared for the four efrotomycin concentrations, untreated control, and sterile control samples of each soil type and treatment regime. One representative sample was also prepared for each soil type for later microbial analysis.

Soil samples were then incubated at 22 ±2°C for a period of 3 days to stabilize the microbial population and to establish a uniform moisture content. Prior to the 3-day pre-treatment period, the sterile control samples were sterilized once for 30 minutes using 15 psi steam at 121°C.

After soil pre-treatment, a representative sample from each soil type was used to estimate the fungal and bacterial population by a modified plate count procedure. Soil samples were serially diluted with phosphate buffer followed by plating on prepared agars. The total colony forming units per gram (CFU/g) of soils of fungi, bacteria and actinomycetes were respectively 6.5×10^4, 7.8×10^5, and 6.0×10^6 for the sandy loam soil, and respectively 6.5×10^4, 1.1×10^6 and 8.0×10^6 for the loam soil.

The test chemical, efrotomycin, was a brown, free flowing powder with an active ingredient (purity) of $\geq$80 percent. The chemical had a reported solubility of 1.0 mg/ml in water at pH 7.0 ambient temperature and was kept in a freezer when not in use.

A stock solution of efrotomycin was prepared in deionized water at a concentration of 0.40 mg/ml for subsequent dosing of soils. Each concentration (0.05, 0.2, 2.0, and 20.0 ppm), sterile controls and untreated controls were made from the appropriate volume of stock solution mixed with the appropriate amount of deionized water to make 1.0 ml final volume in each test vessel containing 20 g (wet weight) of pre-incubated soil.

The test system was each dosed container of 20 g of soil. Each test system was identified with project number, soil type, dosing regime, dose concentration of efrotomycin, and replicate number. The samples were capped immediately after the addition of each dose with rubber septa before placing them back into the 22 ±2°C incubator until analysis.

Water was used as the solvent for the test material in this study. Therefore, the untreated control was equivalent to a solvent control.

Ammonia (100 ppm-N as $(NH_4)_2SO_4$ in 250 µl of water) was applied to each set of soil samples concurrently after 0, 2, and 4 weeks of incubation with test material. Seven-eight days after the last ammonia application 100 ml of 2 Normal aqueous potassium chloride solution was added to each sample followed by shaking for 30 minutes on a mechanical rotary shaker. After shaking, samples were allowed to settle for 15 minutes before a 10 ml sample was decanted and centrifuged at 1000 rpm for 30 minutes to remove particulate matter suspended in the aqueous solution. Three samples of each soil type were spiked with ammonium, nitrate, or nitrite. These were extracted, stored and analyzed along with the day 14 and day 28 samples.

The soil extracts were refrigerated until analyzed. All samples from each treatment regimen were analyzed for ammonia, nitrate or nitrite on the same day. Day 0 samples were analyzed for each parameter within 2 days of extraction, as were day 14 ammonia and nitrite. Analysis of the remaining samples and parameters were delayed 85-135 days.

The centrifuged solution was diluted by a predetermined factor if necessary so that it could be analyzed for each parameter using an autoanalyzer with a nitrate-nitrite manifold for $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ analysis and an ammonia manifold for $N\text{-}NH_4^+$ analysis.

Printouts for each sample were obtained from the autoanalyzer in the form of peaks. The height of these peaks was converted to ion concentration by direct comparison to a standard curve derived from a set of standards of different known concentrations. These standards were run before and after each set of test samples to give a representative calibration curve for the autoanalyzer over the time of analysis.

A conversion from printout via the standard curve linear regression for the autoanalyzer gave a result of µg of $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, or $N\text{-}NH_4^+$ per ml of solution. To convert to ppm or µg of $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, or $N\text{-}NH_4^+$ per gram of soil, it is noted that there was a dilution factor to be taken into account and that 100 ml of 2 Normal solution was used to extract the 20 g of soil in each container. Thus the ppm of

nitrates, nitrites, and ammonia in the soil can be represented by:

$$\text{ppm in the soil} = \text{ppm in the solution} \times \text{dilution factor} \times 5$$

$$5 = \frac{\text{100 ml aqueous KCl extracted solution}}{\text{20 g wet soil}}$$

$$\text{where ppm in the soil} = \frac{\mu\text{g of } N\text{-}NO_3^-,\ N\text{-}NO_2^- \text{ or } N\text{-}NH_4^+}{\text{g wet soil}}$$

$$\text{ppm in the solution} = \frac{\mu\text{g of } N\text{-}NO_3^-,\ N\text{-}NO_2^- \text{ or } N\text{-}NH_4^+}{\text{ml analyzed solution}}$$

The results of the nitrification of the two soil types were computed as the mean of replicates versus time for all treatment levels and controls.

Although some of the extracts for the day 14 and 28 samples were held for several weeks before analysis, the recoveries demonstrated that the concentrations did not change on storage. The nitrate recoveries for the day 28 samples could not be very accurately quantitated because the background levels were about the same or higher than the spiking levels of 1.26 and 1.28 ppm. However, the day 14 recoveries were held for a longer time, validating the stability of the extracts during the storage period. The

day 14 recoveries were spiked at 24.5 and 25.3 ppm, making quantitation more accurate. Statistical analyses were conducted on the results.

For each soil type, variable, and treatment regime, the following analyses were carried out:

1) Analysis of variance (ANOVA) A one-way ANOVA was performed with the factor of interest being the dose of the compound (including the non-sterile control as a zero dose) in order to obtain a pooled measure of variability.

2) Comparisons among treatment groups The sterile group was compared with the non-sterile groups using a two sample t-test adjusted for unequal variance when necessary (where the variance estimate for the non-sterile control was the pooled variance estimate obtained from the ANOVA). Each dose level of efrotomycin was compared with the non-sterile control using Williams' test.[11]

3) Trend analysis - A trend test incorporating the non-sterile control[12] was performed to test for progressiveness of response with increasing dose of efrotomycin. If a statistically significant (two-sided $p<0.05$) trend was found when all concentrations were included, the highest concentration was deleted and the test repeated. This process was continued until a concentration was reached where a statistically significant trend was no longer observed.

The analyses results are as follows:

Loam Soil - There were no statistically significant treatment-related effects for nitrate on any of the three ammonia application dates. For the day 0 ammonia application, there was a statistically significant positive trend in ammonia with the 2 and 20 ppm doses being significantly greater than the control. Similar results were observed for the day 14 nitrite levels but these results are somewhat suspect since there was no detectable differences between the sterile and non-sterile (0 dose) control groups.

Sandy Loam Soil - For the day 0 ammonia application, each dose of efrotomycin had a statistically significantly lower mean nitrate level than the non-sterile control. No other statistically significant efrotomycin related differences were observed for this soil type.

Efrotomycin, therefore, did not appear to cause inhibition or enhancement of bacterial nitrification at the 0.05, 0.2, 2.0, or 20 ppm treatment levels in either of the two soils tested. A statistically significatn trend in the ammonia level in the 2 and 20 ppm doses at day 0 in the loam soil and the statistically significantly lower mean nitrate level than the non-sterile control on day 0 in the sandy loam soil were not observed at 14 or 28 days.

g) Acute Toxicity of Efrotomycin to Rainbow Trout (Salmo gairdneri)

The acute toxicity of efrotomycin to rainbow trout (Salmo gairdneri) was assessed. The purpose of this test was to determine the 24-, 48-, and 96-hour LC_{50} levels for efrotomycin to rainbow trout (Salmo gairdneri). Preliminary range-finding studies were conducted to determine the concentration range for the definitive

bioassay. Water quality parameters of temperature, dissolved oxygen and pH were measured throughout the test and were within acceptable limits. Culture and acclimation records indicated the fish were in good condition for testing.

The procedures for status bioassay, as described in Methods of Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians[13] and Standard Methods for Examination of Water and Wastewater[14], were used in this experiment. The rainbow trout were held in culture tanks on a 16-hour daylight photoperiod and observed for at least fourteen days prior to testing. Fish culture techniques used were basically those described by Brauhn et al.[15]. A daily record of fish observations during the holding period, along with any prophylactic or therapeutic disease treatments, was kept. During this period, the fish received a standard commercial fish food occasionally supplemented with brine shrimp nauplii (Artemia sp.) daily until 48-96 hours prior to testing at which time feeding was discontinued. The rainbow trout used for this experiment had a mean weight of 0.43 (±0.10) g and a mean standard length of 32

(±2.1) mm. This gave a test chamber loading biomass of 0.29 g/l for the definitive study. Weight and length measurements were made on the control group of fish at the termination of the test.

The efrotomycin sample was observed to be a yellow powder and was stored in the freezer in the dark. Sample purity was specified as ≥65% of A_1 isomer, total efrotomycin ≥80%.

The definitive test concentrations were obtained by transferring appropriate weights of test compound directly to the test chambers. Before addition to the test chambers, 7.5 ml of acetone was added to each sample weight to increase dispersion of the compound in the dilution water. All test concentrations were based on the total compound, i.e. not corrected for sample purity. The solvent control chamber received a 7.5 ml aliquot of acetone, which was equivalent to the highest amount used in any test solution.

The static fish bioassay was conducted in five gallon glass vessels containing 15 liters of soft reconstituted water composed of the following compounds in the amounts stated per liter of deionized water:

48 mg $NaHCO_3$
30 mg $CaSO_4 \cdot 2\ H_2O$
30 mg $MgSO_4$
2 mg KCl

This reconstituted water was prepared to yield a total hardness of 40-45 mg/l as $CaCO_3$, a total alkalinity of 30-35 mg/l as $CaCO_3$ and an initial pH of 7.2 to 7.6. The 0-hour measured control water parameters of this dilution water were dissolved oxygen 9.6 mg/l and pH 7.4.

The test vessels were kept in a water bath at 12°C (±1.0). The test fish were acclimated to the dilution water and test temperature and held without food for 48-96 hours prior to testing.

Range-finding tests of 96-hours duration were conducted to determine the concentration range for the definitive

study. The preliminary test concentrations were set at 10 and 100 and at 320 mg/l. Based on the results of preliminary testing, five concentrations of the test compound, ranging in a logarithmic series from 10 to 100 mg/l, with ten fish per concentration were selected for the definitive bioassay. Another level of 76 mg/l was also included as this appeared to be the maximum concentration at which no precipitation was observed by visual observation. Also included was a dilution water control and a solvent control chamber. The fish were added to the test chambers by random assignment within 30 minutes after addition of test material. All test organisms were observed once every 24 hours for mortality and abnormal (sub-lethal) effects. Any dead individuals were removed from the test chambers after each 24-hour observation.

Statistical analysis of the concentration vs. effect data (generally mortality) was obtained by employing a computerized LC_{50} program developed by Stephan et al.[16]. This program calculated the LC_{50} statistic and its 95-percent confidence limits using the binomial, the moving average, and the probit tests. However, if no mortality occurred or if a dose response could not be demonstrated over reasonable range (<37 to >63%) an LC_{50} and/or its 95-percent confidence limits could not be

calculated. Three different methods of analyzing the data were used since no one method of analysis is appropriate for all possible sets of data that may be obtained.

The results of the 96-hour static toxicity test with rainbow trout (Salmo gairdneri) exposed to efrotomycin are presented in Table IV. The 24-, 48-, and 96-hour LC_{50} values for efrotomycin were all >100 mg/l. All results were based on the nominal concentrations of 10, 18, 32, 56, 76, and 100 mg/l. The water solubility of the compound appeared to be ≅76 mg/l by visual observation of precipitation at various concentrations. After stirring the test solutions the 10 mg/l solution was pale yellow in color. The density of the color increased with increasing concentrations to the 100 mg/l solution which was dark gold in color. The 100 mg/l solution also had a precipitate at the bottom of the test chamber. After 96 hours of testing the colors and precipitate were as noted at 0-hour. The 96-hour no-observed effect concentration was estimated to be 100 mg/l, the highest concentration tested, based on the lack of mortality or observed abnormal (sub-lethal) effects. There were no abnormal effects observed in any test concentration during the 96-hour exposure period.

The dissolved oxygen concentrations ranged from 9.1 to 9.8 mg/l during the test. These values represented 86 and 92% saturation at 13°C, respectively, and were considered adequate for testing[13]. The pH values ranged from 7.1 to 7.5.

Table IV

The Acute Toxicity of Efrotomycin
to Rainbow Trout (Salmo gairdneri)

Compound	LC_{50} in milligrams/liter (ppm)		
	24 Hours	48 Hours	96 Hours
MK-621 (efrotomycin)[a]	>100[b]	>100[b]	>100[b]

N = 10 fish per concentration

a Bioassay as conducted at 12°C (±1.0), mean fish weight and length, 0.43 (±0.10) g and 32 (±2.1) mm.

b No mortality occurred in any concentration tested, therefore, and LC_{50} could not be calculated.

The 96-hour no-observed effect concentration could be estimated at 100 mg/l, based on the lack of mortality and abnormal effects at the highest concentration tested.

h) Acute Toxicity of Efrotomycin to Algae (Selenastrum capricornutum Printz

The acute toxicity of efrotomycin to algae Selenastrum capricornutum Printz was assessed. The purpose of this test was to determine the 96-hour EC_{50} levels for efrotomycin to Selenastrum capricornutum Printz. A preliminary range-finding study was conducted to determine the concentration range for the definitive bioassay. Temperature and light readings were measured throughout the test and were within acceptable limits.

The procedure for static algal assay was patterned after methods that were formulated by the U.S. Environmental Protection Agency[17], Office of Toxic Substances (OTS)[18] and American Society for Testing and Materials (ASTM)[19]. Algal cultures used for the toxicity test ranged from 7-10 days old.

The algal toxicity study was conducted in 250-ml Erlenmeyer flasks containing 100 ml of synthetic algal nutrient medium.

The deionized water was filtered through a water purification system. This system consists of a prefilter, a carbon filter to remove organics, two ion exchange resin beds, and a final 0.2 micron filter. Resistivity of the water is approximately 17 megohm-cm and organic content is less than 0.1 mg/l. After the media was prepared it was sterilized through a 0.45-μ filter. The definitive 2.0 mg/l test concentrations was prepared directly in a 1000-ml volumetric flask with the sterile media. Each of the triplicate 2.0 mg/l test vessels received 100 ml of this preparation. The four remaining treatment levels were prepared in triplicate as 100 ml proportional dilutions of the 2.0 mg/l test solution and algae nutrient media. To each triplicate control and solvent control test flask, 100 ml of media was added. The solvent control replicates each received 0.010 ml aliquots of acetone which was equivalent to the amount of solvent in the highest test concentration. Each test flask received 1.0 ml of algal inoculum containing approximately 1.0×10^6 cells/ml resulting in approximately 1.0×10^4 cells/ml for each flask. Actual initial cell counts of control flasks resulted in a mean cell count of 1.2×10^4 cells/ml, verifying the initial cell count.

The algal cell counts were accomplished utilizing a hemacytometer and a microscope. The hemacytometer has two chambers each with nine squares, 1-mm on a side. The average number of cells per 1-mm^2 was designated as "Q". The center square also was subdivided into twenty-five 0.04-mm^2 squares. The average number of cells per 0.04-mm^2 square was designated as "R". The cells per ml (d) for a given suspension of algal cells could be calculated from either of the following equations:

A. d(cells/ml) = 10^4 x Q (Average number of cells per 1-mm^2)
B. d(cells/ml) = 10^6 x 1/4R (Average number of cells per 0.04-mm^2)

In general, equation "B" was only used with very dense cell populations such as those encountered with a 7- to 10-day old algal culture used in the preparation of algal test inoculum. Algal counts during the definitive study used equation "A". When the average number of cells per 1-mm square was less than ~ 11 algal cells, all nine 1-mm squares were counted and divided by nine to obtain average number of cells per 1-mm square (Q). For all

suspensions with at least 11 cells per 1-mm square, the four corner 1-mm^2 squares could be counted and averaged to obtain "Q". The cells/ml for each replicate was calculated and recorded.

Following preparation, the test vessels were incubated for 96 hours at 24 ± 1°C under continuous "cool-white" fluorescent light and constant shaking. Light intensity was maintained at 400 ±10% ft-c and shaker speed was 100 rpm. Temperature and light intensity were monitored throughout the study.

A 96-hour range-finding study was conducted to determine the concentration range for the definitive study. Test concentrations for this study were set at 1.0, 10, and 100 mg/l. Based on the results obtained from this preliminary study, five concentrations of the test compound ranging from 0.13 to 2.0 mg/l were selected for the definitive algal assay. Test flasks were prepared in triplicate for each test concentration and the controls. All test flasks were stoppered with a foam plug.

The efrotomycin test material was a yellow powder and was stored at room temperature.

The efrotomycin stock solution used in the preparation of test concentrations was refrigerated after use.

Cell counts for each concentration and control were subjected to analysis of variance (ANOVA) and treatment means were compared using a multiple means test (Tukey's HSD). Differences were considered significant at α=.05. Cell counts for each replicate were first transformed using the square root of the cell count.

EC_{50} values were estimated by regression analysis. The independent variable was the concentration of the test chemical, and the dependent variable was defined as:

$$P_i = 100(\bar{C} - T_i)/\bar{C}$$

where: P_i = % difference from control for treatment replicate i

T_i = the measured cell density for treatment replicate i

$\bar{C}$ = the mean control measurement for cell density

For each set of data (cell count), two regression models were analyzed:

1) Quadratic model of P vs. concentration

$$P = a + b_1 (conc) + b_2 (conc)^2$$

2) quadratic model of P vs. ln concentration

$$P = a + b_1 (\ln conc) + b_2 (\ln conc)^2$$

The best model was then chosen by a combination of least squares and visual techniques. The EC_{50} value was estimated from this "best" model by substituting P = 50 and solving for concentration.

Ninety-five percent confidence limits for the EC_{50} were obtained from the plot of the 95% confidence bounds for the means of the dependent variable adjusted for the contribution of the variability of C. The adjustment factor is derived as follows:

$$\text{Var}(P) = 100^2 \text{Var}(T/\bar{C})$$

$$= 100^2 \left[\frac{\text{Var}(T)}{\bar{C}^2} + \frac{\text{Var}(\bar{C})}{\bar{C}^4} T^2 \right] \quad \text{(by propagation or error method)}$$

$$= 100^2 \left[\frac{\text{Var}(T)}{\bar{C}^2} + \frac{\text{Var}(\bar{C})}{\bar{C}^2} \left(\frac{100-P}{100} \right)^2 \right]$$

$$= 100^2 \left[\frac{\text{Var}(T)}{\bar{C}^2} + \frac{\text{Var}(T)}{3\bar{C}^2} \left(\frac{100-P}{100} \right)^2 \right] \quad [\text{assuming } \text{Var}(\bar{C}) = \text{Var}(T)]$$

$$= \left[100^2 \frac{\text{Var}(T)}{\bar{C}^2} \right] \left[1 + \frac{(100-P)^2}{3(100)^2} \right]$$

The first bracketed term estimates $S^2_{P/Conc}$ (the variance of the regression residuals) when the model fits, and the second bracketed term is the adjustment factor, which changes depending on the predicted value of P at each concentration.

A 96-hour static acute algal study with efrotomycin was successfully completed. The five nominal concentrations of efrotomycin which ranged from 0.13 to 2.0 mg/l were selected from the results of range-finding tests. Cell counts were conducted at 24, 48, 72, and 96 hours for each concentration. Initial cell counts were performed only on control replicates.

The protocol required that dead cells be differentiated from live cells through the use of Evans blue dye (Mortal staining). In an attempt to verify the staining of dead Selenastrum capricornutum cells, cells were subjected to heat (10 and 30 minutes in 90°C water) and formalin for 24 hours before exposure to the dye. It was observed that none of the presumed dead algae cells stained dark blue. The staining process was checked using a saltwater algae (Skeletonema costatum) that was subjected to formalin for 5 minutes. All cells stained dark blue indicating 100% death, thus verifying the staining process. It appears that the mortal staining is not applicable to Selenastrum capricornutum and therefore was not performed for this study. This protocol deviation did not effect the results of the study.

The growth data (cell counts) from the definitive test are presented in Table V. Log phase growth was confirmed at 96-hours with a mean count of 8.2×10^5 cells/ml in the control, which was a 68 x increase from the initial 1.2×10^4 cells/ml. The growth data were subjected to a one-way analysis of variance (ANOVA), which indicated a significant inhibition effect ($\alpha = 0.05$) on growth for

0.50, 1.0, and 2.0 mg/l mean test concentrations of efrotomycin to Selenastrum capricornutum, as compared to the control after 96 hours.

The calculated EC_{50} results are presented in Table VI. The 24-, 48-, 72-, and 96-hour EC_{50} values for efrotomycin based on cell counts, were >2.0, 1.1, 0.49, and 0.52 mg/l. The results indicated a no-observed effect concentration of 0.13 mg/l for the study.

The levels of efrotomycin predicted in water in contact with soils fertilized with swine feces at the "worst-case" levels are below the 96-hour EC_{50} and no-observed effect concentrations except for water in contact with sandy soil, Section 6b. Fertilization of sandy soils at recommended use levels would lead to expected ground-water levels in the range of the no-observed effect concentration.

Table V

Measured Cell Counts for Selenastrum capricornutum
During the exposure to Efrotomycin

Nominal Concentrations	Mean Cell Counts (3 Flasks)[a] Cells/ml x 10^{-4}				
	0 hr	24 hr	48 hr	72 hr	96 hr
Control	1.2	1.6	4.6	20	82
Solvent Control		1.1	3.5	18	80
0.13 mg/l		1.6	4.0	16	72
0.25 mg/l		1.2	4.2	12*	65
0.50 mg/l		1.0	3.3	11*	46*
1.0 mg/l		1.3	2.4*	3.7*	9.8*
2.0 mg/l		1.2	1.4*	2.2*	1.1*

a Rounded to two significant figures.

* Denotes a significant (α=0.05) inhibition effect from control.

Table VI

EC_{50}, 95% Confidence Intervals and No-Effect Level of Efrotomycin to Selenastrum capricornutum

	EC_{50} Based on Cell Counts		
Hours	EC_{50} mg/l	95% Confidence Interval mg/l	No-Effect Level mg/l
24	>2.0	a	2.0
48	1.1(2)	0.77-1.6	0.50
72	0.49(1)	0.42-0.58	0.13
96	0.52(1)	0.46-0.60	0.25

The EC_{50} and 95% confidence levels were calculated using one of the following methods:

(1) Quadratic Regression (Concentration vs. % Difference from Control)

(2) Quadratic ln Regression (Concentration ln vs. % Difference from Control)

a A 95% confidence interval could not be calculated.

i) Environmental effects

Based upon the laboratory data, the effects upon animals, plants, human, and other organisms can be predicted.

Efrotomycin is extremely non-toxic to animals, as evidenced by the toxicity data from Daphnia, manure worms, rainbow trout, rats, mice, dogs, and rabbits. It is neither a mutagen nor a teratogen. Though it is an

antibiotic, it had no environmentally significant effects on microbial nitrification and caused stimulation of microbial respiration only at high levels. It had no phytotoxic effects on the germination of seeds, though there were stunting and loss of pigmentation effects at high levels in plant growth tests. Its photodegradation products were less toxic to Daphnia than the parent compound. Fertilization of soils with swine feces at "worst-case" levels should not lead to toxic effects on algae, except perhaps in sandy soils. Fertilization of sandy soils at recommended rates should lead to ground water levels on the order of the no-observed effect level for efrotomycin.

At the expected and worst-case use levels, efrotomycin use in swine would not be expected to cause any environmentally significant effects.

9. Use of resources and energy

The raw materials utilized to manufacture efrotomycin and efrotomycin premix are common compounds all of which are in ample commercial supply. Energy commitment for bulk chemical and dosage form production in the United States is nominal and

not excessive. Only very small increases in the utilization of energy is anticipated since production occurs at existing facilities.

No effects upon endangered or threatened species and upon property listed in or eligible for listing in the National Register of Historic Places are anticipated.

10. Mitigation measures

No potential adverse environmental impacts are foreseen with the production and use of efrotomycin drug substance and feed premix.

The manufacture, distribution and use of the drug product takes place under regulated and controlled conditions which further mitigate against negative environmental consequences.

11. Alternatives to the proposed action

No potential adverse environmental impacts are foreseen with the production and use of efrotomycin premix.

The use of efrotomycin feed premix in the rations of swine will permit more rapid production of food (pork) with reduced

cost, making pork products more afforable to the consuming public.

Approval for the use of efrotomycin feed premix is justified from an environmental perspective and is preferable to non-approvals.

12. List of preparers

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13. Certification

The undersigned official certifies that the information presented is true, accurate, and complete to the best of the knowledge of the firm or agency responsible for preparation of the environmental assessment.

Date: June 23, 1986

Signature of responsible official: Kenneth G. Davis
(Kenneth G. Davis, D.V.M.)

Title: Director, Regulatory Affairs

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15. Appendices

a) Data summary charts

Structural formula on p. 4.

Molecular weight 1145.36

Molecular formula $C_{59}H_{88}N_2O_{20}$

Aqueous Stability - Half-lives in days

	Dark	Laboratory Light
pH 5	21.6	--
pH 7	49.2	26.0
pH 9	89.3	--
Methanol	106.5	87.4

Photostability - Half-lives in hours when exposed to sunlight

pH 5	0.25
pH 7	1.54
pH 9	2.52

Octanol/Water Partition Coefficient

pH 5	122
pH 7	59
pH 9	0.033

Vapor Pressures

4×10^{-14} torr at 25°C

Daphnia magna Toxicity, in ppm

	LC50	95% Conf. Interval	NOEC
Efrotomycin A_1	180	140-230	31
Photoirradiation products	---	---	96

Earthworm (Manure worm) Toxicity

Efrotomycin caused no dose-related toxicity or sub-lethal effects up to 1000 ppm. LC_{50} could not be calculated.

Phytotoxicity

Germination -- Efrotomycin had no effects on germination of pinto bean, rye grass, and cucumber, when tested up to 100 ppm versus the solvent and negative controls. For corn, the germination means were statistically different for the two control groups. Efrotomycin had no effect on the germination of corn up to 100 ppm versus the solvent control, but had statisti- cally significant lower germination, at the 5% level, than the negative control at the 60 and 100 ppm levels on day 1 only.

Growth -- Efrotomycin at 20 and 80 ppm affected the growth of the ryegrass and corn shoots and the growth of pinto bean and cucumber roots. Except for the dried root weights of cucumber roots at 4 ppm, efrotomycin caused no adverse growth effects at 0.4 or 4 ppm in any of the four species.

Respiration

Loamy sand -- Efrotomycin increased microbial respiration at 2 ppm and 20 ppm.

Silt loam -- Efrotomycin increased microbial respiration in the range tested, 0.05 to 20 ppm.

Nitrification

Loam and sandy loam soils -- No inhibition or enhancement of nitrification was observed over the range tested, 0.05 to 20 ppm.

Soil Binding

	SOILS				
Soil Texture	Silt Loam	Loam	Sand	Sandy Loam	Clay Loam
Screening Test					
% Sorbed	63	61	17	91	98
% Desorbed	28	30	--	8	1
Advanced Test					
Sorption:					
Freundlich K	13.4	8.0	--	46.4	336
n	1.27	1.10	--	1.03	0.955
k_d	18	8	--	51	294
k_{om}	847	334	--	4639	6390
k_{oc}	1460	576	--	7997	11017
Desorption:					
Freundlich K	24	13	--	86	514
n	1.30	1.03	--	1.04	0.981

Biodegradation -- Percent degraded in 98 days

	Sandy Loam	Clay Loam
^{14}C-efrotomycin, soil unamended	5.9	23.9
^{14}C-efrotomycin, glucose, amended	6.3	19.3
^{14}C-glucose, glucose, amended	45.4	41.3

Time for 50% degradation could not be calculated in either soil.

Rainbow Trout (Salmo gairdneri) Toxicity, in ppm

96-hour LC_{50} >100
96-hour NOEC >100

Algae (Selenastrum capricornutum) Toxicity, in ppm

96-hour EC_{50} 0.52
95% Confidence Interval 0.46 - 0.60
96-hour NOEC 0.13

Supplement to the Environmental Assessment

4. Description of the Proposed Action

Description of the Haarlem, Holland, Plant of Merck & Co., Inc.

Location. The Haarlem MSD plant is located in the municipality of Haarlem, near the North Sea coast, and approximately 20 km west (13 miles) from the city of Amsterdam. The plant is located east of the city Haarlem, on 18 hectare (approximately 45 acres) land near the river Spaarne in the area of the Waarderpolder, which is dedicated to industrial activities only. Population of the city of Haarlem is approximately 150,000.

Weather/Air Resources. Annual rainfall is 0.75 meter (30 inches). Mean July temperature is 18°C (64°F). Mean January temperature is 4°C (40°F). Prevailing wind directions are west and south-west (sea wind) at a windforce of 3 to 8 Beaufort.

Dutch governmental laws prescribe emission standards for hazardous pollutants. No significant air pollution-generating industries are located in the vicinity.

Water Resources. All water used for consumption, process, and sanitary equipment is obtained from the official county supplier. The water quality constantly meets the standards of potable water. For fire fighting, water can be withdrawn from the River Spaarne.

There are no injection wells in the plant property. No private wells are located in the vicinity of the plant. The sanitary and storm sewer systems are directly coupled to the municipal sewer system while the process effluents are treated before discharged into the municipal sewer. The discharge of waste water into the municipal sewer is covered by official permit by the municipality. All waste water from the municipal sewer is treated in the municipal waste water treatment system. No waste water is discharged into the river.

Land Resources

The land of the industrialized zone where the plant is located is reclaimed ("polder"). The soil is composed of layers of clay and sand.

6. Introduction of Substances Into the Environment

a. Substances Expected to be Emitted

PRODUCIL Formulation - Haarlem, Holland

The primary modes of introduction of chemical substances into the environment as a result of PRODUCIL formulation include air emissions, aqueous liquid wastes, and solid waste materials. Little or no introduction of chemical substances will occur via the soil route.

Air Emissions - The emission of dust to the atmosphere may result from the physical operations undertaken in the granulation and formulation of PRODUCIL. The granulation operations include mixing, blending, extrusion, wet milling, drying, and cracking (particle size reduction). The formulation operations include blending of the 20% Efrotomycin Magnesium Alginate Granules with excipients and packaging of the final formulation. The components of the formulation which may be emitted to the atmosphere in the form of dust are listed below:

Substance	CAS Registry Number
Alginic acid	9005-32-7
Wheat Middlings	
Efrotomycin	56592-32-6
Magnesium hydroxide	1309-42-8

Liquid Emissions - The major aqueous liquid waste streams from the formulation of PRODUCIL are the water washings of processing equipment and empty containers. The resulting wastewater may contain any of the components listed above under air emissions and may also include mineral oil (MI 7048).

Solids Emissions - There is no routine hazardous solid waste generated from the formulation of PRODUCIL except for: (1) residue left in equipment at the end of production run; (2) dust collected in dust collectors for air emission control; and (3) used packaging components for raw materials such as plastic bags. All such solid wastes are incinerated.

6b. Control Procedures for the Expected Emissions and Citation of Applicable Emission Requirements

Haarlem, Holland

Merck Sharp, & Dohme B.V. at Haarlem, the Netherlands operates regarding environmental matters within the Environmental Pollution Act.

Air Controls - Emissions to the atmosphere from manufacturing operations are controlled by equipment, such as dust collectors so that the facility conforms to applicable emission requirements. Emissions of dust from solids handling operations are controlled by the use of exhaust hoods and a series of filter bags.

Liquid Controls - All process wastewater and domestic sewage is discharged to the Municipal Sewage Wastewater Treatment Plant.

Solids Controls - All chemical wastes from the manufacturing area are transferred by closed vehicle to the Rotterdam incinerator.

Air Citations - Emissions to the atmosphere fall under the State Rules and Regulations Act with regard to Environmental Pollution.

Liquid Citations - The plant operates under the control of the Hoogheemraadschap Rijnland, which issues the necessary permits to discharge the plant effluent to the Municipal Sewage Water Treatment Plant.

Solids Citations - Permit for incineration is issued by the owner of the Rotterdam incinerator under the Law regulating processing of solid wastes.

6c Effect of Amendment Approval on Compliance with Current Emission Requirements

The formulation of PRODUCIL at Haarlem, Holland will not affect the facility's ability to comply with existing environmental regulations.