Identification and Synthesis of an Oxidation Product of Tilmicosin

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During formulation development of the veterinary macrolide antibiotic tilmicosin, an oxidation product of the parent molecule was identified. Use of liquid chromatography/mass spectrometry and nuclear magnetic resonance spectroscopy characterized the structure as 12,13-epoxytilmicosin, resulting from the oxidation of the double bond at the 12,13-position of the macrolide dienone. The synthesis of this oxidation product for confirmation of structural identity is described. The synthetic compound was identical to the isolated oxidation product, 12,13-epoxytilmicosin.

Keywords: *Tilmicosin; epoxytilmicosin; macrolide; oxidation; synthesis; liquid chromatography/ion spray mass spectrometry*

INTRODUCTION

Tilmicosin is a semisynthetic macrolide antibiotic derived from tylosin. The chemical name for tilmicosin is 20-deoxo-20-(3,5-dimethylpiperidin-1-yl)desmycosin (Figure 1). The synthesis of tilmicosin starts with the removal of the sugar mycarose from tylosin by acid hydrolysis to give desmycosin. Reductive amination of the aldehyde at C-20 of desmycosin with 3,5-dimethylpiperidine via a Wallach reaction or a metal hydride (e.g., $NaBH_3CN$) completes the synthesis to give tilmicosin (Kirst et al., 1989; Debono et al., 1989).

Tilmicosin has been granted market approval in the United States and numerous other countries in two formulations, Micotil (tilmicosin phosphate, injection) and Pulmotil (tilmicosin phosphate, type A medicated article). Micotil is indicated for subcutaneous use in cattle to treat bovine respiratory diseases (BRD) associated with *Pasteurella* species (Galyean et al., 1995; Musser et al., 1996; Scott et al., 1996). Pulmotil is indicated for control of bacterial pneumonia in swine caused by *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, and other organisms susceptible to tilmicosin (Moore et al., 1996a,b).

To obtain market approval for new drug formulations, a detailed package of information is generated by pharmaceutical companies for submission to regulatory authorities. The information thoroughly addresses all aspects of the new drug including efficacy testing, animal safety, residue profile, chemical stability, packaging, analytical methods, and manufacturing. To address the category of chemical stability, development of the drug formulation is critical in providing a form of the active molecule that resists the formation of chemical degradation products. Thus, one of the goals of formulation development is to identify drug degradation pathways and inhibit formation of degradation products.

During formulation development of tilmicosin for the treatment of respiratory disease in animals, an oxidation product of tilmicosin was identified. Initial studies under high-temperature storage conditions indicated a decrease in high-performance liquid chromatography (HPLC) chromatogram peak area corresponding to tilmicosin (potency loss). Examination of chromatograms from a gradient HPLC system to monitor degradation products did not initially reveal peaks that might explain the potency loss. Subsequent formulation studies were conducted that identified conditions that caused the decrease in tilmicosin HPLC peak area, and a formulation was identified that successfully resolved the oxidation issue. This paper describes the discovery of the oxidation product 12,13-epoxytilmicosin from formulation development studies and laboratory synthesis of the oxidation product that confirms the structure.

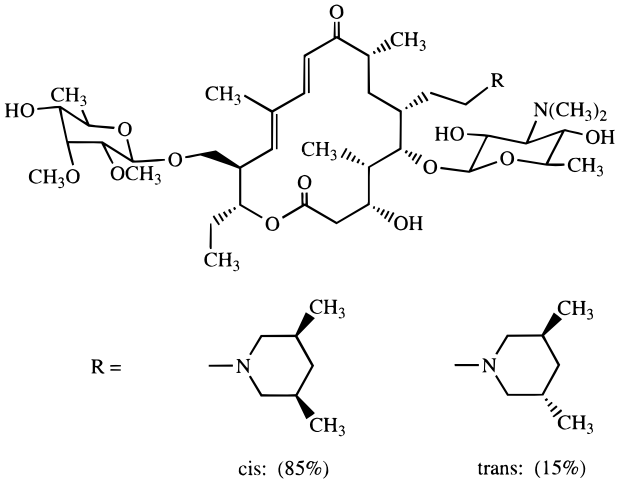

Figure 1. 20-Deoxo-(3,5-dimethylpiperidin-1-yl)desmycosin.

MATERIALS AND METHODS

Formulation Development Studies. Initial formulation studies were conducted at high temperature to gauge the stability of various tilmicosin formulations. Containers of the formulations were placed in temperature-controlled environmental chambers at 5 °C (control samples) and 52 °C (acceler-

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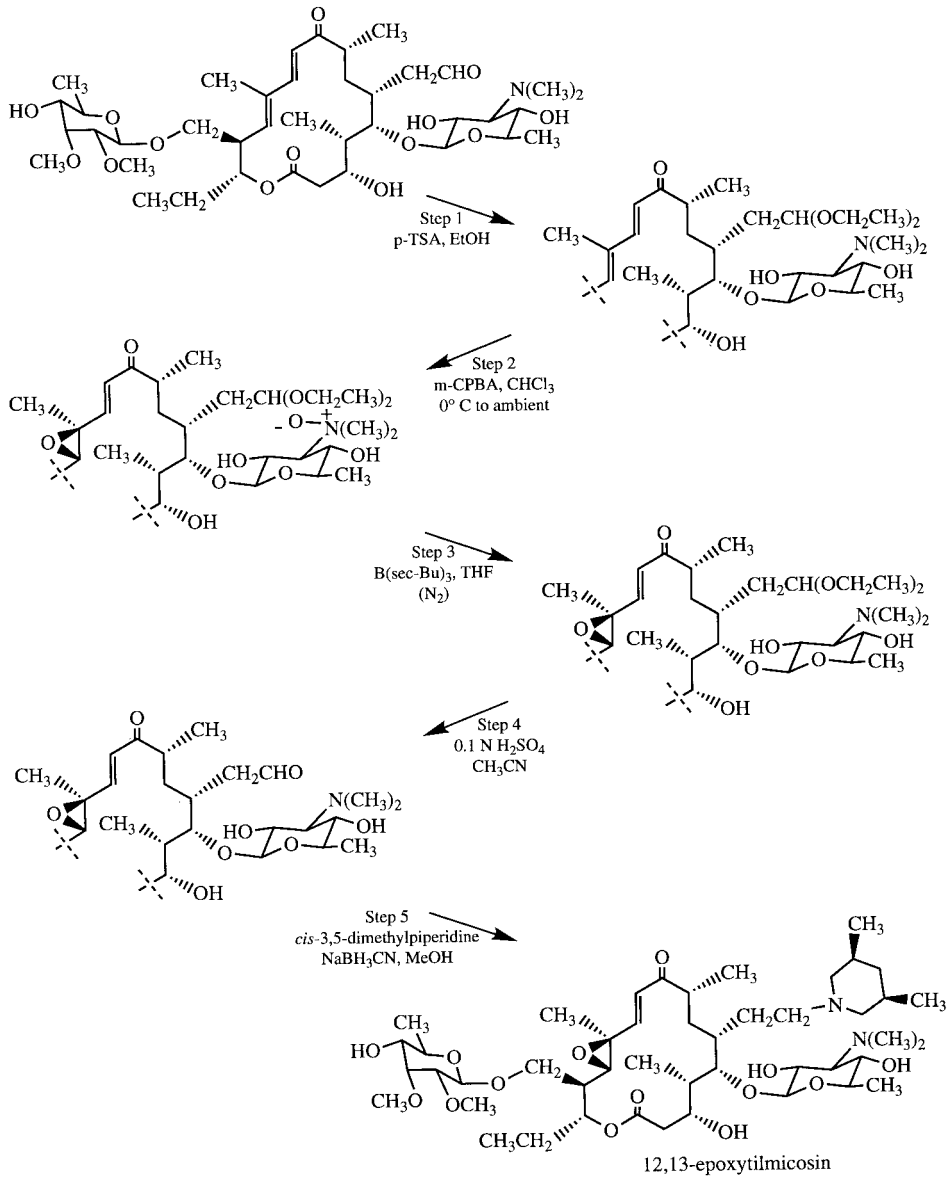

Figure 2. 12,13-Epoxytilmicosin synthetic scheme.

ated condition) for 3 months. Samples were analyzed by HPLC weekly, and results were compared to 5 °C controls for changes in peak area corresponding to tilmicosin.

Liquid Chromatography/Diode Array Detection. A high−low chromatography method (Inman et al., 1988) was used to assay for 12,13-epoxytilmicosin. HPLC was performed on a Beckman System Gold (Beckman Instruments, Inc., Fullerton, CA) equipped with a model 126 solvent pump, a model 507 autosampler, and a model 168 diode array detector. The column used was a 25 cm × 4.6 mm (i.d.) Zorbax Rx-C18 with 5 μm particle size (Rockland Technologies, Inc., Newport, DE). The instrument was operated with a gradient consisting of a 25 mM dibutylammonium phosphate buffer (pH 2.5)/acetonitrile, at 85 and 15%, respectively, held for 7.5 min, followed by a linear ramp to 40% acetonitrile over 30 min. The flow rate was 1.1 mL/min. The column was maintained at 60 °C. The sample injection volume was 10 μL. 12,13-Epoxytilmicosin and tilmicosin were detected at 240 and 280 nm, respectively.

Liquid Chromatography/Ion Spray Mass Spectrometry (LC/ISP-MS). *Liquid Chromatography.* HPLC was performed with a Waters (Millipore Corp., Milford, MA) 600MS solvent delivery system equipped with a Waters model 717 autosampler. The column used was a 25 cm × 4.6 mm (i.d.) Zorbax Rx-C18 with a 5 μm particle size (Rockland Technologies). The instrument was operated with a gradient consisting of a 10 mM ammonium acetate buffer (pH 2.5 with trifluoroacetic acid)/acetonitrile at 75 and 25%, respectively, held for 7.5 min, followed by a linear ramp to 40% acetonitrile over 30 min. The flow rate was 1.0 mL/min. The chromatographic effluent was split (95:5), with 50 μL/min introduced directly into the mass spectrometer interface and the remainder routed to waste. The sample injection volume was 100 μL.

Mass Spectrometry. Mass spectrometric analysis was accomplished using a SCIEX (Thornhill, ON, Canada) API I single-quadrupole mass spectrometer equipped with an ion spray (ISP, pneumatically assisted electrospray) interface. The ion spray needle potential was 5.0 kV, and the voltage in the cone/skimmer region of the ion source (orifice voltage, OR) was set at 150 V to provide conditions conducive to collision-induced dissociation (CID). Scanning MS data were acquired from *m*/*z* 150 to *m*/*z* 1100 (positive ion mode) at a rate of 2.03 s/scan with a dwell time of 1.0 ms.

Synthesis of 12,13-Epoxytilmicosin. Desmycosin was produced at Eli Lilly fermentation facilities at Indianapolis, IN. Sodium cyanoborohydride, *p*-toluenesulfonic acid, *m*-chloroperoxybenzoic acid (m-CPBA), tri-*sec*-butylborane, and 3,5-dimethylpiperidine were purchased from Aldrich Chemical Co., Milwaukee, WI. The *cis*-3,5-dimethylpiperidine was obtained from fractional crystallization of a mixture of 85:15 *cis*- and

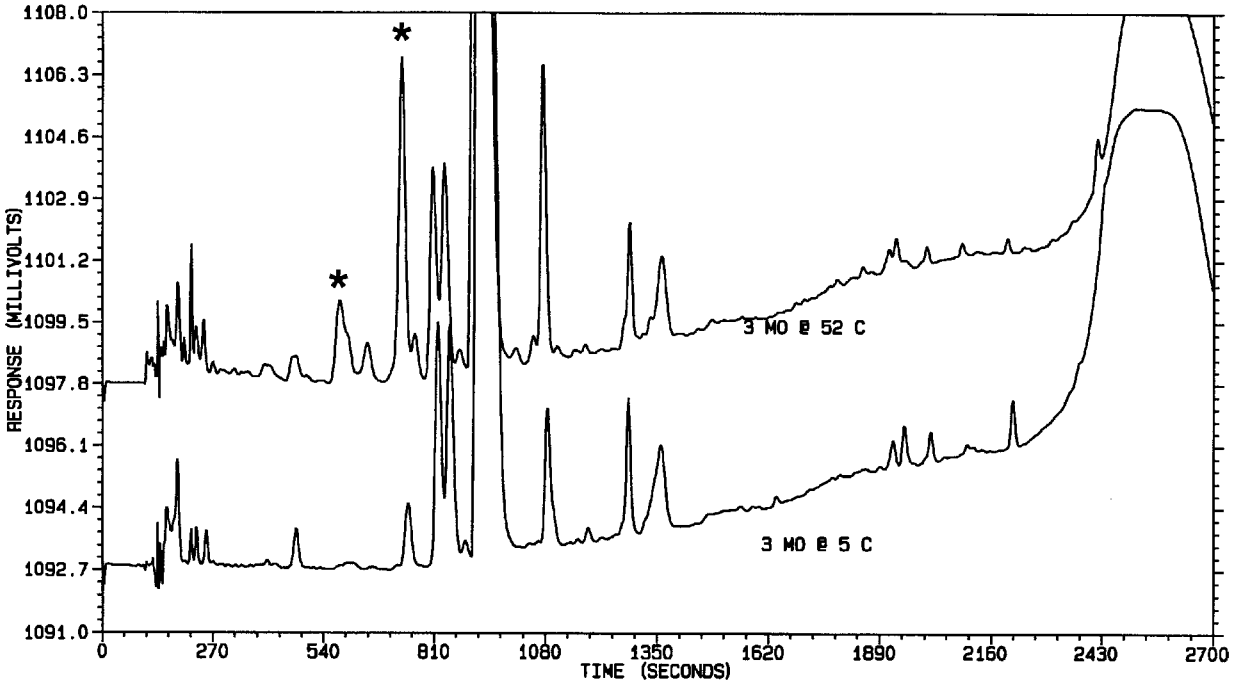

Figure 3. 12,13-Epoxytilmicosin (* epoxides of *trans*-, *cis*-tilmicosin isomers) at 240 nm.

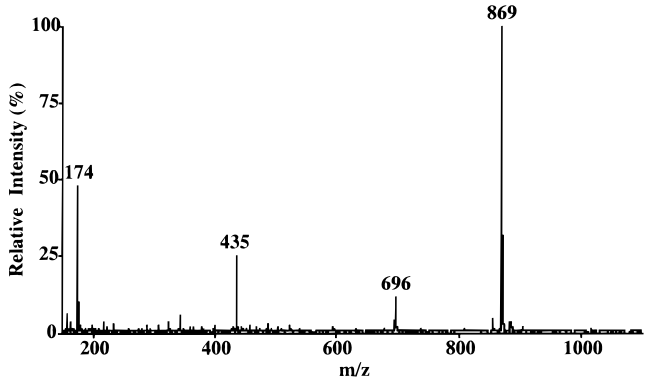

Figure 4. Typical ISP-MS spectrum of tilmicosin.

trans-3,5-dimethylpiperidine hydrochloride (Debono et al., 1989). Thin-layer chromatography was performed using E. Merck silica gel 60 plates with a fluorescent indicator (F-254). Products were purified via silica gel flash chromatography techniques with E. Merck grade 60 silica gel or by use of a Waters Prep LC system. ^{1}H NMR spectra were measured in $CDCl_3$ solution using a General Electric QE-300 NMR. UV spectra were measured in 95% ethanol solution using a Cary 219 UV spectrophotometer. Field desorption mass spectra (FD-MS) were obtained using a Varian-MAT 731 mass spectrometer.

The reaction scheme for synthesis of 12,13-epoxytilmicosin is shown in Figure 2. The procedure involves a five-step synthesis beginning with protection of the aldehyde moiety at the C-20 position of desmycosin by conversion to the diethylketal (Kirst et al., 1988). This is followed by oxidation with m-CPBA to simultaneously form the 12,13-epoxide and *N*-oxide of the dimethylamino group of mycaminose. The *N*-oxide is reduced back to the tertiary amine with tri-*sec*-butylborane (Kabalka et al., 1975), followed by deprotection of the aldehyde to prepare for the reductive amination step. Reductive amination of the C-20 aldehyde with *cis*-3,5-dimethylpiperidine under previously described conditions gives the final 12,13-epoxytilmicosin (Borch et al., 1971; Kirst et al., 1989; Debono et al., 1989).

Step 1. Desmycosin-20-diethylketal. Desmycosin (20 g, 25.9 mmol) was dissolved in 200 mL of absolute ethanol. To this was added *p*-toluenesulfonic acid monohydrate (7.4 g, 1.5 equiv). The reaction mixture was stirred for 1 h, and 4A molecular sieves were added. The reaction mixture was allowed to stand overnight at ambient temperature with the exclusion of moisture. The mixture was filtered to remove the sieves and was then concentrated under reduced pressure to ~100 mL volume. This was diluted with saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to dryness. The solid was purified by preparative liquid chromatography using an 8 L gradient of CH_2Cl_2 to CH_2Cl_2 containing 7.5% methanol and 1% NH_4OH, followed by an additional 2 L of the final gradient solvent composition. Fractions containing the desired product, as determined by thin-layer chromatography, were collected. Combined fractions were evaporated to dryness to yield 16.5 g (75% yield) of desmycosin-20-diethylketal as a white, solid foam: IR ($CHCl_3$, cm^{-1}) 3021, 3010, 2976, 2935, 1592, 1167, 1125, 1081, 1060; FD-MS, *m*/*z* 845 (M^+); UV λ_{max} (ETOH, nm) (ϵ) 282 (23,135); selected ^{1}H NMR ($CDCl_3$) δ (macrolide position) 7.25 (C-11), 6.25 (C-10), 5.85 (C-13), 4.95 (C-15), 4.70 (C-20), 4.00 (C-23), 2.50 (NMe_2), 1.80 (C-22 Me).

Step 2. 12,13-Epoxydesmycosin-20-diethylketal-N-oxide. Desmycosin-20-diethylketal (10.0 g, 11.8 mmol) was dissolved in 120 mL of $CHCl_3$ and then cooled to 0 °C in an ice bath. Upon cooling, m-CPBA (~80%) (10.2 g, 4 equiv) was added in one portion. The mixture was allowed to warm slowly to ambient temperature. The reaction mixture was diluted to 200 mL with $CHCl_3$ and extracted with saturated $NaHCO_3$ solution (2 × 100 mL), 10% Na_2SO_3 (100 mL), and saturated $NaHCO_3$ (100 mL). Crude product was obtained after evaporation of $CHCl_3$ under reduced pressure. Product was purified by preparative liquid chromatography using an 8 L gradient of CH_2Cl_2 to CH_2Cl_2 containing 10% methanol and 1% NH_4OH. Fractions were combined after evaluation by thin-layer chromatography and evaporated to dryness under reduced pressure to yield 5.0 g (49%) of 12,13-epoxydesmycosin-20-diethylketal-*N*-oxide: IR ($CHCl_3$, cm^{-1}) 3005, 2976, 2935, 1183, 1167, 1143, 1127, 1114, 1084; FD-MS, *m*/*z* 878 ($M + H^+$), 862 (M +

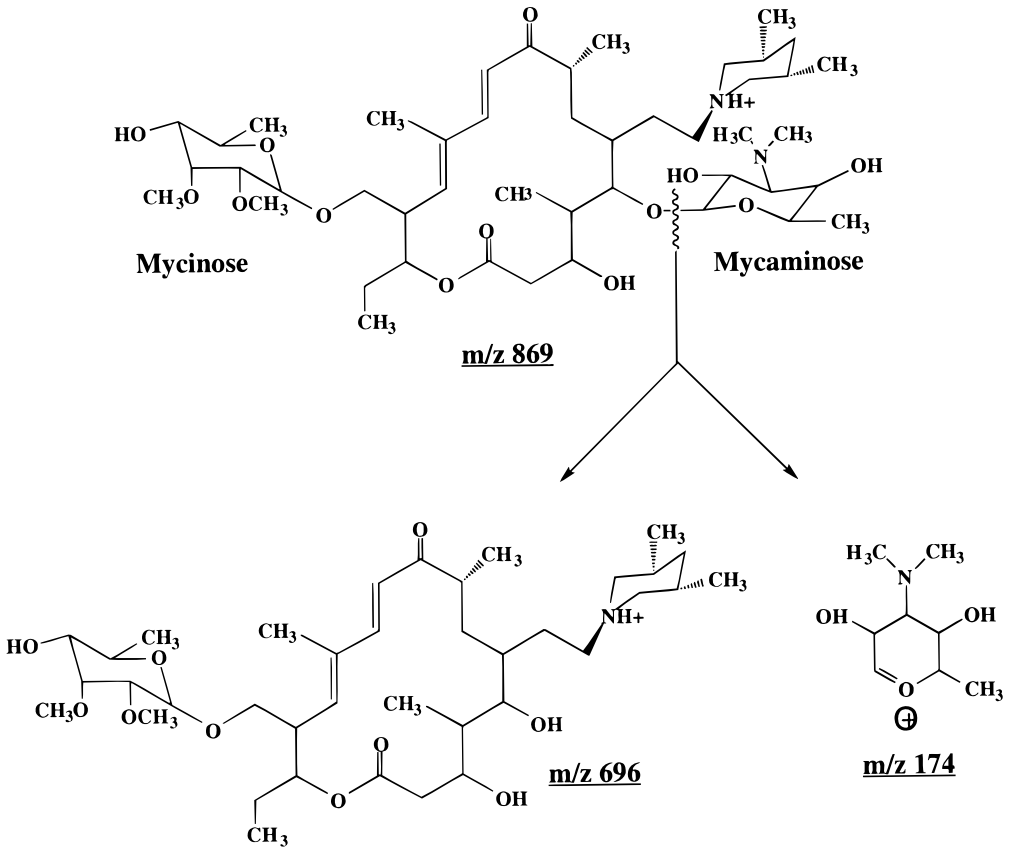

Figure 5. Proposed ISP-MS fragmentation scheme for tilmicosin and related compounds.

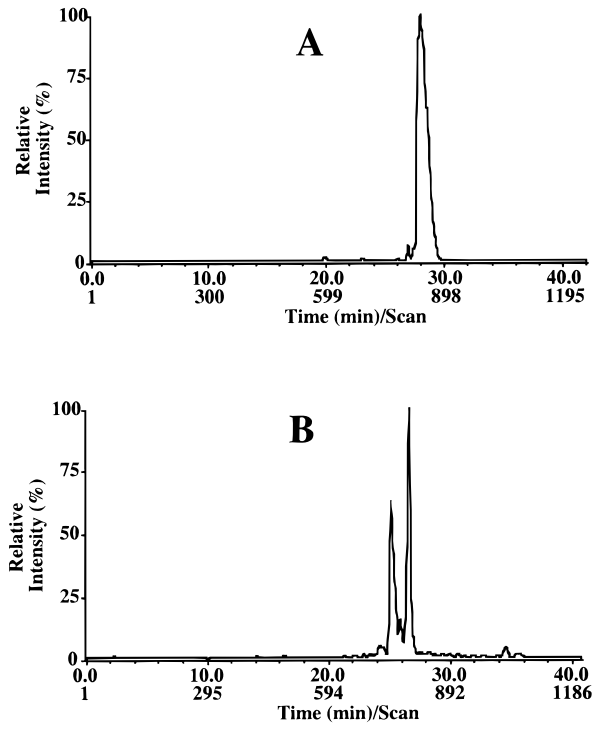

Figure 6. LC/ISP-MS extracted ion chromatograms (*m*/*z* 885) of synthetic 12,13-epoxytilmicosin (A) and the isolated tilmicosin oxidation product (B). MS data collection for (B) was delayed for 1 min, resulting in apparent decreased retention time.

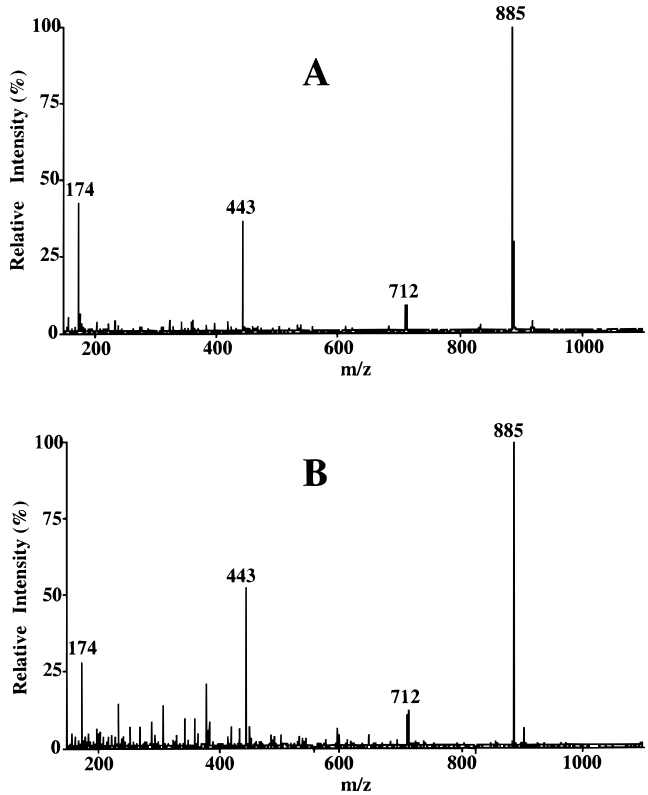

Figure 7. ISP-MS spectra of synthetic 12,13-epoxytilmicosin (A) and the isolated tilmicosin oxidation product (B).

$H^+ - 16$); UV λ_{max} (ETOH, nm) (ϵ) 235 (14,461). Selected ^1H NMR ($CDCl_3$) δ (macrolide position): 6.55 (C-11), 6.45 (C-10), 5.35 (C-15), 4.65 (C-20), 4.15 (C-23), 3.48 (NMe_2) 1.45 (C-22 Me).

Step 3. 12,13-Epoxydesmycosin-20-diethylketal. 12,13-Epoxydesmycosin-20-diethylketal-*N*-oxide (5.0 g, 5.7 mmol) was dissolved in tetrahydrofuran (60 mL) and was overlaid with argon. 1.0 *M* tri-*sec*-butylborane/THF solution (6.2 mL, 1.1

Table 1. In Vitro Activity of 12,13-Epoxytilmicosin and Tilmicosin[a]

	MIC (μg/mL)				
	S. a. C19	*P. m.* 60A	*P. h.* 41D	*M. g.* 29C	*M. h.* S-5972
12,13-epoxytilmicosin	1.56	3.12	3.12	0.78	12.5
tilmicosin	0.78	1.56	1.56	0.19	3.12

[a] Organisms: *S. a., Staphylococcus aureus*; *P. m., Pasteurella multocida*; *P. h., Pasteurella haemolytica*; *M. g., Mycoplasma gallisepticum*; *M. h., Mycoplasma hyopneumoniae.*

eq.) was added to the solution via syringe. The reaction mixture was stirred at ambient temperature under nitrogen. After standing overnight, the reaction was complete as indicated by thin-layer chromatography. The reaction mixture was shaken with saturated $NaHCO_3$ solution (500 mL) and was extracted with CH_2Cl_2 (4 × 50 mL). The organic layer was dried (Na_2SO_4) and was evaporated to dryness under reduced pressure to give a white foam. Product was purified by preparative liquid chromatography. Fractions containing product were combined and evaporated to dryness to yield 2.3 g (48%) 12,13-epoxydesmycosin-20-diethylketal. IR ($CHCl_3$, cm^{-1}) 3018, 3014, 2976, 2936, 1456, 1184, 1167, 1141, 1125, 1082, 1060. FD-MS *m/z* 862 ($M+H^+$). UV λ_{max} (ETOH, nm) (ϵ) 237 (13,260); selected ^1H NMR ($CDCl_3$) δ (macrolide position) 6.55 (C-11), 6.45 (C-10), 5.30 (C-15), 4.70 (C-20), 4.15 (C-23), 2.50 (NMe_2), 1.42 (C-22 Me). Anal. Calcd for $C_{43}H_{75}NO_{16}$: C, 59.34; H, 8.89; N, 1.65. Found: C, 59.57; H, 8.66; N, 1.61.

Step 4. 12,13-Epoxydesmycosin. The deprotection of the aldehyde moiety was achieved by dissolving 12,13-epoxydesmycosin-20-diethylketal (1.0 g, 1.2 mmol) in acetonitrile (10 mL). Sulfuric acid (0.1 N, 25 mL) was added. The reaction mixture was stirred at ambient temperature for ~3 h. The reaction mixture was added to saturated $NaHCO_3$ solution (200 mL) and was extracted with CH_2Cl_2 (4 × 50 mL). The organic phase was dried (Na_2SO_4) and was evaporated under vacuum to give a white foam (0.92 g, 100%): no chromatography was required; IR ($CHCl_3$, cm^{-1}) 2976, 2937, 1722, 1691, 1621, 1186, 1167, 1141, 1083, 1059, 1008; FD-MS, *m/z* 787 (M^+); UV λ_{max} (ETOH, nm) (ϵ) 238 (11,760); selected ^1H NMR ($CDCl_3$) δ (macrolide position) 9.72 (C-20), 6.55 (C-11), 6.45 (C-10), 5.35 (C-15), 4.15 (C-23), 2.50 (NMe_2), 1.45 (C-22 Me). Anal. Calcd for $C_{39}H_{65}NO_{15}$: C, 59.45; H, 8.31; N, 1.78. Found: C, 59.72; H, 8.02; N, 2.04.

Step 5. 12,13-Epoxytilmicosin. The final step of the synthesis involved reductive amination of the 12,13-epoxydesmycosin aldehyde with *cis*-3,5-dimethylpiperidine using sodium cyanoborohydride ($NaBH_3CN$). 12,13-Epoxydesmycosin (1.5 g, 1.9 mmol) was dissolved in methanol. After dissolution, *cis*-3,5-dimethylpiperidine (2.6 mL, 10 equiv) was added. $NaBH_3CN$ (0.12 g, 3 equiv) was added to the solution after ~15 min. The reaction mixture was allowed to stir at ambient temperature with the exclusion of moisture. After 5 h, the volatiles were removed under vacuum and the resultant yellow residue was dissolved in ethyl acetate (50 mL). The desired product was extracted with 0.5 M, pH 5.5, phosphate buffer (3 × 50 mL). The aqueous extracts were combined, and the pH was adjusted to pH 8 with 5 N NaOH. The aqueous portion was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to dryness. The product was purified by silica gel flash chromatography using a 1 L gradient of CH_2Cl_2 to CH_2Cl_2 containing 10% methanol and 1% NH_4OH followed by an additional 1 L of the final gradient solvent composition. Fractions containing the desired product as identified by thin-layer chromatography were combined and evaporated to dryness under reduced pressure to give a white foam, 12,13-epoxytilmicosin (0.58 g, 34%): IR ($CHCl_3$, cm^{-1}) 3019, 2973, 2953, 2934, 1456, 1167, 1141, 1082, 1059; FD-MS, *m/z* 885 ($M + H^+$); UV λ_{max} (ETOH, nm) (ϵ) 238 (12,500); selected ^1H NMR ($CDCl_3$) δ (macrolide position) 6.55 (C-11), 6.50 (C-10), 5.30 (C-15), 4.15 (C-23), 2.50 (NMe_2), 1.45 (C-22 Me), 0.45 (piperidine 4-axial). Anal. Calcd for $C_{46}H_{80}N_2O_{14}$: C, 62.42; H, 9.11; N, 3.16. Found: C, 63.02; H, 9.03; N, 3.58.

Tilmicosin: selected ^1H NMR data for reference, 7.20 (C-11), 6.50 (C-10), 5.87 (C-13), 4.97 (C-15), 3.95 (C-23), 2.48 (NMe_2), 1.84 (C-22 Me), 0.45 (piperidine 4-axial) (Kirst et al., 1994).

RESULTS AND DISCUSSION

A number of naturally occurring 16-membered-ring macrolide antibiotics containing an epoxy moiety at the C-12 and C-13 positions have been isolated from fermentations. Structures and detailed information of these macrolides have been reviewed (Omura, 1984; Kirst, 1992). Among the macrolides containing the 12,13-epoxyenone system within the 16-membered macrolide ring include carbomycin A, deltamycin, angolamycin, cirramycin, and rosaramicin. A key physicochemical characteristic of these epoxy macrolides is their UV absorbance around 240 nm instead of the characteristic 280 nm absorbance for 16-membered macrolides containing the dienone system. This was a contributing factor in recognizing the oxidative degradation product as 12,13-epoxytilmicosin.

Identification of Degradation Product. The identification of the degradation product occurred during accelerated stability studies at high temperature (52 °C). Degradation due to high-temperature storage is not unexpected. Indeed, stability results from accelerated storage conditions are critical in early-stage formulation development to determine product shelf life and potential degradation pathways. These accelerated storage conditions indicated a significant loss of tilmicosin potency, as determined by the decrease in tilmicosin peak area from the HPLC chromatogram, in partially filled containers (25 mL or 10% of fill volume) compared to full containers (250 mL), suggesting oxidation as a contributing factor in the loss of tilmicosin HPLC peak area.

To confirm the role of oxygen in the potency loss, a study was conducted to evaluate the impact of nitrogen versus oxygen in the container headspace. In this study, containers were filled to ~10% of their total volume and the container headspace was replaced with nitrogen, air, or oxygen. The sealed containers were then placed in 52 °C incubators, and contents were monitored by HPLC. Within 7 days, results showed tilmicosin formulation in the nitrogen overlaid containers exhibited essentially no potency loss. Tilmicosin in containers overlaid with air lost ~2%, whereas the oxygen overlaid samples lost nearly 10% tilmicosin. This method was subsequently used to rapidly screen various tilmicosin formulations for their potential to inhibit oxidation.

Once oxidation was established as the cause of tilmicosin potency loss, the next issue to resolve was identification of the oxidation product. Using a Beckman System Gold HPLC equipped with a model 168 photodiode array detector, a gradient system was developed to analyze related substances of the tilmicosin formulation. By monitoring the spectrum of wavelengths, coupled with good peak separation, two new peaks were identified at an absorbance of 240 nm (Figure 3). Because the dienone system of tilmicosin absorbs at 280 nm, absorbance of the new peaks at 240 nm indicated an alteration of the dienone. This, coupled with the role of oxygen, strongly suggested formation of an epoxide

at the 12,13-double-bond position of the dienone of the 16-membered macrolide ring.

Synthesis. The synthetic scheme shown in Figure 2 was successfully followed to synthesize 12,13-epoxytilmicosin. Analysis of the physicochemical data of the final product indicated a mass increase of 16 over parent, a change in UV λ_{max} from 280 to 238 nm, and NMR data consistent with epoxide formation at the 12,13-double bond. The NMR revealed a shift of the doublet of the proton at C-11 to 6.55 ppm from 7.20 ppm and the disappearance of the doublet of the proton at C-13 that resides at 5.87 ppm in tilmicosin. The protons of the methyl group at position C-22 have also shifted from their characteristic 1.84 to 1.45 ppm. Thus, with the chromatographic information and successful synthesis of the 12,13-epoxytilmicosin, liquid chromatography/ion spray mass spectrometry (LC/ISP-MS) was used for final confirmation of the tilmicosin oxidation product.

LC/ISP-MS. Figure 4 shows the most significant ions observed in the ISP-MS spectra of tilmicosin. Modulation of the orifice voltage parameter results in the formation of structure-indicating fragment ions, suggesting the general scheme proposed for the fragmentation of tilmicosin and related compounds as displayed in Figure 5. The most prominent peak in the high-mass region is due to the $[M + H]^+$ ion present at *m/z* 869 ($C_{46}H_{81}N_2O_{13}$). An abundant ion representing $[M + 2H]^{2+}$ is also present at *m/z* 435. The fragment peaks observed at *m/z* 696 ($C_{38}H_{66}NO_{10}$) and 174 ($C_8H_{16}NO_3$) arise from loss of the mycaminose substituent from the protonated macrolide ring structure, with subsequent protonation at the macrolide fragmentation site to form an alcohol.

Figure 6 displays extracted ion chromatograms (EIC) obtained from LC/ISP-MS analysis of the synthetic 12,-13-epoxytilmicosin and the isolated tilmicosin oxidation product. The EIC of the synthetic compound reveals one peak representing the epoxide of the predominant cis form of the tilmicosin, the expected product. The EIC of the isolated oxidation product, however, features two peaks exhibiting identical mass spectra. These two peaks likely represent the epoxides of the trans and cis isomers of tilmicosin, with respect to elution order. This observation is consistent with the fact that commercial formulations generally contain both the trans and cis isomers, with the cis form predominating.

The ion spray mass spectra associated with both the synthetic 12,13-epoxytilmicosin and the isolated tilmicosin oxidation product are shown in Figure 7. Both mass spectra exhibit a similar fragmentation pattern consistent with the epoxidized form of tilmicosin. The $[M + H]^+$ ion at *m/z* 885 ($C_{46}H_{81}N_2O_{14}$) is the base peak, and an abundant peak representing $[M + 2H]^{2+}$ is also present at *m/z* 443. The fragment peaks observed at *m/z* 712 ($C_{38}H_{66}NO_{11}$) and 174 ($C_8H_{16}NO_3$) arise from loss of the intact mycaminose substituent from the protonated macrolide ring structure as previously described. The fragment at *m/z* 712 indicates a 16 u increase in mass located either on the macrolide ring structure or at the dimethylpiperidine or mycinose substituents. The assignment of an additional oxygen atom as an epoxide (12,13-epoxytilmicosin) is supported by the observed shift in UV absorbance characteristics, HPLC retention time matching, and mass spectrometry fragmentation pattern matching with the synthesized 12,13-epoxytilmicosin.

In Vitro Antimicrobial Activity. 12,13-Epoxytilmicosin was compared to tilmicosin for antimicrobial activity against a variety of microorganisms. Table 1 summarizes the in vitro antibacterial activity of these compounds against selected strains. Antibiotic susceptibility data were obtained by broth microtiter dilution conducted according to National Committee for Clinical Laboratory Standards (NCCLS) methods (Ose, 1987; NCCLS, 1997). The formation of the 12,13-epoxide did not cause major reduction of in vitro antimicrobial activity as shown in Table 1.

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