Monteban®

Environmental Assessment for the Use of 100 mg Narasin/kg Feed for Prevention of Coccidiosis in Broiler Chickens

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Monteban®

Environmental Assessment for the Use of 100 Mg Narasin/Kg Feed for Prevention of Coccidiosis in Broiler Chickens

1.0 Introduction

Narasin is the active ingredient in Monteban, a premix that is incorporated into chicken feed. Narasin is already approved for continuous oral use as Monteban (NADA 118-980) in chicken feed for the prevention of coccidiosis at doses up to 80 mg narasin/kg feed.

The following assessment is provided to support an application for the use of narasin (as Monteban) at a higher dose of 100 mg/kg (90 grams/ton) in the feed of broiler chickens for prevention of coccidiosis.

The environmental risk assessment has been conducted based on the VICH guidelines for both phase I (VICH GL6) and phase II (VICH GL38) assessments and on normal use of chicken litter as fertilizer in the United States.

2.0 Pattern of Use and Relevant Exposure Routes

Narasin will be continuously administered to broiler chickens via the feed for the prevention of coccidiosis.

In this risk assessment, the use and exposure scenario is continuous administration of narasin-amended feed during the production period of broiler chickens and subsequent application of chicken litter to agriculture land. This is the primary route for environmental exposure.

3.0 Description of the Product

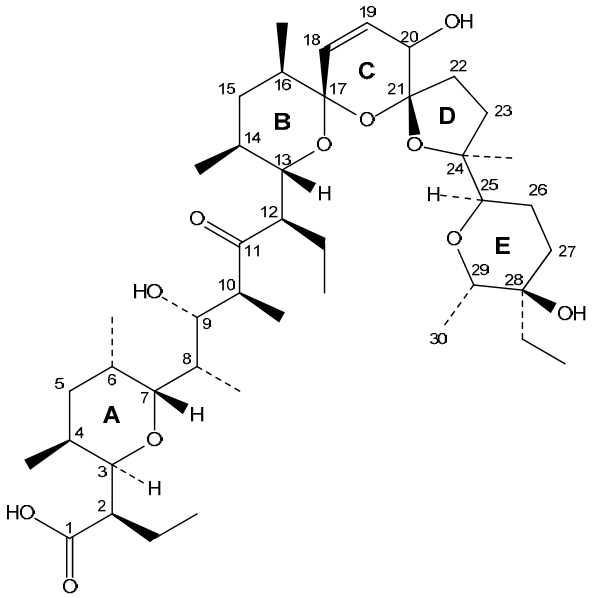
Monteban is a Type A premix to be incorporated into complete feed for broiler chickens. Narasin is the active ingredient in Monteban and is produced in a dried mycelial biomass form by a fermentation process. Monteban contains sufficient quantities of this dried fermentation product to achieve a narasin concentration of 45 gram/pound of premix. Monteban may also contain diluents such as soybean mill run, corn meal, montmorillonite clay, rice hulls, antidusting oil, mineral oil, densifiers, calcium stearate, calcium carbonate, and calcium silicate.

Narasin is produced by the fermentation of a strain of *Streptomyces aureofaciens*. The fermentation culture is harvested such that the narasin is combined with the mycelial cells of the producing organism and unused components of the feed-stock used in the fermentation to achieve growth of the organism. Thus, the dried mycelia or biomass form of narasin contains nutrients which are commonly found in broiler feedstuffs.

Narasin is a monocarboxylic polyether compound which complexes with monovalent alkali cations and has ionophoric activity.

® Monteban (narasin, Elanco Animal Health)

Name:	Narasin
Chemical Name:	(αβ, 2β, 3α, 5α, 6α)-α-ethyl-6-[5-[5-(5α-ethyltetrahydro-5β-hydroxy-6α-methyl-2H-pyran-2β-yl)-3”α,4,4”,5,5”α,6”-hexahydro-3’β-hydroxy-3”β,5α,5”β-trimethylspiro]furan-2(3H),2‘-[2H]pyan-6’(3’H),2”-[2H]pyran]-6”α-yl]-2α-hydroxy-1α,3β-dimethyl-oxoheptyl]-tetrahydro-3,5-di-methyl-2H-pyran-2-acetic acid Or (4*S*)-4-Methylsalinomycin
CAS Number (Narasin):	55134-13-9
Molecular Formula (acid form):	$C_{43}H_{72}O_{11}$
Molecular Weight:	765.03
Structural Formula for Narasin:	



4.0 Phase I Environmental Impact Assessment

Final Guidance for Industry #89 (CVM, 2001) published by the FDA, Center for Veterinary Medicine, and the VICH GL6 Phase I guidance for Environmental Impact Assessments (EIA’s) for Veterinary Medicinal Products (VMP’s) were consulted to conduct the Phase I Environmental Impact Assessment for the use of Monteban in broiler chickens. In this Phase I assessment, the maximum concentration of narasin in the manure and the soil has been considered. No metabolism or degradation in manure is assumed and a total residue approach is taken for the Phase I assessment. The initiation of a Phase II assessment is dependent upon the trigger established in the VICH GL6 guidance: if the predicted environmental concentration of the total residue in soil is greater than 100 µg/kg, then a Phase II assessment is warranted.

4.1 Concentration in Litter

The concentration of narasin in chicken litter was estimated using the following assumptions and calculations:

Concentration in Feed	100 mg/kg
Daily Feed Intake	0.13 kg/day
Duration of Dosing	Continuous
Manure Production Period	50 days
Amount of Diluent in Litter	15%
Daily Manure	0.13 kg

$$Concentration\ in\ Litter = \frac{Total\ Narasin\ Dosed}{Total\ Manure\ Produced \times Adjustment\ for\ Diluent\ in\ Litter}$$

$$= \frac{Concentration\ in\ Feed \times Feed\ Intake\ per\ Day}{Daily\ Manure\ Production \times 1.15}$$

$$= \frac{100\,\frac{mg}{kg} \times \frac{0.13\ kg}{day}}{0.13\,\frac{kg}{day} \times 1.15} = \frac{13\ mg}{0.15\ kg} = 87\,\frac{mg}{kg}$$

4.2 Concentration in Soil

The maximum concentration of narasin in the soil has been calculated using typical agronomy practices for application of poultry litter to land. Litter from poultry is applied to soil at a rate of 9200 kg/acre. Assuming a plow depth of 15 cm, the weight of the soil in an acre is approximately 910,500 kg. Assuming no degradation in manure or soil, the concentration of narasin in soil after application of poultry litter could be as high as 879 µg/kg.

Application Rate of Litter to Soil	9200 kg/acre
Plow Depth	15 cm
Weight of Soil in 1 acre × 15 cm	910,500 kg soil/acre

$$Concentration\ in\ Soil = \frac{Concentration\ in\ Litter\ \times Application\ Rate\ of\ Litter\ to\ Soil}{Weight\ of\ Soil/acre}$$

$$= \frac{87\frac{mg}{kg} \times 9200\frac{kg}{acre}}{910{,}500\frac{kg}{acre}} = 0.879\frac{mg}{kg} = 879\ \frac{\mu g}{kg}$$

Since the initial concentration in the soil is more than 100 µg/kg, a Phase II environmental risk assessment was conducted, as indicated in the FDA CVM Guidance for Industry #89 and the VICH GL6 Final Guidance.

5.0 Phase II Environmental Impact Assessment

Since an initial soil concentration of narasin of 879 µg/kg was calculated in the Phase I assessment, a Phase II environmental risk assessment has been conducted. Final Guidance for Industry #166 (CVM, 2006) published by the FDA, Center for Veterinary Medicine, and the VICH GL38 Phase II guidance for Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) were consulted to conduct the Phase II Environmental Impact Assessment for the use of Monteban in broiler chickens.

5.1 Tier A

5.1.1 Summary of Available Data

This section reviews environmental data that has been collected by the Sponsor and environmental data that has been reported in the published literature. While the Sponsor has collected environmental data with narasin since the late 1970's, only the definitive data used in the risk assessment have been summarized in the appendices and/or submitted in full study reports with the assessment. Information from the published literature is compared to the conclusions drawn from the data collected by the Sponsor.

5.1.1.1 Physical and Chemical Properties

The physical and chemical properties of narasin indicate that the molecule is water soluble and exists as a solid at normal environmental temperatures.

Physical and Chemical Data

Melting Point (Merck Index, 2006)	98 to 100°C		
Aqueous Solubility (Poole et al. 1981, Appendix A)	pH 7.0 102 mg/L pH 9.0 681 mg/L		
Log Kow (n-Octanol/Water Partition Coefficient by Shake Flask Method, OECD 107) (Study 151C-120, 2008, Appendix B)	pH 5	pH 7	pH 9
	4.79	4.85	5.06
Vapor Pressure	As a high molecular weight solid, narasin would be expected to have a negligible vapor pressure.		

5.1.1.2 Fate

5.1.1.2.1 Metabolism and Excretion

The metabolism and excretion of radiolabeled narasin by broiler chickens has been described in Study ABC-0260 (1984, Appendix C) and in Study T4H969301 (1994, Appendix D).

In Study ABC-0260, broiler chickens were administered ^{14}C-narasin in feed at a concentration of 80 mg/kg for 7 days. The excreta from days 4 through 7 was pooled, air-dried, homogenized and then assayed for total radioactivity and for parent using HPLC and microbiological assay. Relative quantities of metabolites were estimated from column and thin-layer chromatography. The concentration of narasin in manure as measured by HPLC was 12.1 mg/kg manure (on an air-dried basis) and 6.5 mg/kg manure on a wet weight basis. The concentration of narasin activity was 11.5 mg/kg (on an air-dried basis) by microbiological assay. Parent narasin accounted for 5% of the total radioactivity in the excreta and accounted for all of the microbiological activity. The remaining radioactivity was characterized by fractionation followed by thin layer chromatography. Several previously identified metabolites were present, two trihydroxylated narasins and four dihydroxylated narasins.

In Study T4H969301, broiler chickens were administered ^{14}C-narasin in feed at a concentration of 50 mg/kg for five days. Residues in the excreta were extracted and subjected to liquid chromatography to isolate narasin and its metabolites. Identification of residues was conducted using high performance liquid chromatography/ion spray/mass spectrometry. Using fractionation on a preparative silica column, fifteen separate metabolite peaks were identified and quantified using the amount of radioactivity in the fraction. The metabolites included a tetrahydroxylated narasin, several di- and trihydroxylated narasins and the corresponding derivatives of narasin factor B. Narasin B is narasin with the hydroxyl group on ring C oxidized to the ketone. About 50% of the total radioactivity in the

excreta was characterized as hydroxylated metabolites and about 3% as parent narasin. The remaining radioactivity included 4% that was non-extractable, 6 to 8% that was more polar and left in the aqueous methanol, and minor fractions in the LC in concentrations too low to characterize by HPLC/ISP/MS/LSC.

5.1.1.2.1.1 Biological Activity of Chicken Metabolites

As discussed above, microbiological characterization in Study ABC-0260 shows that the narasin metabolites (of which the majority are di- and trihydroxylated narasin) are not biologically active, because the amount of microbiological activity in the excreta expressed as narasin equivalents was equal only to the actual amount of narasin itself in the excreta. Therefore, the excreted metabolites do not contribute to the microbiological activity in the excreta.

Wong et al. (1977) demonstrated that the ionophoric activity of narasin could be characterized in isolated rat liver mitochondria. In this assay, ATPase activity, or ATP hydrolysis, induced by the addition of either valinomycin or monazomycin and alkali metal cation, was reduced by narasin. The effects of narasin metabolites on ATPase and oxygen uptake in rat liver mitochondria were then further characterized by Wong (1978, Appendix E). In this study, four narasin metabolites were tested: metabolite F (a dihydroxy narasin), NM-3 (a dihydroxy narasin), NM-2 (a trihydroxy narasin), and the fourth was a mixture of NM-6 (a dihydroxy narasin) and NM-3. These four were individually tested in terms of their effects on ATPase activity and oxygen uptake upon oxidation of malate and glutamate in rat liver mitochondria. The results indicated that the four narasin metabolites exhibited relatively weak effects on ATPase activity and oxygen uptake rates of rat liver mitochondria. The relative ionophoric activity in rat liver mitochondria of the four metabolite preparations was only 0.47% or less compared to narasin (Wong 1978).

Manthey and Goebel (1982, Appendix F) also characterized the biological activity of di- and trihydroxylated metabolites of narasin isolated from excreta from chickens fed ^{14}C-narasin. Six metabolites were evaluated by a standard narasin thin-layer chromatography bioautographic assay system against *Bacillus subtilis*, a microbial species that is susceptible to narasin. Metabolites (equivalent to 500 ng of narasin based on radioactivity) were applied to TLC plates alongside 25 ng of narasin. None of the metabolites exhibited zones of antimicrobial activity while narasin had a zone of inhibition demonstrating its activity. The metabolites were, therefore, at least 20 times less active than narasin.

In summary, narasin is extensively metabolized by chickens to numerous minor metabolites. Narasin makes up 3 to 5% of the excreted residue. The metabolized residue including several di-and trihydroxylated narasin structures is essentially inactive. Therefore, for the purposes of this risk assessment, the measured amount of narasin in chicken manure as measured in Study ABC-0260 will be used to calculate the predicted environmental concentrations. In that study, the narasin concentration in the feed was 80 mg/kg and the amount of narasin in the chicken manure was 6.5 mg/kg on a wet weight basis. For the risk assessment, that number will be adjusted to 8.1 mg/kg to represent a concentration of 100 mg narasin/kg in the feed.

5.1.1.2.2 Degradation

5.1.1.2.2.1 Degradation in Chicken Manure

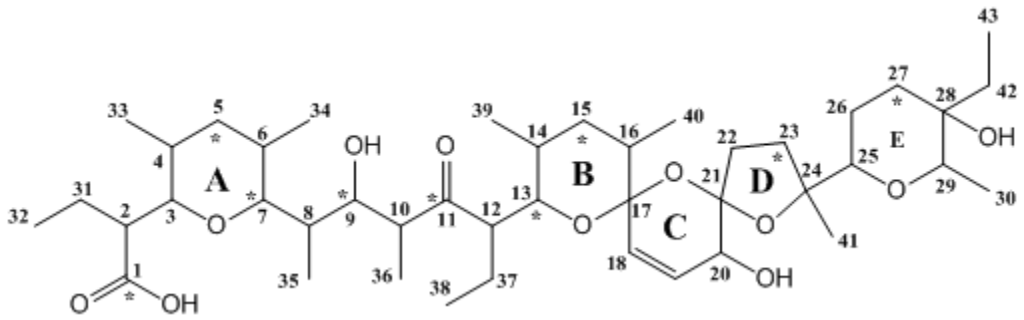
The biodegradation of narasin in fresh chicken manure and in a mixture of fresh chicken manure and litter over 35 days has been examined in Study 151E-125 (2011, Appendix G). In that study, flasks containing ^{14}C-narasin (0.5 mg/kg) were incubated with the chicken manure and with a mixture of chicken manure and litter under aerobic conditions at 20°C for 35 days. At the end of 35 days, only 1.45 to 2.76% of the applied radioactivity was evolved as $^{14}CO_2$ in the manure and manure/litter test systems, respectively. By day 7, the amount of narasin in the test systems was 47 to 56% of the applied radioactivity, and at the end of the study on Day 35, the amount of narasin was 37 to 50% of applied radioactivity (data includes both test systems). There were similar amounts of degradation in the manure and manure/litter test systems. Using fractionation, only two major metabolites (> 10% of applied radioactivity) were observed. HPLC-MS/MS techniques were used to identify these two major metabolites as a trihydroxylated narasin and narasin with the A ring removed. The other degradation products were less than 5% of applied radioactivity.

The disappearance of narasin reached approximately 50% by Day 7, and appeared to plateau for the remainder of the study. While it is unknown as to whether this plateau was due to an artifact of the test system, the parameters used to characterize the viability of the test systems do indicate that there was some decrease in microbial viability over the duration of the study. This degradation study is considered to be a conservative estimate of the potential degradation of narasin in manure because, in a chicken barn, fresh manure would be continuously excreted, replacing the older microbial population in the litter. Additionally, this study was conducted at lower temperatures than those that can exist in the litter matrix of a chicken house or in a composting pile or windrow.

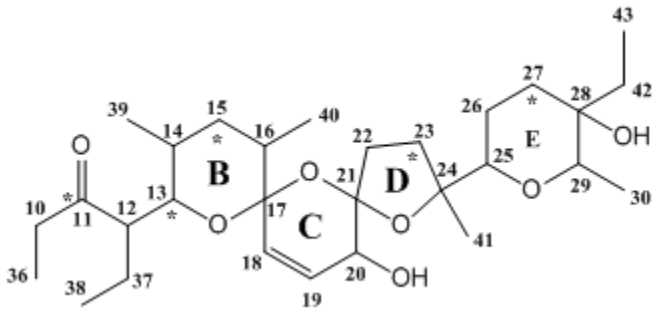
Biological Activity of Narasin Transformation Products formed in Chicken Excreta

The major metabolites identified were a trihydroxy narasin and narasin with the A ring removed. Trihydroxy narasin has already been discussed as having no microbiological activity. Based on the requirement for ionophoric activity for microbiological efficacy and the structure activity relationship of narasin, the degradation product without the A ring is also considered to have no biological activity.

Structure of ^{14}C narasin used in the chicken manure degradation study (asterisks indicate potential sites of ^{14}C incorporation):



Structure of Degradant:



This same structure has been identified as a microbial enzymatic degradation product of the narasin-related ionophore, salinomycin (Vértesy et al. 1987). Narasin differs from salinomycin in only the presence of an additional methyl group on the A ring. In this report, salinomycin was incubated with an enzymatic preparation from *Pseudomonas stutzeri*, a soil bacteria which degrades salinomycin. This soil bacteria is naturally resistant to salinomycin. After incubation with the enzymatic preparation, there was a decrease in both the amount of salinomycin (as observed on thin layer chromatography, TLC) and the microbiological activity. Additionally, a new substance was

observed on TLC. This degradation product was isolated and evaluated with NMR and mass spectrometry. The structure was determined to be the same as that found for the narasin degradant without the A ring in the chicken manure degradation study. The results of this study indicate that the microbiological activity of the degradation product is at least less than that of salinomycin.

Miyazaki et al. (1976) demonstrated the importance of the carboxylic acid associated with the A ring in a structure activity relationship investigation of salinomycin. When Miyazaki et al. substituted a methyl ester or a methyl alcohol for the carboxylic acid group on the A ring, the binding affinity of salinomycin for cations decreased at least two orders of magnitude and the minimum inhibitory concentrations (MICs) for *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, and *Mycobaterium phlei* increased from a range of 1.56 to 12.5 mg/L to more than 100 mg/L for all tested species. Esterification or reduction of the carboxylic acid associated with the A-ring in salinomycin results in extensive or complete loss of biological activity against susceptible bacteria. Since the A ring contains the carboxylic acid in both salinomycin and narasin, loss of the A ring would result in extensive or complete loss of biological activity. Therefore, the narasin transformation product found in chicken manure with loss of the A ring and its associated carboxylic acid is very likely to lack biological activity.

Martinek et al. (2000) used NMR techniques to investigate the three dimensional structure of narasin and how the molecule complexes cations. Six oxygens of narasin were involved in the cation complexation process, including two from the carboxylic acid and the hydroxyl group closest to the A ring. In the identified degradation product, these three oxygens have been removed from the molecule. Without three of the six oxygens involved in complexation of cations, the ionophoric activity in this narasin transformation product is expected to be very low or completely lost.

Therefore, based on the results of Study 151E-125, at least 50% of narasin, and narasin activity, excreted from chickens will be quickly eliminated by microbial degradation in the manure.

5.1.1.2.2.2 Degradation in Soil

The biodegradation of narasin in three soils varying in pH and textural characteristics has been examined for 84 days at 20°C in aerobic conditions (Study 802374, 2002, Appendix H). Soil was treated with approximately 1.6 mg/kg of ^{14}C-narasin. Over the course of the study,

narasin was mineralized, as evidenced by evolution of 64%, 19% and 54% of the applied radioactivity as $^{14}CO_2$ in the sandy loam, silt loam, and clay loam soils, respectively. Several unidentified transformation products were observed, none of which accounted for more than 16% of the applied radioactivity using HPLC with radiometric detection. At the end of the study, the sum of transformation products observed in the extracts made up 9.19%, 34.39%, and 15.56% of the applied radioactivity in the sandy loam, silt loam and clay loam soils, respectively, while 6.91%, 26.22%, and 14.94% of the applied radioactivity was identified as narasin. The half life of narasin ranged from 21 to 49 days in the three soils. The DT90 values were 69 to 162 days. The results of this study demonstrate that narasin degrades in soil and is degraded to CO_2.

In an older study, Study 276A-3480-22 (1977, Appendix I), greenhouse soil fortified with 10 mg/kg narasin (air-dried weight) was incubated under field moisture conditions at temperatures ranging from 21° to 30°C. In this study, the concentration of narasin was determined by microbiological assay. Microbiological activity declined rapidly such that less than 10% of the original level was present after 26 days. The degradation rate was somewhat faster in this greenhouse soil degradation study than in Study 802374. The soil transformation products of narasin that formed in soil did not retain any significant biological activity.

Therefore, narasin degrades in soil and is not expected to accumulate with successive yearly applications of chicken litter.

5.1.1.2.3 Soil Adsorption

The adsorption of narasin to soil was evaluated in five different soils (Study 151E-107, 2008, Appendix J) following OECD Guideline 106. The resulting adsorption Kd values ranged from 5.4 to 150 L/kg and the desorption Kd values ranged from 7.1 to 108 L/kg. The adsorption Koc ranged from 507 to 3670, with a mean value of 1619 (log Koc = 3.2). Even though narasin only moderately adsorbs to soil, significant leaching into groundwater is not expected due to the rapid degradation of narasin in soil.

5.1.1.2.4 Hydrolysis and Photolysis

Narasin is stable in water at pH 7 and 9, but is subject to hydrolysis at pH 5 (Poole et al. 1981, Appendix A). The stability of narasin is corroborated by analytical results in the static aquatic toxicity tests with daphnids and fish over 48- and 96-hour exposure periods. Photolysis of narasin was evaluated when exposed to simulated sunlight (Poole et al. 1981). In that study, the half-life was approximately 1.5 days. However, narasin

concentrations were stable over the 72-hour algae growth inhibition test (Study 802573, 2002, Appendix Q) under constant light conditions. While hydrolysis and photolysis might contribute to the dissipation of narasin in the environment under certain conditions, these routes of dissipation will not be used to calculate predicted environmental concentrations of narasin for the Phase II assessment.

5.1.1.2.5 Bioconcentration

The empirically-determined log n-octanol/water partition coefficient (log Kow) of narasin ranges from 4.79 to 5.06 (shake-flask design, Study 151C-120, 2008, Appendix B). The log Kow value expected for narasin based on its solubility in water is substantially lower than the range found in the shake flask study. The value derived from a comparative HPLC analysis (Study 341587, 2002, Appendix U) is higher than the value determined in the shake flask study. Therefore, estimation of Kow for the ionophorous narasin appears to be influenced by the conditions under which it is evaluated.

Considered alone, the magnitude of its Kow value indicates that there is some potential for narasin to bioconcentrate in tissues. However, narasin is water soluble and extensively metabolized and degraded, indicating that significant bioaccumulation is unlikely to occur. Little tissue accumulation was observed in Study T4H969301 (1994, Appendix D) in which chickens were fed a diet with 50 mg/kg ^{14}C-narasin in their feed for five days. At the end of five days, the highest amount of radioactive residues was in the liver with a mean concentration of 0.319 mg/kg. Fat was the next highest with 0.116 mg/kg. In fat, narasin was the predominant residue representing approximately 60% of the total radioactivity in that tissue. In the excreta only 3% of the total radioactivity was identified as narasin, while a total of 50% was identified as di-, tri-, and tetrahydroxy derivatives of narasin. The remaining radioactivity was spread out among several minor peaks. Tissue residue amounts were similar in cattle as in chickens (Study ABC-0137 1982, Appendix K). Extensive metabolism to several minor hydroxylated narasin metabolites was also observed in cattle, dogs and rats (Studies ABC-0126 and ABC-0127 1986, Appendix L). Therefore, despite its measured octanol-water partitioning coefficients, narasin is not expected to bioaccumulate given its susceptibility to extensive oxidative metabolism and limited accumulation in tissues of several species.

Fate Data

Abiotic Degradation (Poole et al., 1981, Appendix A)	Hydrolysis Stable at pH 7.0 and 9.0 Half-life of ~3.5 days at pH 5.0 Photolysis At pH 7.0 half-life is ~1.5 days		
Degradation in Chicken Excreta (Study 151E-125, 2011, Appendix G)	Incubated aerobically for 35 days Approximately 50% degraded after 7 days		
Soil Adsorption (Study 151E-107, 2008, Appendix J)	Soil	K_d	K_{oc}
	Clay Loam pH 7.2, OC 5%	25	507
	Sandy Clay Loam pH 6.2, OC 1.9%	22	1171
	Clay Loam pH 5.2, OC 4.1%	150	3670
	Loamy Sand pH 5.7, OC 1.3%	26	1971
	Clay pH 7.7, OC 0.7%	5.4	778
	Mean	*46*	*1619*
Degradation in Soil (Study 802374, 2002, Appendix H)	Three soils were dosed with ^{14}C-narasin and incubated aerobically for 84 days.		
	Half-life of narasin	21 to 49 days	
	Mineralization to $^{14}CO_2$	19 to 64 % AR	
	Bound Residues Day 84	18 to 25 % AR	
	Narasin Day 84	6.91 to 26.22 % AR	

%AR: Percent of applied radioactivity

5.1.1.3 Toxicity

5.1.1.3.1 Soil Organisms

Definitive studies in soil microflora, plants, and earthworms have been conducted (Studies 802458, 802442, 802568, and 1982.6391), are described below, and are considered for the PNEC determination.

In a study following OECD guidelines 216 and 217 (Study 802458, 2002, Appendix M), soil was amended with narasin at two concentrations, 3784 and 17,430 µg/kg. There were no biologically important effects on carbon or nitrogen transformation by soil microflora at either concentration.

In a phytotoxicity test (Study 802442, 2002, Appendix N) following OECD guideline 208, winter oat, mung bean and radish were exposed to narasin incorporated in a sandy loam soil at concentrations of 375, 3381, and 29260 µg/kg. Endpoints were number of seedlings that emerged and the fresh weight of seedlings (shoot only). In winter oat, there was no effect on emergence at any concentration. The growth of seedlings in the 29260 µg/kg treatment was significantly decreased compared to control by 38%. Thus, for winter oat, both the LC50 based on emergence and the EC50 based on growth were greater than the highest concentration tested, 29260 µg/kg. In mung beans, there was a concentration-dependent decrease in the percent emergence compared to control, the decrease in emergence was 40% at the highest concentration. Likewise, there was a concentration-dependent decrease in shoot weight of mung beans, the decreases were 15%, 27%, and 67% compared to control in the 375, 3381, 29260 µg/kg treatments, respectively. Thus, for mung beans, the LC50 based on emergence and the EC50 based on growth were >29260 µg/kg and 8990 µg/kg, respectively. In radish, there was no effect on emergence at the two lower concentrations while no seedlings emerged at the highest concentration, 29260 µg/kg. In the report for this study, an LC50 of 5.07 µg/kg is calculated using the probit method. However, given the lack of partial responses, the probit method is not appropriate. Instead, a binomial method, taking the mean of the 3381 and 29260, estimates an LC50 of 16300 µg/kg. For radish seedling weight, concentrations of 375 and 3381 µg/kg resulted in 13% and 25% decrease in weight compared to control (no seedlings emerged at 29260 µg/kg). The EC50 for growth is, therefore, greater than 3381 and less than 29260 µg/kg. Using 100% growth inhibition for seedlings that did not emerge, including those at the highest concentration, a more precise EC50 can be calculated using the ICp approach (Norberg-King, 1993). The resulting EC50 is 6183 (see discussion in Appendix N). The lowest median effect concentration for plant toxicity is, therefore, 6183 µg/kg for growth in radish.

Supplementary information from older studies provides additional context for the potential of narasin to have phytotoxic effects. In 14 test species, narasin had no effects on plants at 150 µg/kg, slight effects at 1500 µg/kg and severe phytotoxicity at 10000 and 40000 µg/kg. In another older study, litter from the pens of broilers fed with ration containing 100 mg narasin/kg was applied to a field prior to cultivation. When the seeds were planted, the narasin concentration was estimated to be 50 to 100 µg/kg (as measured by microbiological assay) and there was no phytotoxicity observed in the seven plant species evaluated.

The effects of narasin on earthworms in subchronic and chronic exposures were evaluated in Study 802568 and Study 1982.6391. In Study 802568 (2002, Appendix O), earthworms (*Eisenia fetida*) were exposed to soil fortified with narasin at concentrations ranging from 4300 to 270,900 µg/kg for 14 days following OECD guideline 207. The endpoints

were survival and body weight. The percent mortality in the solvent and blank controls was 5% and 7.5%, respectively. The percent mortality in the narasin treatment levels was 12.5%, 22.5%, 87.5%, 100%, and 100% for the 4300, 34300, 67700, 137700, and 270900 µg/kg, respectively. The LC50 was determined to be 46400 µg/kg and the no observed effect concentration for survival was 4300 µg/kg. The mean weight change in the solvent and blank controls was -9.7 and -8.7%, respectively. The mean weight change in the 4300, 34300, and 67700 was -8.1, -4.7 and -30.7%, respectively. The EC50 for weight change was greater than 67700 µg/kg and the NOEC was 34300 µg/kg.

In an earthworm reproduction study following OECD guideline 222, mature *Eisenia fetida* were exposed to narasin incorporated in soil for 28 days (Study 1982.6391, 2011, Appendix P). Soil concentrations of narasin were 3100, 6300, 13000, 25000, and 50000 µg/kg. At the end the 28-day exposure, the adult worms were removed, assessed for survival and weighed. For another 28 days, the soil was incubated under the same conditions allowing any cocoons deposited by the adult worms to hatch. At the end of the second 28 days, the young worms were separated from the soil by means of a Berlese funnel and enumerated. Endpoints of the study include survival and body weight change in the adults and number of young worms produced per surviving female. There was a significant effect on survival at 50000 µg/kg, with only 25% of adult worms surviving this exposure. The LC50 was calculated to be 41000 µg/kg. There was no significant decrease in body weight or offspring. Therefore, the NOEC for the study was 25000 µg/kg based on survival in the adult worms. Supplementary subchronic data from older studies indicate that *Lumbricus terrestris* are slightly more sensitive than *E. fetida* with LC50 values between 17900 and 40000 µg/kg.

5.1.1.3.2 Aquatic Organisms

The toxicity of narasin has been assessed in invertebrates, fish, and algae.

The green alga, *Pseudokirchneriella subcapitata (*formerly *Selenastrum capricornutum)*, was exposed to narasin under static conditions for 72 hours in Study 802573 (2002, Appendix Q) that followed OECD guideline 201. Concentrations of narasin in the treatment levels were fairly stable over the study: 100% to 108% of theoretical concentrations at test initiation and 89% to 103% of theoretical at the end of the study. The mean measured concentrations ranged from 35 to 4170 µg/L (the lowest treatment levels, 35 and 230 µg/L, were below the quantification limit and the theoretical concentrations were used). Concentration-dependent decreases in biomass and growth rate were observed. The EC50 values for biomass (area under the curve) and growth rate were 770 and 2920 µg/L, respectively. The NOEC value for both biomass and growth rate was estimated to be 230 µg/L.

In Study C01883 (1983, Appendix R), *Daphnia magna* were exposed to narasin in a 48-hour static toxicity test using procedures that generally followed guidelines available at the time (ASTM, 1980). Procedures were similar to those in the current OECD guideline 202. The treatment levels of narasin ranged from mean measured concentrations of 4690 to 42180 µg/L. The 48-hour median effective concentration for immobilization was determined to be 20560 µg/L.

In Study F05283 (1983, Appendix S) rainbow trout, *Oncorhynchus mykiss*, were exposed to narasin in a static toxicity test using procedures that generally followed guidelines available at the time (ASTM, 1980). Procedures were similar to those in the current OECD guideline 203. The exposure duration was 96 hours to a mean measured concentration range of 103 to 5260 µg/L. Sublethal effects and mortalities were noted for fish exposed to 316 µg/L and higher. The LC50 was determined to be 2230 µg/L.

In Study F05183 (1983, Appendix T) bluegill, *Lepomis macrochirus*, were exposed to narasin in a static test toxicity test for 96 hours using procedures that generally followed guidelines available at the time (ASTM, 1980). Procedures were similar to those in the current OECD guideline 203. The mean measured concentrations, which were stable over the duration of the test, ranged from 880 to 9550 µg/L. Mortality and sublethal effects including hypoactivity and labored respiration were observed in fish at concentrations of 2800 µg/L and higher. The LC50 was determined to be 5020 µg/L.

The fish and daphnid studies described above are used in the risk assessment because they were conducted using standard guidelines and in compliance with Good Laboratory Practices. These studies were conducted using mycelial narasin. There are older, supplementary studies that were conducted with crystalline narasin that give results that are consistent with the studies described above.

Ecotoxicity Data

Terrestrial Effects Studies				
Respiration and Nitrogen Transformation Tests (28 days) (Study 802458, 2002, Appendix M)	Exposure to concentrations up to 17,430 µg/kg had results that varied less than 25% from controls for carbon or nitrogen transformation			
Terrestrial Plants – Seedling Growth (Study 802442, 2002, Appendix N)		Emergence	Growth (Shoot Weight)	
		LC50 µg/kg	EC50 µg/kg	NOEC or LOEC µg/kg
	Winter Oats	>29,260	>29,260	NOEC 3,381
	Mung Beans	>29,260	8,990	LOEC 375
	Radish	16,300	6,183	NOEC 375
Earthworm 14-day Growth and Survival (Study 802568, 2002, Appendix O)	LC50 46,400 µg/kg NOEC 4,300 µg/kg			
Chronic Earthworm Reproduction (Study 1982.6391, 2011, Appendix P)	LC50 41,000 µg/kg NOEC 25,000 µg/kg			
Aquatic Effects Studies				
Algal Growth Inhibition (Study 802573, 2002, Appendix Q)	Biomass EC_b50 770 µg/L $NOEC_b$ 230 µg/L Growth Rate EC_r50 2,920 µg/L $NOEC_r$ 230 µg/L			
Daphnia immobilization (Study C01883, 1983, Appendix R)	*Daphnia magna* EC50 20,560 µg/L			
Fish Acute Toxicity (Study F05283, 1983, Appendix S)	Rainbow Trout LC50 2,230 µg/L			
Fish Acute Toxicity (Study F05183, 1983, Appendix T)	Bluegill LC50 5,020 µg/L			

5.2 PEC Calculations and Refinements (Exposure Assessment)

5.2.1 Soil

The $PEC_{soil\text{-}initial}$ for continuous administration to broiler chickens of 100 mg narasin/kg feed for prevention of coccidiosis was calculated in the Phase I assessment as 879 µg/kg using total narasin residues in litter of 87 mg/kg. Per the VICH guideline (GL38), this value can be refined using metabolism, degradation in manure and degradation in soil.

5.2.1.1 Refinement by metabolism

Because narasin is extensively metabolized by broiler chickens to many minor metabolites which do not have biological activity, the amount of narasin measured in manure based on Study ABC-0260 will be used. The amount of narasin in chicken manure is 8.1 mg/kg (on a wet weight basis).

5.2.1.2 Refinement by degradation in excreta

Narasin is degraded in chicken manure under aerobic conditions such that after 7 days approximately 50% of narasin and narasin activity is gone. Two major degradation products are formed, but since they are not biologically active, they will not be considered in the risk assessment. Additionally, because excreted narasin is such a small percent of dosed narasin, these degradation products are a small part of the total inactive residue. A cycle of broiler chickens is typically in the barn for 50 days. It will be assumed that at least 50% of the remaining narasin in the excreta is degraded after excretion and in the barn.

The concentration of narasin in litter at the end of the broiler cycle can be calculated to be 3.5 mg/kg as shown below:

$$Litter\ concentration_{end\ of\ 50-day\ cycle} = \frac{Manure\ Concentration\ \times M}{Litter\ Dilution}$$

Where:

Manure Concentration:	8.1 mg/kg
M:	Manure Degradation Removal: 50%
Litter Dilution:	1.15
t:	Time for degradation, 25 days

$$Litter\ concentration_{end\ of\ 50-day\ cycle} = \frac{8.1\ mg/kg\ \times 0.5}{1.15} = 3.5\ mg/kg$$

5.2.1.3 Refinement by degradation in soil

Narasin degraded rapidly in soil. However, a conservative approach will be taken assuming that crops are sown immediately after litter containing narasin residues is applied to soil. This approach is also appropriate for assessing risk to earthworms and soil microflora, species which will be present in soil at the time of application. Therefore, the soil concentration will not be refined for degradation in soil.

5.2.1.4 Calculation of $PEC_{soil\text{-}refined}$

Using the refined litter concentration, the $PEC_{soil\text{-}refined}$ can be calculated:

$$Concentration\ in\ Soil = \frac{Concentration\ in\ Litter\ \times Application\ Rate\ of\ Litter\ to\ Soil}{Weight\ of\ Soil/acre}$$

$$= \frac{3.5\frac{mg}{kg} \times 9200\frac{kg}{acre}}{910{,}500\frac{kg}{acre}} = 0.035\frac{mg}{kg} = 35\ \frac{\mu g}{kg}$$

5.2.2 Groundwater

Substantial proportions of narasin are degraded to carbon dioxide in soil, with most of the remaining residues undergoing primary metabolism and/or sorption. Narasin itself is moderately sorbed to soil, with a mean Kd value of 46 in various soils. Given these characteristics and the very low initial concentration of narasin in soil, it is very unlikely that significant levels of narasin would be found in groundwater.

5.2.3 Surface Water

Movement of narasin from soil to surface water may occur through runoff following rainfall events. A scenario of 1% runoff of compound from 10 acres of soil into a one-acre pond which is 2 m deep was considered. A one-acre pond that is 2 m deep has a volume of 8,100,000 L. Using the concentration of narasin in litter and the application rate of litter per acre, the following calculation was performed to estimate the concentration of narasin residues in the pond:

$$[Narasin]_{pond} = \frac{[Narasin]_{litter}\ \times kg\ manure\ per\ acre\ \times 10\ acres\ \times 0.01}{8{,}100{,}000\ L}$$

$$[Narasin]_{pond} = \frac{3.5\frac{mg}{kg} \times 9200\frac{kg}{acre}\ \times 10\ acres\ \times 0.01}{8{,}100{,}000\ L} = 0.398\ \mu g/L$$

Therefore, the narasin $PEC_{surface\ water}$ refined for metabolism and degradation in excreta is 0.398 µg/L. Because narasin has only a moderate propensity to adsorb to soil and sediment, all of the loss is assumed be present in the aqueous layer in the pond.

Reports of narasin in surface water have been published. In a survey of several sites of the Cache la Poudre River in Colorado, including ones located in agricultural areas, Kim and Carlson (2006, 2007) report the highest narasin concentration in surface water as 0.038 µg/L.

Reports from the published literature, therefore, support the use of 0.4 µg/L as an appropriately conservative estimate of the maximum possible surface water concentration of narasin.

5.3 PNEC Calculations (Effect Assessment)

5.3.1 Terrestrial

The assessment factors applied to the toxicity values and the PNECs calculated for terrestrial species are tabulated below. The assessment factors are from the VICH GL38 Phase II guidance for Environmental Impact Assessments.

Terrestrial PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Soil Microflora	<25% change from control = 17,430 µg/kg	1	17,430 µg/kg
Plants, growth	EC50 = 6,183 µg/kg	100	61.8 µg/kg
Earthworms	NOEC = 25,000 µg/kg	10	2500 µg/kg

Plants are the most sensitive species tested in the terrestrial compartment.

5.3.2 Aquatic

The assessment factors used and the PNECs calculated for aquatic species are tabulated below.

	Toxicity endpoint	Assessment Factor	PNEC
Algal Growth	EC50 = 770 µg/L	100	7.7 µg/L
Daphnia acute	EC50 = 20,560 µg/L	1000	20.6 µg/L
Fish Acute	LC50 = 2230 µg/L	1000	2.2 µg/L

The lowest predicted no effect concentration in the aquatic environment is 2.2 µg/L based on fish.

5.4 Risk Characterization

5.4.1 Soil

The predicted concentration of total residues of narasin in soil (PEC_{soil}) after application of litter from broilers treated narasin at 100 mg/kg in feed for prevention of coccidiosis is 879 µg/kg. When metabolism and degradation in chicken excreta is considered, the $PEC_{soil\text{-}refined}$ can be calculated to be 35 µg/kg.

Of the terrestrial species tested, plants were the most sensitive to exposure to narasin. Using an application factor of 100 on the lowest median effect concentration, the predicted no-effect concentration for plants is 61.8 µg/kg. The

PEC/PNEC ratio for terrestrial organisms using the refined PEC_{soil} (35 µg/kg) is less than 1. This is corroborated by the older phytotoxicity data in which 14 species tested had no effects at 150 µg/kg and only slight effects at 1500 µg/kg.

5.4.2 Surface Water

The maximum predicted concentration of total residues of narasin in surface water ($PEC_{refined\text{-}surface\ water}$) is 0.4 µg/L. Algae were the most sensitive aquatic species tested with narasin. However, the lowest predicted no effect concentration was 2.2 µg/L for fish. The PEC/PNEC ratio for aquatic organisms using the refined $PEC_{surfacewater}$ is less than 1.

Because the lowest PNEC for each environmental compartment is higher than the PEC in that compartment, there is no significant risk to organisms in that compartment.

PEC/PNEC Ratios

Compartment	Species	PEC*	PNEC	PEC/PNEC Ratio
Terrestrial	Plants	35 µg/kg	61.8µg/kg	0.57
Surface Water	Fish	0.4 µg/L	2.2 µg/L	0.2

* refined for metabolism and degradation in excreta

5.5 Conclusions of Tier A

The environmental impact from the continuous use of Monteban Type A premix in chicken feed at a concentration of 100 mg narasin/kg feed in broiler chickens to prevent coccidiosis in high intensive rearing situations has been evaluated. The pathway for introduction of narasin into the environment considered in this risk assessment was via the application of chicken litter as fertilizer to soil. Runoff to surface water from that soil was also considered.

The predicted environmental concentrations were refined to consider metabolism and degradation in excreta during the litter holding period. The predicted environmental concentration of narasin in soil is 35 µg/kg after refinement. The predicted environmental concentration in surface water following rainfall events is 0.4 µg/L after refinement. This predicted surface water level was higher than concentrations found in surface water and reported in published literature. These predicted environmental concentrations of narasin in soil and surface water are lower than the predicted no-effect concentrations for terrestrial and aquatic organisms. Since narasin is extensively metabolized by animals, and degraded in manure and soil, it is not expected to persist in the environment or accumulate in environmental species.

Treatment of broiler chickens with Monteban Type A premix to prevent coccidiosis is not expected to result in any substantial effects on terrestrial or aquatic organisms exposed to narasin from chicken litter used to fertilize cropland soil.

6.0 Information on Environmental Assessment Expert

The following individual is responsible for the information in the Environmental Assessment Report for narasin used as Monteban for prevention of coccidiosis:

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Date: 23 September 2011

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Degrees:

BS	Chemistry, Tulane University	1988
PhD	Pharmacology/Toxicology, University of Mississippi	1996

Current and Previous Appointments:

Principal Research Scientist, Environmental Risk Assessment, Lilly Research Laboratories (2010 to present)

Senior Research Scientist, Environmental Risk Assessment, Lilly Research Laboratories (2005 to 2010)

Senior Toxicologist, Senior Research Scientist, Environmental Science/Nonclinical Safety Assessment, Lilly Research Laboratories
(2000 to 2005)

Editorial Board of Environmental Toxicology and Chemistry

Research Scientist, Research Institute of Pharmaceutical Sciences, U. Mississippi
(1996 to 1999)

Publications:

Fifteen publications and numerous presentations and posters in the field of environmental toxicology.

7.0 References

Note: References are found in Attachment 1.

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Appendices

Appendix A – Poole et al. 1981. Solubility, hydrolysis, & photolysis of narasin. Report Date: 1981.

Performing Laboratory: Lilly Research Laboratories

Methods:
Solubility: An excess of narasin was added to sterile 0.01 M pH 7.0 and pH 9.0 buffers and stirred at 25°C. At 24-hour intervals, samples were removed, filtered, and assayed.

Hydrolysis: Narasin solutions were prepared in sterile 0.01M pH 5.0, 7.0, and 9.0 buffers, sealed in glass ampoules and stored in the dark at 25°C. Samples were removed and assayed at various intervals up to 30 days.

Photolysis: Narasin solution was prepared in sterile 0.01M pH 7.0 buffer and exposed to a combination of sunlamps and black lights which produced an ultraviolet spectral energy distribution similar to natural sunlight. Samples were withdrawn and assayed a various time periods.

Results:
Solubility: Narasin was soluble in water to a maximum concentration of 102 mg/L at pH 7.0 and 681 mg/L at pH 9.0 in 24 hours or less at 25°C.

Hydrolysis: Narasin was very stable in water at pH 7.0 and 9.0 but hydrolyzed at pH 5.0 with a half-life of approximately 3.5 days.

Photolysis: Narasin photodegraded in water at pH 7.0 with a half-life of approximately 1.5 days.

Appendix B – Study 151C-120: Narasin – Determination of the n-Octanol/Water Partition Coefficient by the Shake Flask Method. OCED Guideline 107. Report Date: January, 2008

Performing Laboratory: Wildlife International, Ltd.

Test Article: Crystalline narasin

Methods:
The octanol/water partition coefficient (P_{ow}) of narasin was determined at pH 5, 7, and 9. A stock concentration of 1000 mg narasin/L was prepared in n-octanol. Solutions were prepared in duplicate for each pH using the volume ratios of 1:7, 1:3, and 1:1 of n-octanol:aqueous buffer. The mixtures were placed in centrifuge tubes with Teflon®-lined caps, placed in an agitating water bath maintained at 25°C, agitated for five minutes, centrifuged for 10 minutes at 3000 rpm. Each phase was then analyzed by LC/MS.

Results:
The partition coefficients were independent of pH and concentration.

	Mean P_{ow} (range)	**Log P_{ow}**
pH 5	66094 (48983 to 103112)	4.79
pH 7	75027 (43908 to 109947)	4.85
pH 9	131032 (41130 to 209502)	5.06

Appendix C – Study ABC-0260: Chemical & Radiochemical Characterization of ^{14}C Residues in Excreta from Chickens Dosed with Ration Containing 80 ppm ^{14}C Narasin. Report Date: 1984.

Methods:
Male and female broiler chickens were fed a ration containing 80 ppm ^{14}C narasin for seven days. Excreta produced during the fourth through the seventh day was collected, air-dried, and ground in a laboratory mill to give a homogenous sample. The excreta was assayed for total radioactivity by combustion analysis with liquid scintillation counting (LSC). The excreta was extracted and assayed for parent narasin by HPLC and microbiological turbidimetric assay. Relative quantities of metabolites were estimated from column and thin-layer chromatography (TLC) data.

Results:
Total radioactivity in the air-dried excreta was equivalent to 237 ppm ^{14}C narasin equivalents. Parent narasin assay values were 12.1 ppm by HPLC and 11.5 ppm by microbiological (turbidometric) assay (on a fresh weight basis, concentrations were 6.5 mg/kg narasin by HPLC and 6.2 mg/kg narasin activity by microbiological assay). Thus, parent narasin accounted for only approximately 5% of the total radioactivity. The good agreement between HPLC and microbiological assays indicates that the antimicrobial activity is accounted for by the parent narasin, and the narasin metabolites have no appreciable antimicrobial activity in this assay system. Column and thin-layer chromatography of an extract of the excreta gave the array of narasin metabolites which have been reported in other studies. The primary metabolites, NM-1, NM-2, NM-3, NM-4, NM-5, NM-6, and NM-7, were estimated to constitute approximately a total of 25% of the excreta radioactivity.

Appendix D – Study T4H969301: A Comparative Metabolism Study in Tissues & Excreta of Chickens Dosed with ^{14}C-Narasin with & without Nicarbazin. Report Date: April, 1994.

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{14}C Narasin

Methods:
Two groups of five chickens each were fed either 50 ppm ^{14}C-narasin rations or 50 ppm ^{14}C-narasin and 50 ppm nicarbazin rations. After five days, the two groups were slaughtered, and the tissues and excreta were collected for assay. Liver, kidney, muscle, fat, and skin/fat were assayed for total radioactivity by solubilization and liquid scintillation counting (LSC). Residues in liver, fat and excreta were profiled by extraction and fractionation using silica gel chromatography. Metabolites in excreta were isolated and characterized by HPLC/ISP/MS/LSC.

Results:

Mean ppm Narasin Equivalents at Slaughter

Treatment	Liver	Kidney	Muscle	Fat	Skin/fat
50 ppm ^{14}C-narasin	0.32	0.04	<0.04	0.12	0.08
50 ppm ^{14}C-narasin + 50 ppm nicarbazin	0.27	0.05	<0.02	0.07	0.05

Characterization of the fat showed that the predominant radioactive residue was narasin. The residues in the liver were more widely distributed over the fractions, but no residues were identified

From the excreta, approximately 90% of the radioactive residues were fractionated by chromatography. Fifteen metabolites along with parent narasin were identified from the excreta from both treatments. These metabolites were predominately di- and tri-hydroxylated narasin and di- and tri-hydroxylated narasin B. The distribution and magnitude were similar for both treatments. All of the identified hydroxylated metabolites represented a total of almost 50% of the total radioactivity in the excreta. Another 3% of the radioactivity was identified as parent narasin. The remaining radioactivity was distributed in minor fractions from the chromatography and was in concentrations too low to characterize.

Appendix E – Wong 1978. Effect of narasin metabolites on ATPase & oxygen uptake in rat liver mitochondria. Report Date: 1978

Performing Laboratory: Lilly Research Laboratories

Test Article: Narasin metabolite preparations from chicken and cattle excreta

Methods:
Four narasin metabolite preparations were evaluated for determination of ionophorous properties by measurement of their effects on ATPase activity and oxygen uptake in rat liver mitochondria.

Results:
All four of the metabolite preparations were at least 200 times less active as ionophores than narasin.

Appendix F – Manthey & Goebel, 1982. Isolation & characterization of narasin metabolites derived from excreta of orally dosed chickens. Report Date: 1982

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline ^{14}C Narasin

Methods:
Excreta from broiler chickens which had been fed a ration containing 100 ppm ^{14}C narasin was extracted with methanol to recover ^{14}C narasin and metabolites. The extract was subjected to fractionation by liquid-liquid partitioning, silica gel column chromatography, thin-layer chromatography and reversed phase HPLC to characterize the distribution of narasin metabolites. Specific metabolite fractions were purified and subjected to analysis by mass spectrometry.

Results:
Six labeled narasin metabolites were isolated from chicken excreta as described above. The metabolites were found to be di- or trihydroxylated narasin in which the hydroxy groups were substituted for hydrogen in various positions on the rings of the narasin molecule. Thus, in chickens a primary mode of narasin metabolism is hydroxylation of the narasin molecule.

All six metabolites were evaluated by thin-layer bioautography to determine their antimicrobial activity relative to narasin. The metabolites were inactive. The detection limit for each metabolite was 5% activity when compared to equal amounts of narasin.

Appendix G – Study 151E-125. Narasin – Aerobic mineralization & transformation in chicken manure. Report Date: March 2011.

Performing Laboratories: Wildlife International, Ltd. and Analytical Bio-Chemistry Laboratories, Inc.

Test Article: Crystalline ^{14}C Narasin

Methods:
The degradation of ^{14}C narasin was evaluated in two test systems: fresh chicken manure and fresh chicken manure mixed with used litter in a 4:1 ratio (based on dry weight). The test systems were placed in the test chambers, dosed with 0.5 mg [^{14}C]-narasin/kg (oven-dry weight), and incubated in air-flow-through systems under aerobic conditions at approximately 20°C for 35 days. Laboratory air was pulled through the chamber by means of a vacuum to provide aerobic conditions and air exiting the test chambers was passed through a sorbent material and then a KOH solution to trap organic volatiles and $^{14}CO_2$, respectively.

Duplicate test chambers were sacrificed on Days 3, 7, 14, 28 and 35 and contents were extracted with methanol. The methanol extracts were concentrated by partitioning into chloroform. The concentrated extracts were analyzed with liquid scintillation counting (LSC) and High Performance Liquid Chromatography (HPLC/β-RAM).

Matrix controls and viability controls (test systems dosed with ^{14}C glucose) were incubated under the same test conditions as the test chambers. Evolved $^{14}CO_2$ was trapped in KOH traps. Microbial viability was also assessed by measurement of respiration (total CO_2 evolved from the test systems), microbial biomass and enumeration of colony-forming units.

Concentrated samples of the methanol extract collected on Days 7 and 14 were profiled for identification and quantification of parent material and degradation products using LSC, HPLC/β-RAM, HPLC/MS/MS, HPLC Ion-trap MS/MS, and HPLC Time of flight/MS.

Results:
Microbial biomass, glucose mineralization rate, and enumeration of colony-forming units provided evidence that microbial population was viable and metabolically active at the beginning of the test and trended down at the end. Viability and metabolic activity of two test systems were similar.

The amount of narasin in the test systems declined to about 50% of the initial dose levels by test Day 7 and remained around that level for the rest of the study. About 2% of the dosed radioactivity was captured as carbon dioxide or volatile organics by the end of the study. Between about 10 and 15% of the radioactivity could not be extracted from solids throughout the study. The distribution of radioactivity in the test systems is included in the table:

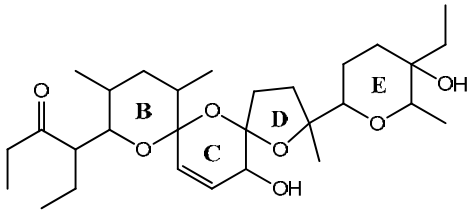
Test Day Sampled	Test System	Mean Recovery of applied radioactivity (%)				
		Total recovered	Total captured as CO_2 plus Volatiles	Non-extractable residues in solid	Total extracted in methanol	Total in extract identified as narasin
0	Manure	113	na	14.3	99	91.6
	Manure+Litter	120	na	12.5	107	102
3	Manure	105*	0.033	9.7	96	85.5
	Manure+Litter	112	0.017	11.7	100	72.2
7	Manure	103	0.065	14.3	89	48.1
	Manure+Litter	107	0.113	15.5	91	55.4
14	Manure	110	0.111	13.8	96	52.6
	Manure+Litter	111	0.150	14.3	97	51.8
28	Manure	106	0.872	12.8	92	52.9
	Manure+Litter	110	0.678	15.3	94	47.6
35	Manure	107	2.196	13.5	91	49.0
	Manure+Litter	108*	1.800	14.6	91	36.9

*Rounded mean values in table do not exactly add up to the rounded mean total recovery values

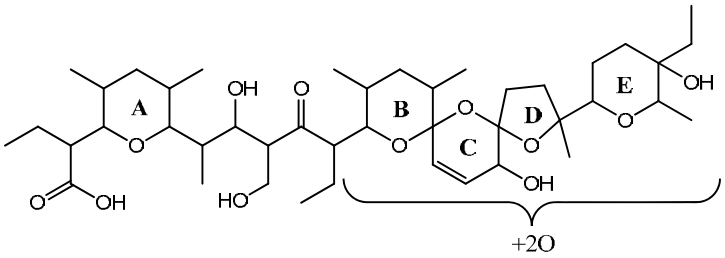
Degradation profiling analysis was performed by ABC Laboratories, Inc. A total of nine transformation product peaks were resolved in a concentrated sample of the methanol extract, along with narasin. These peaks were quantified in the Day 7 and Day 14 chicken manure and manure/litter extracts by fractionation and liquid scintillation counting. The other peaks of radioactivity in the extracts were very minor.

Among the nine degradation products, Peak 3 and Peak 9 were the largest and accounted for approximately 27-29% and 19-24% of the radioactivity in the concentrated sample of extract, respectively; while the other seven quantified peaks were each less than 6%. Parent narasin accounted for approximately 38 to 41% of the radioactivity in the concentrated methanol extract. This profile was consistent in both Day 7 and Day 14 samples.

The isolated components in Peaks 3 and 9 were investigated using LC-MS/MS techniques. The data for Peak 3 are consistent with narasin without the A ring. A proposed structure for degradation product Peak 3 is shown below:



The data for Peak 9 are consistent with a trihydroxylated narasin. A proposed structure for degradation product Peak 9 is shown below with an indication that two additional oxygen atoms exist in hydroxyl groups somewhere in the indicated portion of the molecule:



Appendix H – Study 802374: The Degradation of [14C]-Narasin in Soil under Aerobic Conditions. OECD Guideline 307. Report Date: June, 2002.

Methods:
^{14}C-Narasin (approximately 1.6 mg/kg) was incubated in 3 soils in the dark at 20°C for 84 days under aerobic conditions. Test vessels were connected to volatile traps. Duplicate vessels were sacrificed on Days 0, 7, 14, 21, 28, 42, 56, 70 and 84. Soil samples were extracted and analyzed by liquid scintillation counting (LSC), high performance liquid chromatography with radiochemical detection (HPLC), and thin layer chromatography (TLC).

Results:

	Sandy Loam pH 7.8 1.5% OC	**Clay Loam pH 8.0 1.6% OC**	**Silt Loam pH 7.9 2.4% OC**
DT_{50} of narasin (days)	21	29	49
Mineralization (% AR)	64	54	19

%AR = Percent of applied radioactivity

Narasin degraded to several unidentified components; individually no component accounted for >16% of the applied radioactivity.

Appendix I – Study 276A-3480-22. Decline of narasin in greenhouse soil. Report Date: 1977

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline Narasin

Methods:
Crystalline narasin, dissolved in a methanol solution, was blended with air-dried potting soil in order to record the disappearance of narasin activity from soil over time in a relatively controlled situation. The initial nominal concentration of narasin in the soil was 10 ppm. A soil sample was taken on day 0 to confirm this concentration. An initial assayed value of 9.83 ppm indicated that narasin was readily extracted from the soil. The soil consisted of a 1/1 mixture of sand and Brookston loam and it was not sterilized prior to use. The soil was placed in a plastic-lined metal flat to eliminate the possibility of leaching. The moisture level of the soil was brought to field capacity by adding water. The whole metal flat was then placed in a large plastic bag which was subsequently closed to prevent evaporation of the water. The covered soil was then held in a greenhouse for 41 days. Soil samples were taken on days 11, 26, and 41, air dried and assayed for narasin activity. Residue data from soil samples were fitted to a line using a non-linear least-squares procedure for regression analysis and the model $C_t = C_0 e^{-rt}$. The rate constant, r, was used to calculate the degradation half-life for narasin.

Results:
Narasin activity rapidly dissipated in the soil (Table 1). Less than 7% of the original narasin activity remained after 26 days and less than 3% remained after 41 days. Because of the experimental design, narasin could not leach out of the sampled soil and a good extraction efficiency was demonstrated on day 0. The dissipation of active narasin from the soil was then presumably due to degradation of narasin into inactive substances, based on microbiological activity.

Table 1

Sampling Period (Days after initiation)	Narasin Concentration In Soil (ppm)
0	9.83
11	4.70
26	0.62
41	0.26
“r”, degradation rate constant (day^{-1})	0.079
Model correlation coefficient (R^2)	0.99
Degradation half-life (days)	8.8

Appendix J – Study 151E-107: Narasin – Adsorption / Desorption Characteristics in Five Representative Soils Following OECD Guideline 106. Report Date: January, 2008

Performing Laboratory: Wildlife International, Ltd.

Test Article: Crystalline Narasin

Methods:
Five soils varying in pH, organic carbon, and texture were used to obtain isotherms of narasin adsorption and desorption to soils, to estimate narasin distribution coefficients, and to calculate the coefficients of the Freundlich adsorption and desorption isotherm equations.

In the study, duplicate containers of narasin in five different soils in a solid:solution ratio of 1:5 were incubated for approximately 4 hours at 20°C. Samples of the equilibrium solution after centrifugation were assayed by LC/MS, and Kd, Koc, and Freundlich isotherms were constructed from the sorption/desorption data.

Results:

	Clay Loam pH 7.2 OC* 5.0%**	**Sandy Clay Loam pH 6.2 OC 1.9%**	**Clay Loam pH 5.2 OC 4.1%**	**Loamy Sand pH 5.7 OC 1.3%**	**Clay pH 7.7 OC 0.7%**
Adsorption					
Kd	25	22	150	26	5.4
Koc	507	1171	3670	1971	778
Desorption					
Kd	42	27	108	24	7.1
Koc	840	1423	2628	1841	1016
Freundlich Log Kf (Sorption)	1.7074	1.2833	2.0606	1.2310	0.9625
Freundlich Log Kf (Desorption)	1.6670	1.2848	1.9718	1.3605	1.2861

*OC = Organic Carbon

** Measured in 0.01 M $CaCl_2$, 1:2

Appendix K – Study ABC-0137: A ^{14}C Narasin tissue residue & comparative metabolism study in cattle. Report Date: 1982.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline ^{14}C Narasin

Methods:
Hereford cattle, six steers and three heifers, ranging in weight between 218 to 319 kg were dosed with ^{14}C narasin equivalent to a feeding level of approximately 18 g/ton of feed. ^{14}C Narasin was administered in gelatin capsules morning and evening for three, five or seven days. Twelve hours after the last dose, edible tissues were collected from 2 steers and 1 heifer and then assayed for radioactivity. Parent narasin in liver was determined by microbiological assay.

Results:
Radioactivity in liver was approximately 0.8 ppm calculated as narasin equivalents and there was no significant difference between animals in the three dosing groups. Therefore, steady state concentrations were approximated within three days' dosing. Muscle, kidney and fat residues were all less than 0.025 ppm (mean of all nine animals). Narasin concentrations in livers of the cattle dosed for seven days were approximately 8% of the total radioactivity. Chromatographic profiles of liver radioactivity from the seven-day animals were similar.

Appendix L – Studies ABC-0126 & ABC-0127. Comparative metabolism of ^{14}C narasin in orally dosed cattle, dog & rats. Report Date: September 1986.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline ^{14}C Narasin

Methods:
Cattle, a dog, and rats were dosed orally for up to 7 days with ^{14}C narasin. The radiochemical residues extracted from the liver and feces were subjected to fractionation by solvent partitioning, silica gel column chromatography and thin-layer chromatography (TLC). Cattle liver metabolites were visualized by TLC autoradiography and relative quantities of individual metabolites were estimated by liquid scintillation counting of zones scraped from TLC plates.

Results:
All three species produced multiple narasin metabolites. Fecal extracts contained more than twenty radioactivity metabolites and no single metabolite constituted a significant proportion of the total radioactivity. Comparison of column elution profiles and TLC autoradiograms indicated that metabolite patterns were qualitatively similar among cattle, rats, and dogs. There were, however, some quantitative differences among these three species.

Eight of the di- and trihydroxy derivatives of narasin have been previously identified in chickens. Two novel metabolites, a monohydroxylated narsin (NM-12) and a dihydroxylated narasin (NM-13), were identified in this study. These two were produced by all three species but were most prevalent in cattle.

Quantitative fractionation of radioactivity in cattle liver indicated that metabolite NM-12 was the most abundant (approximately 16%) and that metabolites NM-3 and NM-6, which are dihydroxy narasin derivatives, each accounted for approximately 4% of the total. The remainder of the liver radioactivity consisted of several metabolites with low relative abundance, approximately 10% nonextractable radioactivity, and 10 to 15% intractable polar residues. Very little (<3%) of the liver residue was parent narasin.

Appendix M – Study 802458. Soil Microorganisms: Carbon & Nitrogen Transformation Tests with Narasin. OECD Guidelines 216 & 217. Report Date: July, 2002

Methods:
Narasin was incorporated into sandy loam soil at nominal concentrations of 3.5 and 17.5 mg/kg (dry weight). Soils were incubated under aerobic conditions for 28 days in the dark at 20°C. On days 0, 7, 14, and 28, vessels were removed for evaluation of carbon respiration and nitrogen transformation. Carbon respiration was evaluated by adding glucose and monitoring CO_2 production. Nitrogen transformation was evaluated by analyzing concentrations of ammonium, nitrite and nitrate. Only changes greater than 25%, when compared to control, were considered biologically significant by these test guidelines.

Results:

Carbon transformation

	Mean Calculated Carbon Respiration Rates (mL CO_2 kg^{-1} hr^{-1}, (standard deviation)	**Deviation from Control %**
Control	5.02 (0.17)	
Narasin @ 3.784 mg/kg	4.43 (0.14)	-11.8
Narasin @ 17.43 mg/kg	4.86 (0.48)	-3.2

Nitrate transformation

	Mean Concentration of Nitrate mg N kg^{-1} (standard deviation)	**Deviation from Control %**
Control	68.3(0.4)	
Narasin @ 3.784 mg/kg	71.9* (0.3)	5.3*
Narasin @ 17.43 mg/kg	72.6* (1.3)	6.3*

*Statistically different from Control

After 28 days, narasin had no effect on carbon respiration and nitrogen transformation that was greater than 25% deviation from the untreated soil controls at either treatment level.

Appendix N – Study 802442: Terrestrial Plant Growth Test with Narasin. OECD Guideline 208. Report Date: June, 2002

Methods:
A seedling emergence and growth study following OECD Guideline 208 was conducted to determine the effects of narasin on emergence and growth of winter oat (*Avena sativa* cv Jalna), radish (*Raphanus sativus* cv French Breakfast 3, and mung bean (*Phaseolus aureus*). Seeds of each species were planted in 75% loamy sand soil and 25% horticultural grade sand. Solutions of narasin in acetone were applied to the sand and the sand premixes were then blended with the appropriate amount of soil to produce the desired concentration of narasin in the soil-sand mixture. Five seeds were planted per replicate 1.1-liter pot, and there were 4 pots per treatment level for each species. The nominal concentrations tested were 0.35, 3.5 and 35 mg narasin/kg (dry weight soil). HPLC analysis of the narasin dosing solutions in acetone was performed to confirm concentration. The seeds and seedlings were observed for 14 days after at least 50% emergence had been observed in the control pots. This corresponded to 19, 19, and 20 days after sowing for winter oats, radish, and mung bean, respectively. At test termination, the stems of the plants were snipped at the soil surface, and the total fresh weight of seedlings from each pot was measured.

Results:
Measured concentrations in the dosing solutions were 107.1, 96.6, and 83.6% of nominal for the 0.35, 3.5 and 35 mg/kg treatment levels. Therefore, the actual tested concentrations were considered to be 0.375, 3.381, and 29.26 mg narasin/kg dry weight soil.

Winter Oat

	Number of Seedlings that Emerged	Mean Weight per Seedling, g (standard deviation)	Inhibition of Growth %
Control	20	0.399 (0.056)	
0.375 mg/kg	19	0.447 (0.022)	--
3.381 mg/kg	20	0.530* (0.076)	--
29.26 mg/kg	19	0.248* (0.041)	38%

*Statistically different from control.

No adverse effects were observed at the 0.375 and 3.381 mg/kg levels. However, there was reduced growth at the highest concentration.

Phytotoxicity of Narasin to Winter Oat	
LC50 (emergence)	>29.26 mg/kg
EC50 (growth)	>29.26 mg/kg

Mung Bean

	Number of Seedlings that Emerged	Mean Weight per Seedling, g (standard deviation)	Inhibition of Growth %
Control	20	0.580 (0.059)	
0.375 mg/kg	20	0.494* (0.084)	15%
3.381 mg/kg	16	0.422* (0.018)	27%
29.26 mg/kg	12	0.194* (0.033)	67%

*Statistically different from control

There was significantly reduced growth at all treatment levels.

Phytotoxicity of Narasin to Mung Bean	
LC50 (emergence)	>29.26 mg/kg
EC50 (growth)	8.99 mg/kg*

*Estimated by probit after data transformations

Radish

	Number of Seedlings that Emerged	Mean Weight per Seedling, g (standard deviation)	Inhibition of Growth %
Control	19	0.696 (0.057)	
0.375 mg/kg	19	0.603 (0.058)	13%
3.381 mg/kg	18	0.522* (0.097)	25%
29.26 mg/kg	0	--	--

*Statistically different from control

There was no effect on emergence at the lower two concentrations and 100% inhibition of emergence at the highest concentration. In the study report, the LC50 was calculated as approximately 5 mg/kg using a probit method. However, this is inappropriate given the lack of partial effects. Therefore, to estimate the LC50 based on emergence, a simple binomial method was used by taking the mean of 3.381 and 29.26 mg/kg.

No calculation of the EC50 for growth was made for radish in the report. However, an EC50 can be calculated for growth inhibition of radish with the ICp method. To use this method, a value of 100% growth inhibition was used for seedlings that did not emerge, including those at the highest concentration (Norberg-King, 1993). The replicate weights were recalculated to include fresh weight values of 0 g for seeds that did not emerge. The recalculated mean seedling weights per treatment level are below:

	Mean Weight per Seedling (emerged and non-emerged), g (standard deviation)	Inhibition of Growth %
Control	0.661 (0.087)	--
0.375 mg/kg	0.571 (0.071)	14%
3.381 mg/kg	0.459 (0.083)	31%
29.26 mg/kg	0 (0)	100%

Using the recalculated replicate means, the EC50 was calculated to be 6.183 mg/kg.

Phytotoxicity of Narasin to Radish	
LC50 (emergence)	16.3 mg/kg*
EC50 (growth)	6.183 mg/kg**

*Calculated from the binomial method

**Calculated from the ICp method

Appendix O – Study 802568: Narasin – Determination of Acute Toxicity (LC_{50}) to Earthworms. OECD Guideline 207. Report Date: July, 2002.

Methods:
Earthworms, >2 months of age, were exposed for 14 days to narasin incorporated in soil at nominal concentrations of 320, 160, 80, 40 and 5 mg/kg. Narasin was fortified into artificial soil using an acetone/sand premix. Dosing solutions of narasin in acetone were added to sand, the acetone allowed to evaporate and then the sand premix mixed with the remaining components of the artificial soil. HPLC analysis of the narasin dosing solutions in acetone was performed to confirm concentration.

Four vessels containing 750 g soil were prepared for each control and treatment group. Ten earthworms were placed on the soil surface of each vessel after being rinsed, blotted dry, and weighed.

Results:
Measured concentrations in the dosing solutions were 95.5, 97.2, 95.5, 96.8, and 96.2% of nominal for the nominal treatments of 320, 160, 80, 40, and 5 mg/kg. Therefore, the actual tested concentrations were considered to be 270.9, 137.7, 67.7, 34.3 and 4.3 mg narasin/kg.

Cumulative Percent Mortality

	Measured Concentrations (mg/kg)						
Day	**Untreated Control**	**Solvent Control**	**4.3**	**34.3**	**67.7**	**137.7**	**270.9**
7	2.5	2.5	5	15	82.5	97.5	100
14	7.5	5	12.5	22.5	87.5	100	100

Mean Percent Body Weight Change at Day 14

Measured Concentrations (mg/kg)						
Untreated Control	**Solvent Control**	**4.3**	**34.3**	**67.7**	**137.7**	**270.9**
-8.7	-9.7	-8.1	-4.7	-30.7	NA	NA

Based on mortality, the LC50 was 51.1 and 46.4 mg/kg for Days 7 and 14, respectively. Based on both body weight and survival the 14-day NOEC is 4.3 mg/kg.

Appendix P – Study 1982.6391: Narasin – Chronic Toxicity & Reproduction Test Exposing the Earthworm *Eisenia fetida* in Artificial Soil, Based on OECD Guideline 222. Report Date: February, 2011.

Methods:
The potential effect of narasin on the growth, reproduction and development of earthworms (*Eisenia fetida*) was evaluated by exposed mature worms to concentrations of narasin in artificial soil of 3.1, 6.3, 13, 25, and 50 mg/kg. A blank and a solvent control were included in the test. There were 8 replicates of 10 worms each for the blank control and 4 replicates of 10 worms each for the solvent control and narasin treatment levels. Adult worms were weighed (as a group) and then added to the fortified soil treatments. After 28 days, adults were removed from the soil and weighed again. The fortified soil treatments were held under the same conditions (17 to 21°C) for an additional 28 days to allow cocoons to hatch. At the end of this time, young worms were separated from the soil and counted. Endpoints included survival and weight change of adults and number of offspring per surviving adult.

Results:

Mean Percent Survival, Weight, and Weight Change for the F_0 Generation (Day 28)

Nominal Concentration (mg narasin/kg)	Percent Survival @ Day 28	Day 0 Mean Individual Weights (g)	Day 28 Mean Individual Weights (g)	Percent Weight Change
Control	100	0.3177	0.3841	21
Solvent Control	100	0.3116	0.3740	22
3.1	100	0.3107	0.3715	20
6.3	100	0.3230	0.3577	11*
13	100	0.3105	0.3642	18
25	95	0.3105	0.4057	31
50	25	0.3036	0.4218	37

*Significantly different from control, but based on lack of dose-response, not considered to be treatment related.

The NOEC for adult survival was 25 mg narasin/kg. The 28-day LC50 value was 41 mg/kg (95% confidence limits of 37 to 46 mg narasin/kg). There was no effect on weight change.

Number of Offspring Produced During Exposure

Nominal Concentration (mg narasin/kg)	Mean Number of Surviving Offspring per Replicate	Number of Offspring per Surviving Adult
Control	74	7.4
Solvent Control	68	6.8
3.1	77	7.7
6.3	90	9.0
13	83	8.3
25	54	5.6
50	16	9.2

There was no statistically significant effect on the number of offspring per surviving adult.

The lowest NOEC observed for the study was 25 mg/kg.

Appendix Q – Study 802573: Narasin – Alga, Growth Inhibition Test (72 h, EC50). OECD Guideline #201 and EC Guideline C3. Report Date: July, 2002

Methods:
A static toxicity test was conducted to evaluate the effects of narasin on green algae, *Selenastrum capricornutum*, now known as *Pseudokirchneriella subcapitata*. Algal cells were cultured for 72 hours in a liquid nutrient medium that contained nominal concentrations of 0, 0.05, 0.32, 0.75, 1.5, 3, and 6 mg narasin/L. Concentrations of narasin were measured at the beginning and end of the exposure. Each narasin treatment consisted of three replicate vessels and the control consisted of six replicate vessels. All vessels contained 100 ml of nutrient medium with an initial algal density of 10,000 cells/ml. The vessels were incubated in continuous light in an environmental chamber at a temperature range of 21- 23°C. The number of cells was determined at 24, 48 and 72 hours using a compound microscope and Improved Neubauer Counting Chambers.

Results:
The test solution concentrations were similar between the initiation and termination of the exposure. The mean measured concentrations were 4.17, 2.16, 1.06, 0.54, 0.23 and 0.035 mg/L. The lowest two concentrations were below the quantitation limit, the values of 0.23 and 0.035 mg/L were calculated based on dilution of the higher measured concentrations.

Mean Biomass and Growth Rate:

Treatment (mg/L)	Area Under the Curve ($\times 10^5$ cells.h.mL^{-1})		Mean Growth Rate (day^{-1}) (0-72hr)	
	Mean	% Inhibition	Mean	% Inhibition
Control	3.23	NA	1.54	NA
0.035	2.94	9	1.53	1
0.23	2.47	24	1.45	6
0.54	1.85*	43	1.37*	11
1.06	1.45*	55	1.32*	14
2.16	0.69*	79	1.06*	31
4.17	0.11*	97	0.47*	69

*Significantly different from control
NA = Not Applicable

The 0-72 hr EC_{50} values for biomass and growth rate were 0.77 and 2.92 mg/L, respectively. The NOEC for both parameters was 0.23 mg/L

Appendix R – Study C01883: The acute toxicity of narasin to *Daphnia magna* in a static test system. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Mycelial narasin

Methods:
Groups of 30 *Daphnia*, ≤24 hours old, were exposed to each of the following average assayed narasin concentrations for 48 hours: 0.0 (water control), 4.69, 7.86, 12.45, 18.96, 35.08, and 42.18 mg/L. The dissolved oxygen concentration, pH, and temperature of each test solution were measured and recorded at test initiation and at each 24-hour interval. Total alkalinity, total hardness, and conductivity of the dilution water were determined once during the test. Daphnia were assessed for hypoactivity, prostration and immobility.

Results:
Based on immobility, the 48 hr EC50, the 95% confidence limits and the slope of the concentration response curve were 20.56 mg/L, 9.19 to 68.1 mg/L, and 4.23. The following behavior was noted at the end of the study:

Concentration	Normal	Hypoactive	Immobile
0.0	30	-	-
4.69	27	2	1
7.86	22	4	4
12.45	9	21	-
18.96	7	14	9
35.08		4	26
42.18	-	-	30

Appendix S – Study F05283. The acute toxicity to rainbow trout (*Salmo gairdneri*) of narasin (Compound 79891). Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Mycelial narasin

Methods:
Groups of ten juvenile rainbow trout, 0.74 to 2.05 g per fish, were exposed to the following average assayed narasin concentrations for 96 hours: 0.0 (water control), 0.103, 0.190, 0.316, 0.561, 1.00, 1.82, 3.04, and 5.26 mg/L. Jars with 15 L of test or control solution were used to contain each group of ten fish. Dissolved oxygen concentration, pH and temperature were recorded daily for each jar. Total hardness, total alkalinity and conductivity were recorded once for the dilution water. Behavioral signs of toxicity (sluggish, hypoactivity, swimming impaired, labored respiration and minimal voluntary movement, and prostration) and mortalities were noted for fish in each jar on a daily basis.

Results:
The temperature, total hardness, total alkalinity, and conductivity of the test solutions were 13°C, 120 mg/L (as $CaCO_3$), 148 mg/L (as $CaCO_3$), and 225 µmhos/cm, respectively. Dissolved oxygen concentrations averaged 10.2 mg/L and were at least 92% of saturation. Measured pH values ranged from 8.2 to 8.6.

No mortalities or behavioral abnormalities were found for fish in control water or for fish in solutions with average narasin concentrations of 0.103 and 0.190 mg/L. Fish exposed to 0.316 mg/L exhibited hypoactive behavior by the end of the study. Fish exposed to narasin concentrations ≥ 0.561 mg/L displayed behavior which ranged from sluggish movement to labored respiration and minimal voluntary movement. At least some mortalities were noted for fish exposed for 96 hours to average narasin concentrations ≥ 1.82 mg/L. The 96 hour LC50, the 95% confidence limits and the slope of the concentration-response curve were 2.23 mg/L, 1.84 to 2.71 mg/L, and about 7.0, respectively.

Appendix T – Study F05183. The acute toxicity to Bluegill (*Lepomis macrochirus*) of Narasin (Compound 79891). Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Mycelial narasin

Methods:
Groups of ten juvenile bluegill, 0.89 to 1.82 g per fish, were exposed to the following average assayed narasin concentrations for 96 hours: 0.0 (water control), 0.88 1.66, 2.80, 4.68, 6.00, 6.74, 7.80, 8.70, and 9.55 mg/L. Jars with 15 L of test or control solution were used to contain each group of ten fish. Dissolved oxygen concentration, pH and temperature were recorded daily for each jar. Total hardness, total alkalinity and conductivity were recorded once for the dilution water. Behavioral signs of toxicity (sluggish, hypoactivity, swimming impaired, labored respiration and minimal voluntary movement, and prostration) and mortalities were noted for fish in each jar on a daily basis.

Results:
Narasin concentrations were stable throughout this study. The ranges in the water quality characteristics were as follows: pH, 8.05 to 8.6; temperature, 20 to 20.5°C; total hardness, 120 mg/L (as $CaCO_3$); total alkalinity, 152 mg/L (as $CaCO_3$); and conductivity, 250 µmhos/cm. Dissolved oxygen concentrations averaged 8.75 mg/L and were at least 83% of saturation in all test solutions.

No mortalities or behavioral abnormalities were found for fish in control water or for fish in solutions with average narasin concentrations of 0.88 and 1.66 mg/L. Fish exposed to 2.80 mg/L of narasin exhibited sluggish behavior. The behavior of fish exposed to narasin concentrations ≥4.68 mg/L ranged from hypoactivity to labored respiration with minimal voluntary movement. At least some fish died in narasin concentrations ≥4.68 mg/L. The 96 hr LC50, 95% confidence limits, and slope of the concentration response curve were 5.02 mg/L 4.61 to 5.46 mg/L, and about 18.1, respectively.

Appendix U – Study 341587. Physico-Chemical Testing with Narasin: Partition Coefficient. OECD Guideline 117. Report Date: June 2002.

Methods:
The octanol/water partition coefficient, P_{ow}, of narasin was estimated using high performance liquid chromatography (HPLC) with a C18 reversed phase column and a methanol/citrate buffer mobile phase. A series of reference chemicals with well-characterized octanol/water partition coefficients were injected onto the analytical column followed by an injection of the test substance. The retention time of each chemical is in proportion to its octanol/water partition coefficient, with water-soluble chemicals eluting first and oil-soluble chemicals last. The octanol/water partition coefficient of the test substance was interpolated from its capacity factor k (based on retention time), using a calibration plot of log k versus log P_{ow} of the reference chemicals.

Results:
Narasin eluted after the reference standard with the highest log P_{ow} value. The log P_{ow} value for narasin was estimated to be greater than 6.2, the log P_{ow} for the most hydrophobic reference standard. Narasin eluted as a split peak.