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Probing the Substrate Specificity of an Enzyme Catalyzing Inactivation of Streptogramin B Antibiotics using LC–MS and LC–MS/MS†

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LC–MS and LC–MS/MS analyses indicated that an enzyme responsible for inactivating the antibiotic etamycin is specific for streptogramins and acts on both type B-I and B-II streptogramin subgroups. No enzymatic activity was detected for other cyclodepsipeptides such as surfactins and viscosin. It was demonstrated using analogs of etamycin that the picolinyl moiety is essential to obtain enzyme-generated ring-opened compounds. Because the picolinyl moiety is also essential for the biological activity of streptogramins, it is proposed that this residue is a distinctive topographic feature in the binding of this group of antibiotics to enzyme active sites. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Antibiotic resistance is now recognized as an area of concern in the health sciences, and the increased frequency with which it is encountered in pathogenic organisms has been referred to as a 'crisis',[1] an 'incipient public health emergency'[2] and a 'medical disaster'.[3] Understanding the mechanisms of antibiotic resistance in bacteria is critical for a rational approach to the design of new antimicrobial agents.[4] Recently we reported a novel mechanism for bacterial resistance to etamycin,[5] a macrocyclic peptidolactone antibiotic that belongs in the streptogramin B group.[6] Resistance was linked to antibiotic inactivation by an enzyme-catalyzed ring opening, which was elucidated with the aid of liquid chromatography (LC) coupled with electrospray mass spectrometry (ESMS) and on-line tandem mass spectrometry (LC–MS/MS).[5]

The peptidolactone ring of etamycin and other type B-II streptogramins is composed of seven amino acid residues, while that of B-I streptogramins has only six residues (Fig. 1). Structural variation within each subgroup occurs in the side chain substituents. The streptogramins show antibiotic activity against most major Gram-positive, some Gram-negative bacterial pathogens, including *Mycobacterium tuberculosis*, and against some fungi.[7] Representatives of the B-I subgroup have been used for over 25 years to treat staphylococcal infections in humans.[8] Like erythromycin, the streptogramins interfere with protein synthesis by blocking ribosomal function. Production of water soluble, semisynthetic streptogramin derivatives has renewed interest in these antibiotics for more general therapeutic usage,[9] and a recent mongraph is entirely devoted to the properties of RP59500, a mixture of synergic components.[10]

A common feature of the streptogramins is the presence of a substituted threonine at the N-terminus of the peptide which has been cyclized via a lactone linkage. The amino acid is N-acylated with 3-hydroxypicolinic acid and esterified via its 3-hydroxyl group with the C-terminal carboxyl group of either a phenylglycine or phenylsarcosine residue. The remaining amino acids in the peptide can vary and are not restricted to the 20 commonly found in proteins (Fig. 1). We have investigated whether the enzyme-catalyzed inactivation observed with etamycin[5] extends to other streptogramins. Both types B-I and B-II streptogramins, as well as non-streptogramin peptidolactones and cyclic depsipeptides, were incubated with a partially purified extract of *Streptomyces lividans* that inactivated etamycin, and the reaction was monitored using LC–MS and LC–MS/MS to investigate the specificity of enzyme activity. The importance of the common structural features of streptogramins has been investigated using analogs of etamycin bearing a modified picolinyl moiety.

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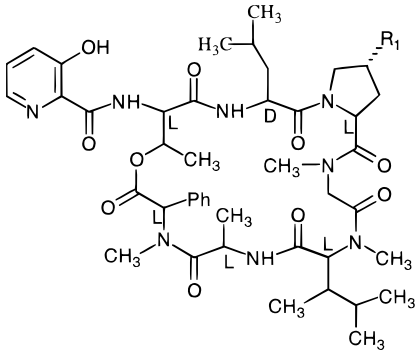
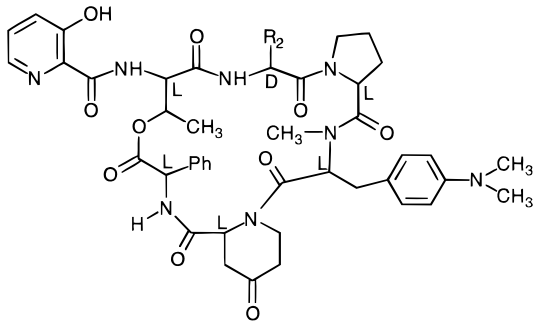


Type BII

	R_1
Etamycin	OH
Etamycin B	H



Type BI

	R_2
Vernamycin Bα	CH_2CH_3
Vernamycin Bγ	CH_3

Figure 1. Structures of streptogramin B-I and B-II antibiotics investigated in this study.

EXPERIMENTAL

Enzyme Substrates

Enniatins and sporidesmolides were previously isolated at the Institute for Marine Biosciences.[11,12] Viscosin and related surfactins were isolated as described elsewhere.[13] Vernamycins were obtained as vernamycin B_α from E. R. Squibb and Sons (New Brunswick, NJ). Etamycin and etamycin B were isolated by methods described previously.[14,15]

Des-3-hydroxypicolinoyl etamycin

The 3-hydroxypicolinoyl residue was cleaved from etamycin by a modification of the method described for pristinamycins.[16,17] Zinc dust (13.4 mg) was added in small portions with manual shaking over a 30 min period to etamycin (2.4 mg) dissolved in an acidic solution of deionized water (250 µl) and 12 M HCl (22 µl). The mixture was shaken on an orbital mixer for another 30 min and neutralized with 1.0 M NaOH (~100 µl). CH_2Cl_2 (80 µl) was added and the mixture was filtered through a 0.22 µm Ultrafree MC centrifuge filter unit (Millipore Corp., Bedford, MA). The layers were separated and the aqueous layer was re-extracted with CH_2Cl_2 (80 µl). The organic layers were combined, evaporated to dryness and redissolved in 50% methanol for LC–MS/MS analysis of the reduction products. No products were detected by LC–MS analysis of the aqueous layer.

Enzyme extract

Culture conditions for *Streptomyces lividans* strain ESR and the partial purification of the etamycin-inactivating enzyme were described previously.[5]

Enzyme Incubations

Partially purified enzyme in 50 µl Hepes buffer[5] was mixed with an equal volume of substrate solution (0.5–2.5 mg ml^{-1} in distilled H_2O) and incubated at 37 °C for 10–180 min.

LC

All LC separations were carried out with a Hewlett-Packard 1091 Series II liquid chromatograph equipped with a ternary DR5 solvent delivery system and a 2.1 mm × 250 mm Vydac 218TP52 column. Samples (10 µl) were eluted over 20 min using a linear gradient of 20%–90% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml min^{-1}.

Mass Spectrometry

The LC–MS experiments were carried out using a Perkin-Elmer SCIEX API/III$^+$ triple-quadrupole mass spectrometer with an Ionspray™ interface. A post-column split delivered 15 µl min^{-1} of the column flow rate to the mass spectrometer. Full-scan mass spectra (1 Da step and 3.5 ms dwell) were acquired over 300–1000 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 tandem mass spectra were acquired using a 0.2 *m/z* unit step with a 3 ms dwell time.

RESULTS AND DISCUSSION

Incubation of etamycin B, the proline analog of etamycin (Fig. 1), with the partially purified enzyme resulted in a product with a shorter retention time and the same mass as etamycin B (data not shown). Given the

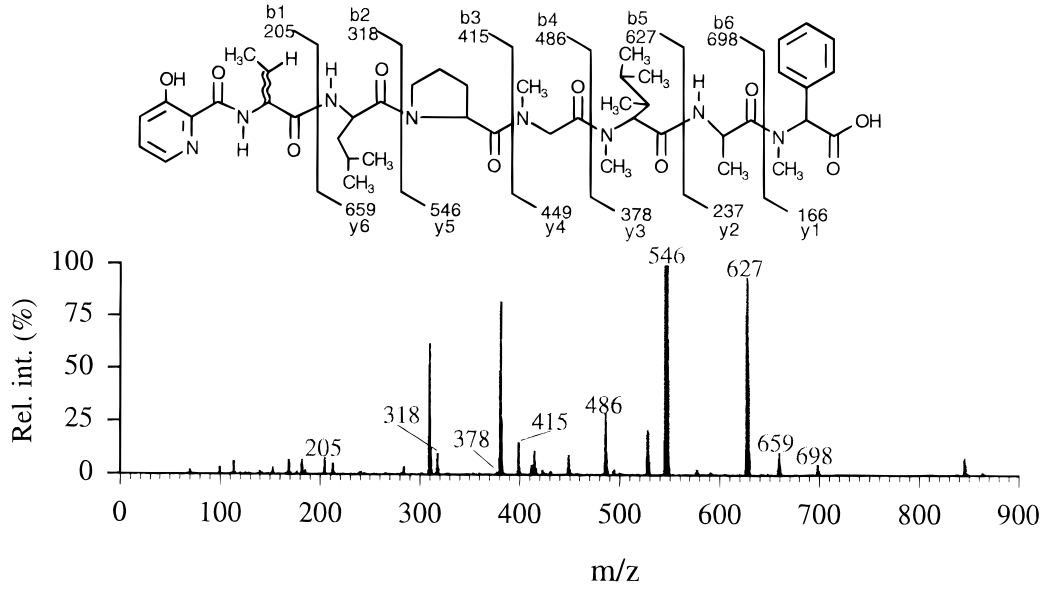


Figure 2. LC–MS/MS fragment ion spectrum of *m/z* 864 from enzyme reaction product of etamycin B.

relatively small structural difference between etamycin and etamycin B, the reaction was expected to follow the same course as with etamycin. The structure of the product was confirmed by LC–MS/MS to be the linear peptide (Fig. 2) generated by the enzyme-catalyzed intramolecular elimination mechanism.[5] This reaction is similar to that catalysed by muconate-lactonizing enzyme, an intramolecular lyase.[18,19]

Type B-I and B-II streptogramins (Fig. 1) have several structural differences, particularly the size of the macrocyclic ring (six amino acids versus seven) and the presence of two unusual amino acids, *p*-dimethylamino-*N*-methyl-L-phenylalanine and 4-oxo-pipecolic acid, in the B-I streptogramins. Other differences in the B-I compounds included the replacement of phenylsarcosine by phenylglycine and variability of the alkyl side chain in the residue adjacent to threonine. Biological activity is conserved in all these compounds, indicating that the mode of action is not crucially related to these variable structural features.[6]

The type B-I streptogramin vernamycin B_α was incubated with the etamycin-inactivating enzyme and the products were characterized by LC–MS and LC–MS/MS. The total ion chromatogram (TIC) of the vernamy-

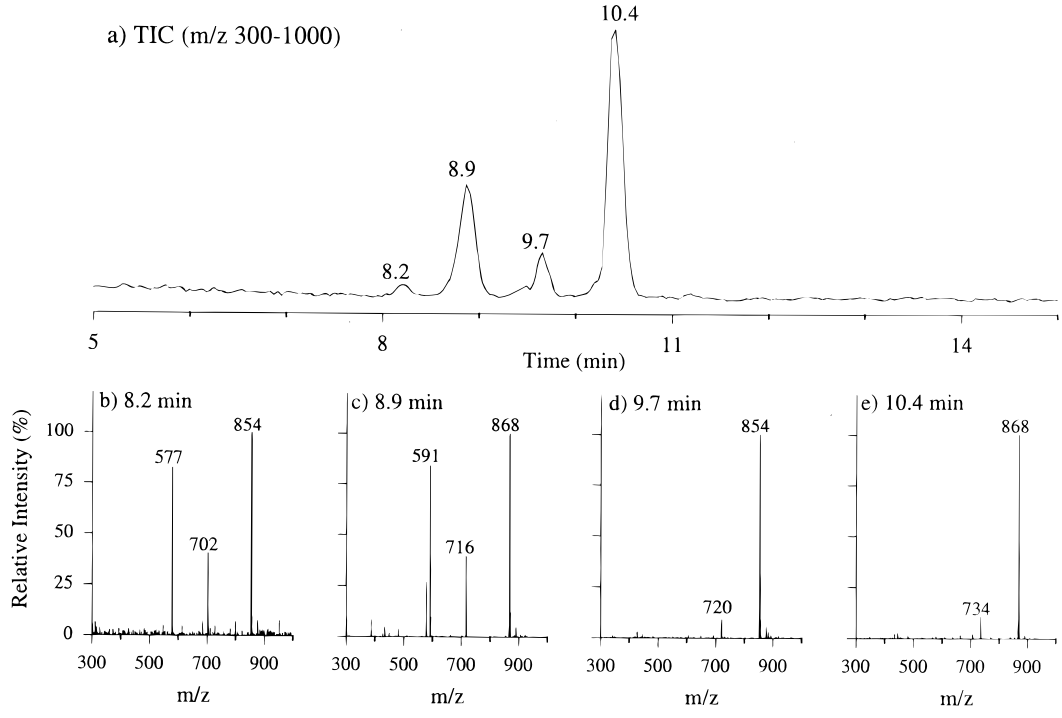


Figure 3. LC–MS analysis of vernamycins incubated with enzyme: (a) total ion chromatogram; (b)–(e) mass spectra of peaks at 8.2, 8.9, 9.7 and 10.4 min respectively. Conditions: linear gradient 20–90% aqueous acetonitrile (0.1% TFA) in 20 min, Vydac 218TP52 C18 column, flow rate split ratio of 1 : 10 to Ionspray interface.

cin B_α incubation mixture contained four peaks (Fig. 3(a)); the last two eluting peaks were due to the antibiotic substrate. The background-subtracted mass spectrum for the 10.4 min component (Fig. 3(e)) showed an $[M + H]^+$ ion at *m/z* 868 consistent with the expected value of vernamycin B_α. The minor component at 9.7 min, with an $[M + H]^+$ ion at *m/z* 854, corresponded to the mass of vernamycin B_γ.[6] The identities of these two compounds as vernamycin B_α and B_γ were confirmed by LC–MS/MS (not shown).

The peaks at 8.2 and 8.9 min in the TIC (Fig. 3(a)) corresponded to the expected enzyme products of vernamycin B_γ and B_α respectively. The background-subtracted mass spectra obtained for these peaks (Figs 3(b) and 3(c)) have $[M + H]^+$ ions at the same *m/z* values as the substrates (Figs 3(d) and 3(e)), suggesting that the mechanism of lactone ring opening is similar to that described previously for etamycin.[5] Based on product-to-substrate peak area ratios, the percentage conversion of each vernamycin substrate to a linear peptide product was similar to that of etamycin incubated with the same enzyme preparation.

Conversion of the macrocyclic peptidolactone substrates to the corresponding linear analogs is apparent from the fragment ions in the mass spectra of the enzyme products (Figs 3(b) and 3(c)). Under electrospray ionization conditions, most cyclic peptides do not fragment readily, whereas linear peptides can give fragment ions by collision-induced dissociation (CID) in the orifice/skimmer region of the mass spectrometer. The fragment ions at *m/z* 577 and 591 for vernamycin B_γ and B_α (Figs 3(b) and 3(c) respectively) correspond to cleavage of the linear peptide at the amide bond of the 4-oxo-pipecolic acid residue. Cleavage at a tertiary nitrogen is a common effect observed in proline-containing peptides.[20,21] The structures of the ring-opened B-I streptogramins detected after enzyme treatment were confirmed using LC–MS/MS. Figure 4 shows the tandem mass spectra of the fragments of enzyme action on vernamycin B_α and vernamycin B_γ.

To assess the substrate specificity of the enzyme, a variety of non-streptogramin peptidolactones were investigated. Viscosin and related surfactins are structurally similar to the streptogramins in that they are

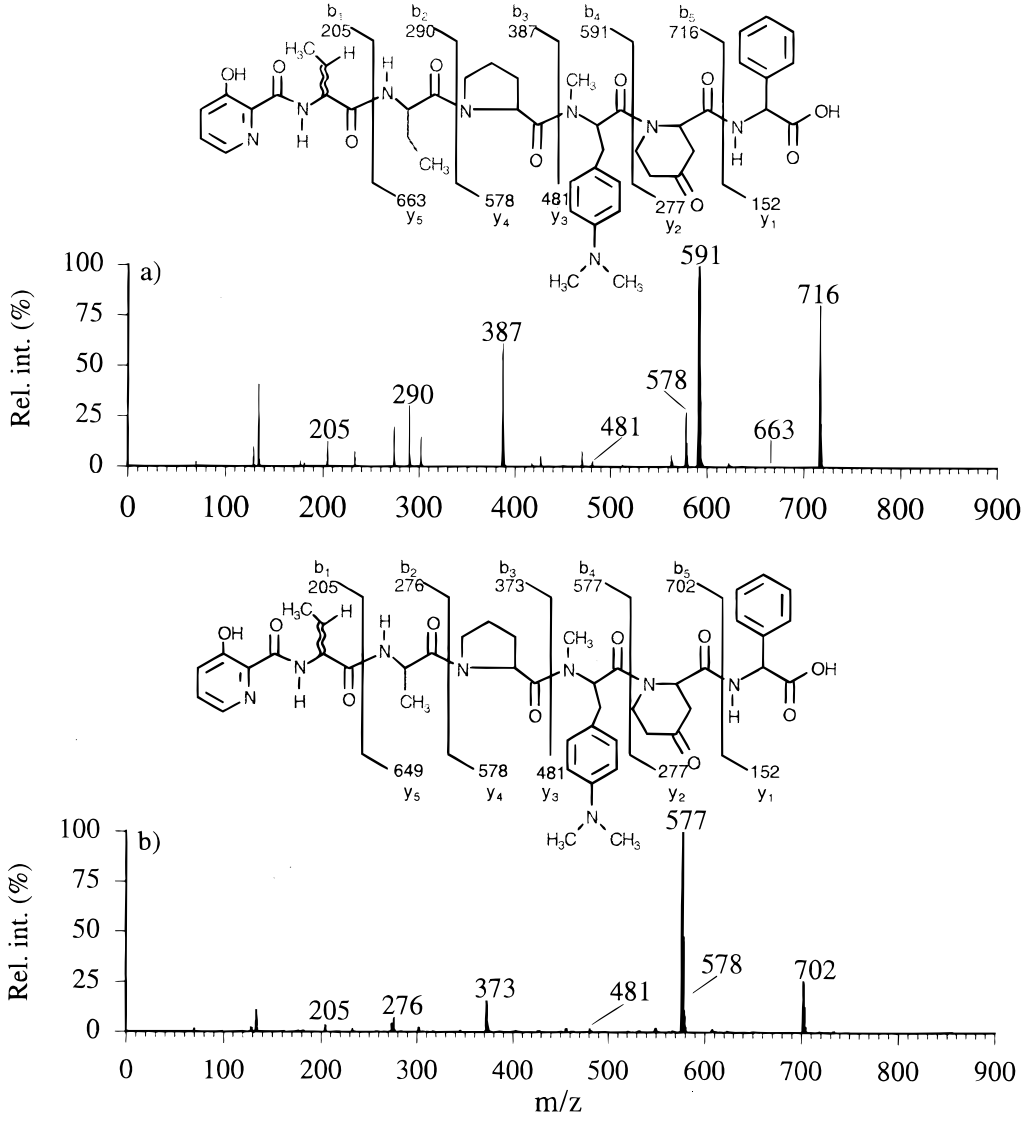


Figure 4. Fragment ion spectra of enzyme reaction products from vernamycins obtained by LC–MS/MS: (a) vernamycin B_α; (b) vernamycin B_γ.

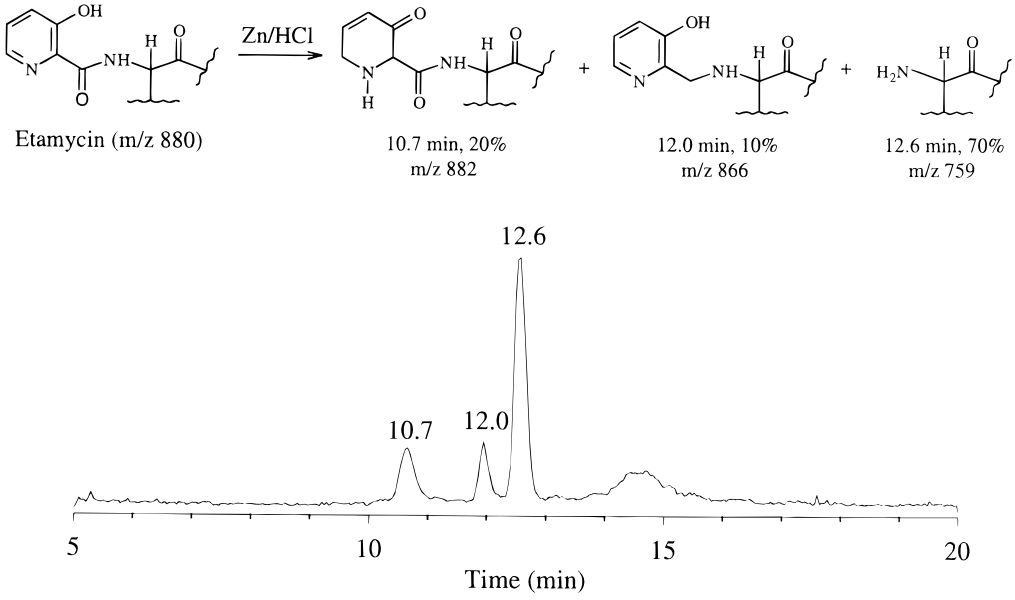


Figure 5. Total ion current from LC–MS analysis of etamycin reduction products. Conditions as for Fig. 3.

Table 1. Fragment ion assignment for LC–MS/MS analysis of reduced etamycin (time annotation based on Fig. 5)

Assignment[a]	10.7 min	12.0 min	12.6 min
$[M + H]^+$	882	866	759
(Ala) or (Sar)	72	72	72
(Hyp) or (Leu)	114	114	114
(dMeLeu)	142	142	142
(Hyp–Sar)	185	185	185
(dMeLeu–Ala) or (Sar–dMeLeu)	213	213	213
(Sar–dMeLeu–Ala)	284	284	284
(Hyp–Sar–dMeLeu)	326	326	326
(Hyp–Sar–dMeLeu–Ala)	397	397	397
(Hyp–Sar–dMeLeu–Ala–PheSar)	544	544	544
(Hyp–Sar–dMeLeu–Ala–PheSar–OH)	562	562	562
$[M + H]^+$–rPic1	759	—	—
(rPic1–Thr)	207	—	—
(rPic1–Thr–Leu)	320	—	—
(rPic1–Thr–Leu–Hyp)	433	—	—
(rPic1–Thr–Leu–Hyp–Sar)	504	—	—
(rPic1–Thr–Leu–Hyp–Sar–dMeLeu)	645	—	—
(rPic1–Thr–Leu–Hyp–Sar–dMeLeu–Ala)	716	—	—
$[M + H]^+$–rPic2	—	759	—
(rPic2–Thr)	—	191	—
(rPic2–Thr–Leu)	—	304	—
(rPic2–Thr–Leu–Hyp)	—	417	—
(rPic2–Thr–Leu–Hyp–Sar)	—	488	—
(rPic2–Thr–Leu–Hyp–Sar–dMeLeu)	—	629	—
(rPic2–Thr–Leu–Hyp–Sar–dMeLeu–Ala)	—	700	—
(Thr)	—	—	86
(Thr–Leu)	—	—	197
(Thr–Leu–Hyp)	—	—	310
(Thr–Leu–Hyp–Sar)	—	—	381
(Thr–Leu–Hyp–Sar–dMeLeu)	—	—	522
(Thr–Leu–Hyp–Sar–dMeLeu–Ala)	—	—	593

[a] Hyp, 3-hydroxyproline; dMeLeu, *N*,*β*-dimethylleucine; PheSar, phenylsarcosine; rPic1, reduced 3-hydroxypicolinic acid form 1; rPic2, reduced 3-hydroxypicolinic acid form 2.

macrocyclic peptidolactones containing seven amino acids with a bridging threonine.[13] However, the threonine residue in the surfactins is linked via an amide bond to a two-residue side chain and is D-*allo*-threonine, not L-threonine as in the streptogramins. Incubation with the enzyme for up to 90 min gave no detectable products from these compounds.

As representative cyclodepsipeptides, enniatins and sporidesmolides[11,12] were also tested as potential enzyme substrates. These compounds are cyclized oligomers of alternating amino and hydroxy acids linked by peptide and ester bonds.[21] The depsipeptides bear little similarity in structure to the streptogramins and, as expected, incubation for up to 160 min with the enzyme gave no detectable products corresponding to the ring-opened analogs.

A feature common to all type B-I and B-II streptogramins is the threonine residue which is N-acylated with 3-hydroxypicolinic acid. The presence of the 3-hydroxypicolinyl residue has been shown to be essential for the biological activity of the streptogramin antibiotics.[22–26] We evaluated the importance of this moiety for the inactivating enzyme by using analogs of etamycin in which the 3-hydroxypicolinyl residue has been chemically altered or completely removed. Reduction of pristinamycin I (vernamycin B_α) electrochemically or with zinc generates products in which only the picolinyl moiety has been reduced.[16,17] When etamycin was treated with zinc and HCl, three products were detected by LC–MS (Fig. 5). The extracted mass spectra indicated that each chromatographic peak corresponded to a single component and that the $[M + H]^+$ ions of the zinc reduction products had *m/z* values different from that of etamycin. The fragmentations observed in the tandem mass spectra of the $[M + H]^+$ ions (Fig. 6) were assigned (Table 1) and used to generate the structure of each reduction product (Fig. 5). Based on this analysis, it is likely that etamycin was converted to products of comparable structure to those obtained from pristinamycin. On incubation of the

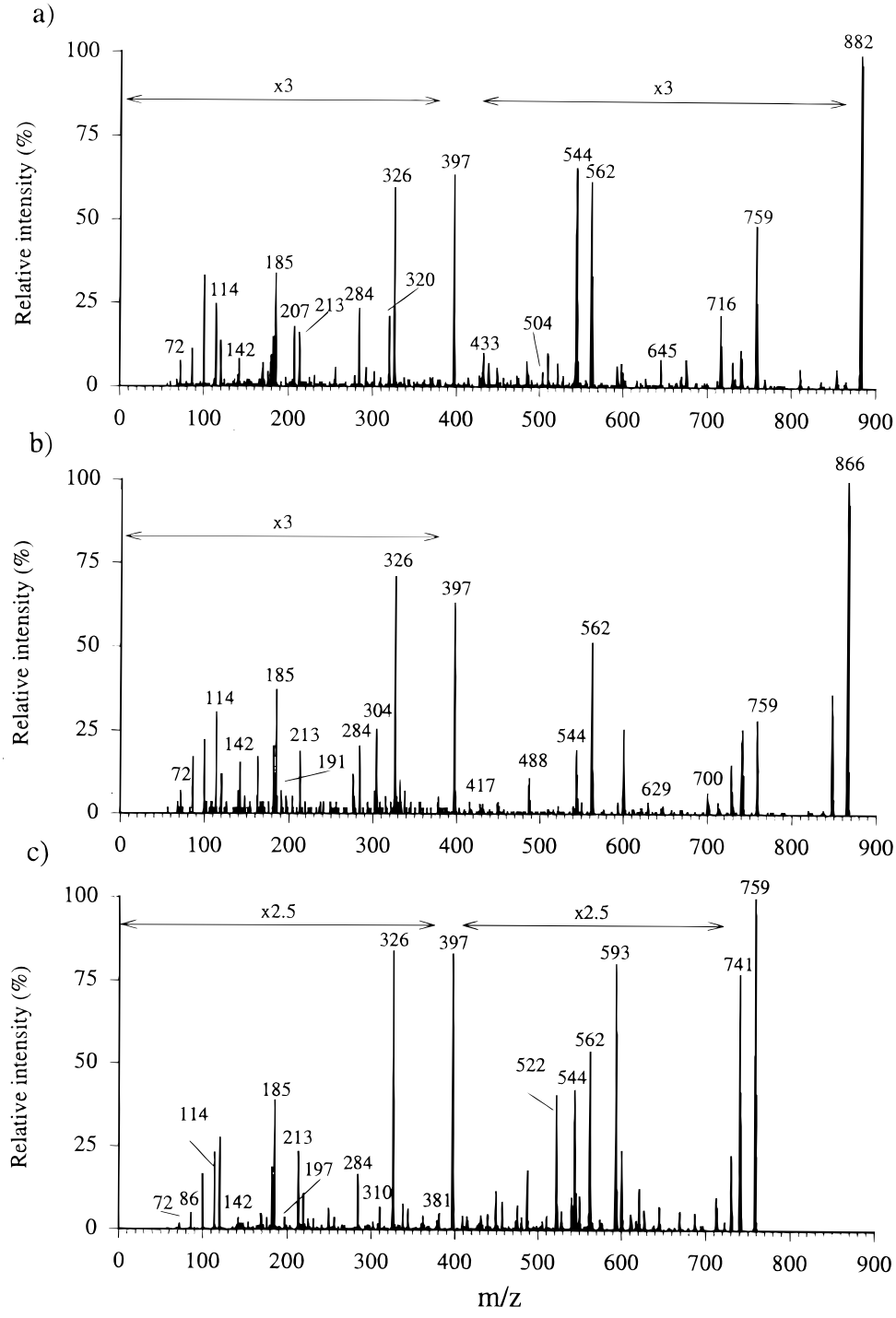


Figure 6. Fragment ion spectra of etamycin reduction products. Precursor ions *m/z* 882 (a), *m/z* 866 (b) and *m/z* 759 (c) obtained using combined LC–MS/MS. Chromatographic conditions as for Fig. 3.

mixture of etamycin analogs with enzyme for up to 20 min, no linear peptide was detected by LC–MS (not shown).

Solution conformations of streptogramins determined by ^{1}H and ^{13}C NMR show that the OH of the picolinic acid residue is involved in an internal hydrogen bond with the carbonyl oxygen of threonine.[27–30] Crystallographic studies have confirmed this observation.[8] The intramolecular hydrogen bond cannot form in two of the etamycin analogs, and this may prevent these compounds from serving as substrates for the enzyme.

The fact that the picolinyl moiety is essential for the biological activity of the streptogramins and is also required for them to serve as substrates for the inactivating enzyme implies that this structural entity has a key role in both processes. The antibiotic activity of the streptogramins depends upon the binding of these molecules to ribosomes through the formation of a salt bridge between the picolinyl residue and an Mg^{2+} ion.[25] The active site of the enzyme may therefore have a structure similar to that of the binding site on the ribosome.

CONCLUSIONS

The enzyme responsible for inactivating the antibiotic activity of etamycin is specific for streptogramins and acts on both type B-I and B-II antibiotics. Presumably essential mechanisms in the enzyme-catalyzed ring opening are not blocked by changes in the number or type of amino acids contained in these antibiotics, but reaction rates may be affected. The enzyme did not show activity towards compounds outside the streptogramin family, although only a limited number of substrates were examined. The use of specifically modified etamycin indicated that the picolinyl moiety is essential to obtain enzyme-generated ring-opened compounds. Because the picolinyl residue is also essential for the antibiotic activity of streptogramins, it is postulated that this moiety is needed for recognition of the substrate by the inactivating enzyme.

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