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Inactivation of Etamycin by a Novel Elimination Mechanism in *Streptomyces lividans*

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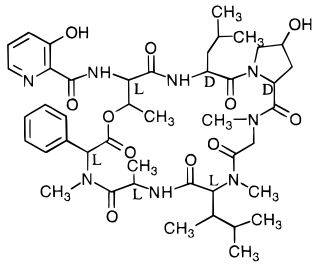
Abstract: An enzyme responsible for inactivating the peptidolactone antibiotic etamycin was partially purified from extracts of *Streptomyces lividans*. On-line liquid chromatography−electrospray mass spectrometry showed that the product of the enzyme-catalyzed reaction had a different retention time but the same mass as etamycin. Reaction of etamycin in 1.0 M NaOH gave a mixture consisting of the product expected from hydrolysis of the lactone bond and a product corresponding to that from the enzyme reaction. Label from $H_2{}^{18}O$ was incorporated into the hydrolysis product but not into the product of the enzyme reaction. An enzyme-catalyzed elimination reaction is proposed, and is supported by evidence for the structure of the resulting dehydrobutyrine peptide from combined liquid chromatography−tandem mass spectrometry (LC−MS−MS) and ^{1}H NMR spectroscopy.

Introduction

Antibiotics of the streptogramin B group are macrocyclic peptidolactones in which an N-terminal threonine is N-acylated with 3-hydroxypicolinic acid and esterified via its 3-hydroxyl group with a C-terminal phenylglycine or phenylsarcosine.[1] Type B-I streptogramins have six amino acids; etamycin (**1**) with seven amino acids[2] is a type B-II streptogramin. The streptogramins inhibit most major Gram-positive and some Gram-negative bacterial pathogens,[3] and for over 25 years have been produced for human therapeutic use in the treatment of staphylococcal infections.[4] Streptogramins have not found more general therapeutic use because of their limited water solubility, but the introduction of a water-soluble, semisynthetic streptogramin RP59500 has renewed interest in their potential applications.[5]



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The development of resistance by members of the Streptococci, Staphylococci, Enterobacteriaceae, and Pseudomonas

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families to virtually all of the older antibiotics has created a need for effective new antibiotics,[6] and an understanding of the antibiotic resistance mechanisms in bacteria is critical for a rational approach to the design of such drugs.[7] Three mechanisms are reported to mediate bacterial resistance to the streptogramins: (i) target modification by enzymes that methylate 23S rRNA; this confers cross-resistance to macrolides and lincosamides as well as among other streptogramin B-type antibiotics (the MLS phenotype),[8] (ii) active efflux of the antibiotics probably mediated by an ATP-binding protein,[9] and (iii) inactivation by a lactone hydrolase.[10]

During the course of experiments aimed at cloning an etamycin resistance gene from an etamycin producer, we detected enzyme-catalyzed inactivation of etamycin in the cloning host *Streptomyces lividans*. The characterization of the inactivated product reported here supports lactone ring scission by an elimination reaction as the inactivation mechanism, in contrast to the hydrolysis route previously reported for the structurally related antibiotics dihydrostaphylomycin S, mikamycin B, pristinamycin 1A, and virginiamycin B.[10]

Experimental Section

Bacterial Strains and Culture Conditions. Etamycin superresistant (ESR) strains (minimum inhibitory concentration (MIC) >100 μg/mL) of *S. lividans* TK24 (SLP1$^-$, SLP2$^-$ *str*; MIC 30 μg/mL; derived from *S. lividans* 66)[11] were isolated from regenerated protoplasts resistant to high levels of etamycin. Protoplasts were prepared and regenerated on R5 medium.[11] MIC values were determined by spreading spores of the TK24 or ESR strains on the surface of MYM[12] agar containing 5−200 μg/mL etamycin, and incubating at 30 °C for 72 h. The etamycin-sensitive *Micrococcus luteus* (FDA strain PC1 1001) used to assay for etamycin was grown in GNY medium[13] at 37 °C for 24 h. Standard samples of etamycin were obtained from Parke, Davis and Co., Ann Arbor, MI.

Partial Purification of the Inactivating Enzyme. YEME medium[11] (500 mL) was inoculated with spores of *S. lividans* ESR1 and incubated with orbital shaking (220 rpm) for 48 h at 27 °C. The mycelium harvested by centrifugation (5000*g*, 10 min) was washed twice and resuspended in Hepes buffer (25 mL, 50 mM containing 10 mM $MgCl_2$, 60 mM NH_4Cl, 4 mM mercaptoethanol, and 10% (v/v) glycerol and supplemented with 0.5 mM phenylmethanesulfonyl fluoride after the pH was adjusted to 7.6 with KOH). The mycelial suspension was sonicated (Branson Sonifer, six pulses of 6 s at 50 MHz), heated at 95 °C for 5 min, and centrifuged (11000*g*, 30 min). Fractionation of the supernatant with ammonium sulfate precipitated an active fraction at 45−65% saturation.

Enzyme Incubations. Typically, partially purified enzyme in Hepes buffer (50 μL) was mixed with an equal volume of etamycin solution (500 μg/mL in distilled H_2O) and incubated at 30 °C for 30 min. For the experiment in labeled water, partially purified enzyme (20 μL) was mixed with Hepes buffer (80 μL) and taken to dryness (Speedvac). The solid was resuspended in $H_2{}^{18}O$ (100 μL, 98.6 atom % ^{18}O, Isotec Inc., Miamisburg, OH), mixed with etamycin dissolved in [^{18}O]water (25 μL, 0.5 mg/mL), and incubated at 37 °C for 10 min. Control incubations under basic conditions were carried out by dissolving etamycin (84 μg) in 1 M NaOH in [^{18}O]water (100 μL). The mixture was incubated at 37 °C for 12 h, neutralized with 1 M HCl, and taken to dryness (Speedvac). The solid was redissolved in water (100 μL) for LC−MS analysis.

The incubation mixtures were analyzed for etamycin and its reaction products by thin-layer chromatography (Sil 60 F254, E. Merck, Darmstadt, Germany, benzene−methanol−water (12:6:1), R_f 0.23 and 0.31, respectively) or liquid chromatography. The latter was carried out using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a ternary DR5 solvent delivery system and a 2.1 × 250 mm Vydac 218TP52 column. Samples (10 μL injection) were eluted over 20 min using a linear gradient of 20−90% acetonitrile (0.1% trifluoroacetic acid). Residual antibiotic activity was determined by adding the incubation mixture (20 μL) to sterile filter paper disks (13 mm diameter) placed on MYM agar seeded with *M. luteus*. After 24 h at 37 °C, inhibition zone diameters were measured.

Purification of the Enzyme Reaction Product. A reaction mixture in which 20 mg of etamycin had been incubated with 10 mL of partially purified cell extract was acidified to pH 4.5 and extracted with ether (3 × 50 mL). The residue obtained by evaporation of the extract was suspended in methanol−water (7:3), and the leachate was applied to a column (0.9 × 22 cm) of C_{18} reversed phase silica (Bakerbond, 40 μm particle size) equilibrated with methanol−water (7:3). The column was developed with a stepped methanol−water gradient, and fractions of the eluate were monitored by HPLC. The enzyme reaction product was eluted as a single peak with methanol−water (9:1). Evaporation of one of the peak fractions yielded a colorless residue (2.1 mg). Etamycin and purified enzyme reaction product were dissolved in CD_3-OD (approximately 1 mg/mL), and the ^{1}H-NMR spectra were acquired at 500.13 MHz on a Bruker AMX-500 spectrometer.

Mass Spectrometry. The LC−MS experiments were carried out using a Perkin-Elmer SCIEX API/III$^+$ triple quadrupole mass spectrometer with an electrospray interface. A postcolumn split delivered 15 μL/min to the mass spectrometer. Full-scan MS spectra (1 Da step and 3.5 ms dwell) were acquired over 400−1100 Da. Tandem mass spectrometry employed a collision energy of 25 eV in the laboratory frame of reference using argon at a collision gas thickness of 3.5 × 10^{15} atoms/cm^2. Full-scan MS−MS spectra were acquired in 0.2 Da steps with a 3 ms dwell time.

The high-resolution mass measurement was performed on a VG AutoSpec M OA-TOF using positive electrospray ionization at an accelerating voltage of 4000 V nominal. Spectra ($n = 3$) were obtained by a source voltage scan over a mass range of 925−825. An internal calibrant (PEG1000) was used for peak matching to obtain the exact mass of the unknown. Matching of the empirical formula was performed with an OPUS data system.

Results and Discussion

Antibiotic resistance in the ESR strains was due to elevated levels of an etamycin-inactivating enzyme that was also detectable in the wild type organism. Extracts of *S. lividans* mycelium (but not culture supernatant) converted etamycin (**1**) to a compound lacking activity against *M. luteus*, and analysis by liquid chromatography (LC) detected a single product at a retention time (RT) lower than that of the substrate. Separation achieved using on-line LC−MS showed that both the enzyme reaction product (RT 13.0 min) and etamycin (RT 15.6 min) had an $[M + H]^+$ ion at *m*/*z* 880 (Figure 1). Accurate mass measurements confirmed that the $[M + H]^+$ ions of etamycin (879.457 ± 0.008) and its enzyme reaction product (879.460 ± 0.004) are identical within experimental error and correspond to the theoretical monoisotopic molecular mass for the $[M + H]^+$ ion of etamycin (879.4616).

Treatment of etamycin with 1.0 M NaOH (37 °C, 12 h) yielded a product at RT 13.0 min and $[M + H]^+$ at *m*/*z* 880, corresponding to the enzyme-catalyzed reaction product, as well

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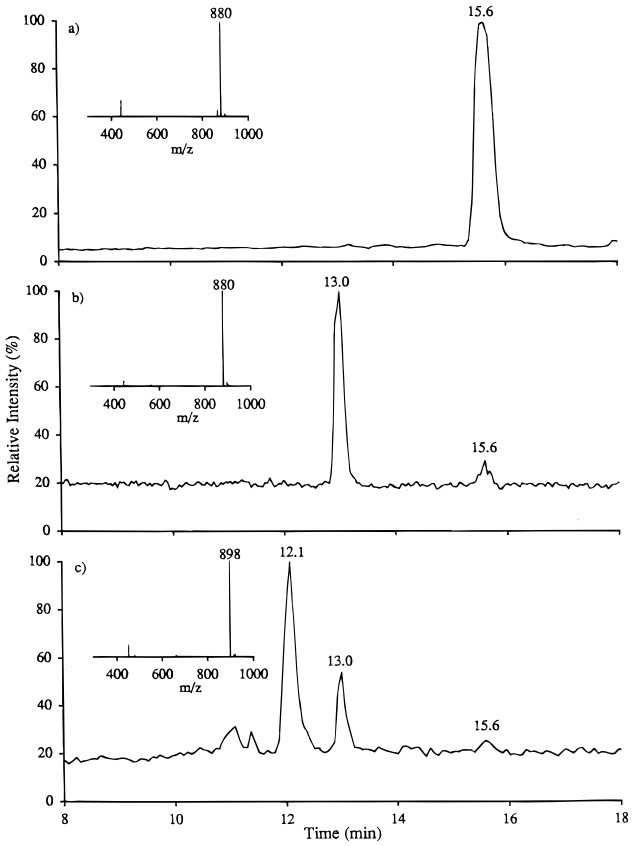

Figure 1. LC−MS analysis: total ion chromatograms and extracted mass spectra (shown as insets); (a) etamycin (15.6 min); (b) enzyme-catalyzed reaction (extracted mass spectrum for product at 13.0 min); (c) base-catalyzed reaction (extracted mass spectrum for product at 12.1 min).

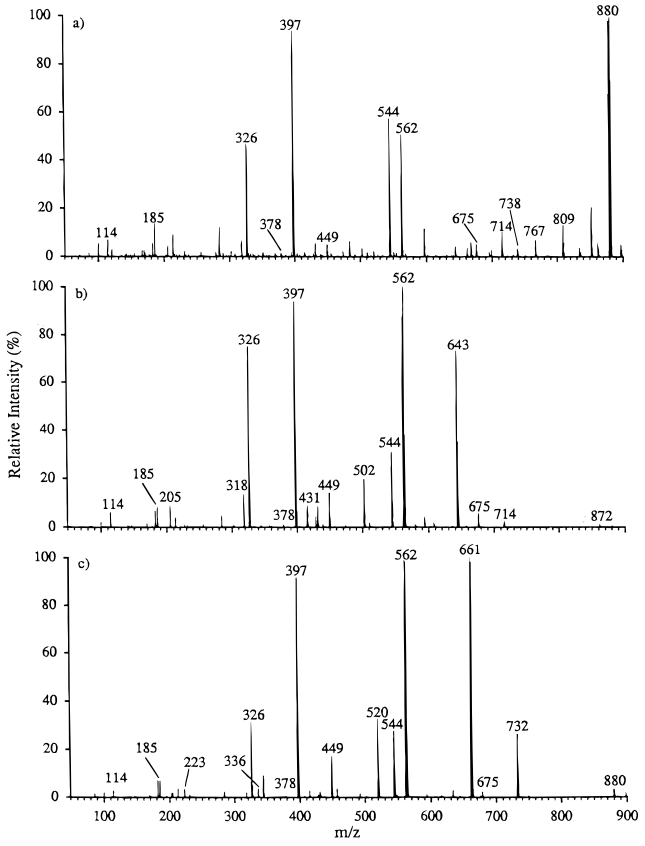

Figure 2. Product ion spectra: (a) etamycin (precursor ion at *m*/*z* 880); (b) enzyme reaction product (precursor ion at *m*/*z* 880); (c) base-catalyzed hydrolysis product (precursor ion at *m*/*z* 898).

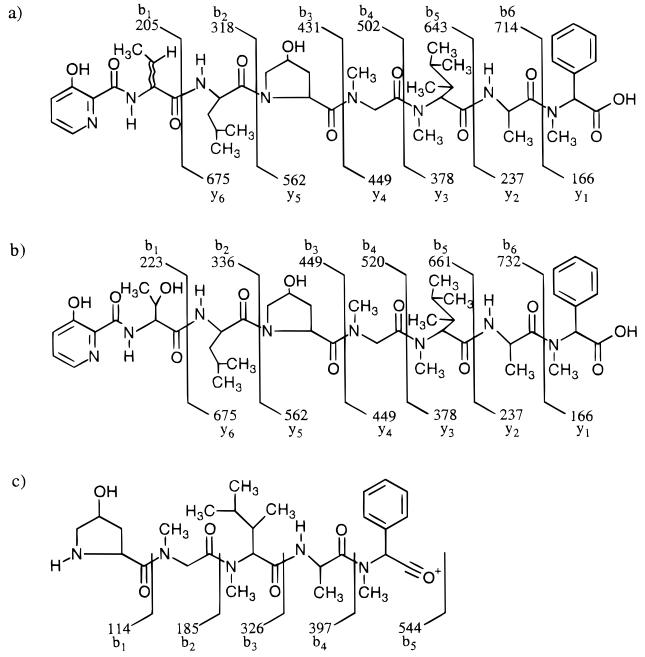

Figure 3. Structures and fragment ions: (a) enzyme reaction product; (b) base-catalyzed hydrolysis product (the y-series ions show a 2-Da shift in the isotope incorporation experiment); (c) fragment common to all due to facile cleavage at the hydroxyproline residue.

as a more abundant species at RT 12.1 min (Figure 1c). The $[M + H]^+$ ion for the latter product was at *m*/*z* 898, the expected mass for the product of a hydrolysis reaction. When the reaction was repeated in ^{18}O-enriched water (>90% ^{18}O), the $[M + H]^+$ ion of the latter product shifted from *m*/*z* 898 to *m*/*z* 900, consistent with incorporation of one oxygen from the labeled water. No shift occurred in the peaks for the product at *m*/*z* 880 or for the residual etamycin. Moreover, when the enzyme-catalyzed reaction was carried out in $H_2{}^{18}O$ (>98% ^{18}O), no incorporation of isotope was observed in the product or substrate.

The results, which do not support enzyme-catalyzed hydrolysis of etamycin (mechanism iii above), are consistent with lactone ring scission by an elimination mechanism yielding a linear dehydrobutyrine peptide with the same mass as etamycin but without incorporation of water. A possible hydrolysis/elimination reaction sequence is ruled out by the isotopic experiments, the absence of a hydrolysis product in the enzyme-catalyzed reaction, and evidence that the elimination and hydrolysis products are formed in a constant ratio over time during the base-catalyzed reaction.

The structures of the enzyme and base-catalyzed products were examined by LC with tandem mass spectrometry. The product ion spectra for the $[M + H]^+$ ion of etamycin, its enzyme reaction product, and its hydrolysis product are presented in parts a−c, respectively, of Figure 2. Fragment ions at *m*/*z* 714 and 675 (Figure 2b, loss of phenylsarcosine and *N*-(3-hydroxypicolinyl)dehydrobutyrine residues, respectively) and *m*/*z* 732 and 675 (Figure 2c, loss of phenylsarcosine and *N*-(3-hydroxypicolinyl)threonine residues, respectively) confirmed that linear peptides resulted from lactone cleavage in each reaction. In contrast, ring scission at different locations in the cyclic etamycin generated fragment ions at *m*/*z* 809, 767, and 738 (Figure 2a) due to the loss of an alanine (or sarcosine, leucine or hydroxyproline), and dimethylleucine residues, respectively. The characteristic b-type sequence ions[14] of the hydrolysis product (Figures 2c and 3b) are 18 Da higher than

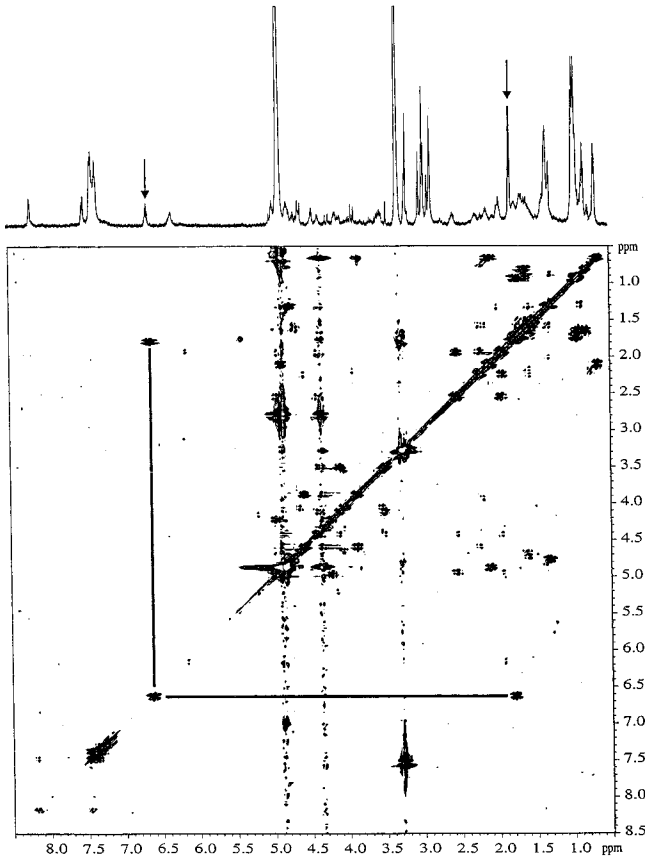

Figure 4. COSY and 1-D ^{1}H-NMR spectra of the enzyme reaction product. Coupling between the methyl and vinyl protons of the dehydrobutyrine residue is indicated.

those of the enzyme reaction product (Figures 2b and 3a), consistent with the dehydrobutyrine residue in the latter. The y-type sequence ions[14] (*m*/*z* 675, 562, 449, and 378) and b-type ions (*m*/*z* 544, 397, 326, 185, and 114) obtained after initial cleavage of the amide bond of the hydroxyproline residue (Figure 3c) are common to the mass spectra of both reaction products and etamycin (Figure 2). Abundant fragment ions of the latter series are formed due to the facile cleavage of amide bonds at proline residues.[15] A 2-Da shift in the y-ion series but not the b-ion series demonstrated that isotope from ^{18}O-enriched water was incorporated into the phenylsarcosine residue of the hydrolysis product (data not shown).

The presence of a dehydrobutyrine residue in the enzyme-catalyzed product was further supported by coupling ($J = 6.8$ Hz) between a vinyl proton at 6.63 ppm and the protons of a methyl group at 1.80 ppm in the 1-D ^{1}H-NMR spectrum. Confirmation of this coupling was obtained in COSY (Figure 4) and TOCSY experiments. The shift of the threonine methyl signal from 1.28 ppm in the etamycin spectrum to 1.80 ppm in the spectrum of the enzyme reaction product is also consistent with the formation of a double bond. The assignment of the etamycin methyl group was based on previous NMR studies of other streptogramins.[16]

Dehydrobutyrine and dehydroalanine residues are found in some peptide antibiotics, including the streptomycete products berninamycin, nosiheptide, and thiostrepton. Threonine and serine have been identified as the precursors to these units,[17] but it is not known whether an ester is an intermediate of the dehydration step in these pathways. On the other hand, analogous eliminations of acyl groups have been documented for the bacterial biosynthesis of cysteine from *O*-acetylserine[18] and methionine from either *O*-acetylhomoserine or *O*-succinylhomoserine.[19]

In summary, enzyme-catalyzed ring opening of the macrocyclic peptidolactone by an elimination mechanism confers resistance to etamycin on *S. lividans*. The reaction contrasts with the previously reported hydrolytic inactivation.[10] The generality of the resistance mechanism is under investigation with other peptidolactone antibiotics.

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