Vgb from *Staphylococcus aureus* Inactivates Streptogramin B Antibiotics by an Elimination Mechanism Not Hydrolysis†

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ABSTRACT: The streptogramin antibiotics were identified almost 50 years ago but have only recently found clinical use as a consequence of the increase in multidrug-resistant bacteria. Despite the fact that these antibiotics have historically not found intense clinical use, resistance to streptogramins exists. Streptogramins consist of a mixture of two components: cyclic polyunsaturated macrolactones (group A) and cyclic hexadepsipeptides (group B). The latter are cyclized through an ester bond between the hydroxyl group of an N-terminal threonine and the C-terminal carboxyl. Resistance to the B streptogramins can occur through the production of enzymes such as Vgb from *Staphylococcus aureus*. This enzyme had been assumed to be a lactonase that inactivates the cyclic antibiotic by linearization through hydrolytic cleavage of the ester bond. We have expressed recombinant Vgb in quantity and, using a combination of mass spectrometry, NMR, and synthesis of model depsipeptides, show unequivocally that streptogramin B inactivation does not involve hydrolysis of the ester bond. Rather, the hexadepsipeptide is linearized through an elimination reaction across the ester bond generating an N-terminal dehydrobutyrine group. Therefore, Vgb is not a hydrolase but a lyase. We also have explored the activity of Vgb orthologues present in the chromosomes of various bacteria including *Bordetella pertussis* and *Streptomyces coelicolor* and have determined that these enzymes also show streptogramin B inactivation through an elimination mechanism indistinguishable to that used by Vgb. These results demonstrate that Vgb is a member of a large group of streptogramin B lyases that are present not only in resistant clinical isolates but also in the chromosomes of many bacteria. There is therefore a significant reservoir of streptogramin resistance enzymes in the environment, which has the potential to impact the long-term utility of these antibiotics. This research establishing the molecular mechanism of streptogramin resistance therefore has the potential to be exploited in the discovery of inhibitory compounds that could rescue antibiotic activity even in the presence of resistance elements.

The emergence of strains of Gram-positive bacteria resistant to front line antibiotics such as methicillin and vancomycin is a major problem affecting the clinical outcome of infected patients (*1*). As a result, the search for novel antibiotics active against problematic bacteria such as the *Staphylococci* and *Enterococci* has intensified over the past decade. The streptogramin antibiotics were originally discovered in the 1950s yet have only recently been introduced in North America as a new strategy for the treatment of infections caused by multidrug-resistant Gram-positive bacteria (*2*, *3*). Despite their limited use until now in the clinical setting, these antibiotics face a preexisting background of resistant isolates. This resistance may be a result of the extensive use of streptogramins as animal feed additives over the past several decades and also their limited use as antistaphylococcal drugs in Europe but also is the consequence of some overlapping resistance to the macrolide and lincosamide antibiotics such as erythromycin and clindamycin.

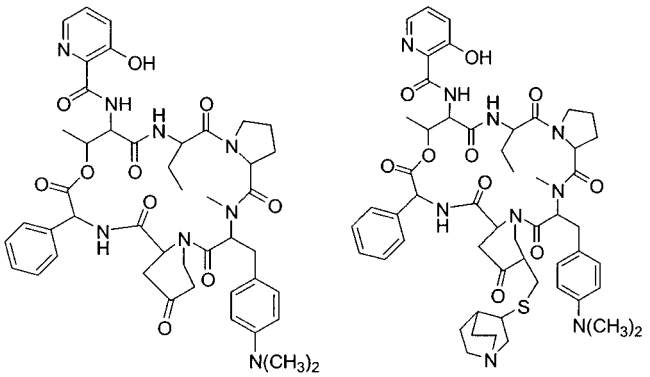

FIGURE 1: Structures of B streptogramins: the natural product pristinamycin Ia and the semisynthetic derivative quinupristin.

The streptogramin family of antibiotics consists of two distinct components, A and B. Group A streptogramins are cyclic polyunsaturated macrolactones, and group B are hexadepsipeptides cyclized through the C-terminal carboxylic acid of a phenylglycine or phenylsarcosine residue and the secondary alcohol of the N-terminal Thr residue (Figure 1)

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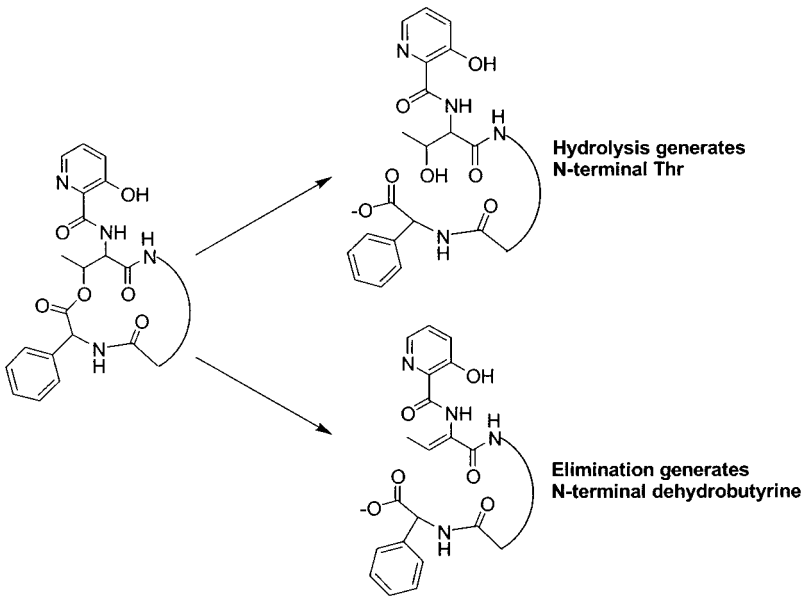


FIGURE 2: Possible products of streptogramin B lactone cleavage.

(*3*). The remaining amino acids can vary and include nonproteinaceous amino acids (*4*).

The mode of action of streptogramins is accepted to be inhibition of peptide bond formation in the 50S ribosome peptidyl transferase center. Streptogramins are unique in that the two components (A and B) are independently bacteriostatic. In combination, however, they act in synergistic fashion, resulting in a bactericidal effect (*5*). The molecular basis for this synergism likely resides in the observation that initial binding of an A-type streptogramin to the ribosome enhances subsequent binding of a group B streptogramin (*6*) and in the determination that the antibiotics bind to distinct but overlapping regions of the ribosome P-site (*7*).

Streptogramins show poor solubility in aqueous solution, and this has limited their clinical utility. The preparation of semisynthetic, water-soluble, derivatives of the natural products pristinamycin IA and pristinamycin IIA gave rise to quinupristin and dalfopristin, respectively, which, when administered in a 3:7 ratio, comprise the recently approved drug Synercid (*8*). This combination has proven to be very effective against most Gram-positive bacteria as well as a select number of Gram-negative bacteria (*9*).

Resistance to streptogramin antibiotics occurs through three mechanisms: (1) active efflux of the drug from the cell, (2) modification of the ribosome target, and (3) antibiotic inactivation. The latter include modification of type A streptogramins by *O*-acetyltransferases and inactivation of type B streptogramins by linearization of the cyclic depsipeptide. Enzymes that catalyze cleavage of the lactone linkage of type B streptogramins have been identified in cell extracts of various streptogramin-producing bacteria (*10*, *11*).

Plasmid-mediated hydrolysis of pristinamycin IA was first characterized from cell extracts of a multiantibiotic-resistant *Staphylococcus aureus* clinical isolate in 1977 (*12*), and the gene sequence of a virginiamycin B hydrolase (*vgb*)[1] from *S. aureus* was subsequently reported (*13*). The presence of *vgb* alone while sufficient for conferring resistance to B streptogramins is, however, not adequate to confer resistance to the combination drug Synercid (*14*). Full resistance to Synercid requires the presence of a streptogramin A acetyltransferase (*14*). Recently, Bateman et al. reported an enzyme activity responsible for inactivating the B streptogramin etamycin in *Streptomyces lividans* cell extracts (*15*). Careful study of the reaction products determined that the inactivation occurring in *S. lividans* was actually an elimination across the ester bond generating an N-terminal dehydrobutyrine and not threonine, which is predicted from a simple hydrolysis mechanism (Figure 2) (*15*).

In an effort to establish the molecular mechanism of *S. aureus* Vgb, we have expressed, purified, and characterized this enzyme and orthologues from *Bordetella pertussis* and *Streptomyces coelicolor* and have demonstrated that elimination rather than hydrolysis is the general mechanism of streptogramin B inactivation.

MATERIALS AND METHODS

Overexpression and Purification of Vgb. The *vgb* gene was amplified in a polymerase chain reaction using the primers listed in Table 1 and using plasmid pIP524 as template. This plasmid was purified from *S. aureus* BM30002/pIP524, which was the gift of Dr. P. Courvalin, Institut Pasteur. The amplified product was digested with *Nco*I and *Xho*I and subcloned into plasmid pET15b (Novagen), creating the plasmid pVGB suitable for overexpression in *Escherchia coli* BL21(DE3).

A 1 L culture of *E. coli* BL21(DE3)/pVGB was incubated at 37 °C and 250 rpm in the presence of 100 μg/mL ampicillin until the culture broth reached an optical density of 0.6 at 600 nm. The culture was then cooled to room temperature on ice and induction of *vgb* initiated with addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. The culture was incubated for an additional 5 h at 23 °C, and the cells were harvested by

[1] Abbreviations: Vgb, streptogramin B lyase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; L-Pgh, L-phenylglycine; Pic, picolinic acid; Cbz, benzyloxycarbonyl.

Table 1: DNA Primers Used for Gene Amplification

organism	primer	sequence[a]
S. aureus	5′	5′-TCTAGACCATGGAATTTAAATTACAAGAATTAAATCTTA
	3′	5′-CCCTCGAGTCACTCCATATTATCCTTAATGAGAGTTAAT
S. aureus His6 fusion	5′	5′-GAATTCGCTAGCATGGAATTTAAATTACAAGAATTAAATCTTACTAAC
	3′	5′-CCCTCGAGTCACTCCATATTATCCTTAATGAGAGTTAAT
B. pertussis	5′	5′-GAATTCATATGAACCAAGCTGAGATGACGGAG
	3′	5′-AGAATTCAAGCTTGGGCGCCAGGCTCAGTC
S. coelicolor	5′	5′-GGAATTCATATGAACGAAATCAACGAGAGCTACGACACC
	3′	5′-AGAATTCAAGCTTCCGAGTCGGTCAAACGAGTGTGT

[a] Underlined sequences identify restriction enzyme sites.

centrifugation at 10000*g* and washed with 0.85% NaCl. The cell paste was resuspended in 10 mL of lysis buffer (50 mM HEPES, pH 7.5, 1 mM EDTA) and disrupted by three passes through a French pressure cell at 20 000 psi. The lysate was clarified by centrifugation at 12000*g* and applied to a 50 mL Q Sepharose column (Pharmacia), equilibrated with 50 mM HEPES, pH 7.5. A linear gradient of 0−1 M NaCl in equilibration buffer over a period of 100 min was applied, and Vgb eluted at approximately 15% NaCl. Vgb-containing fractions were determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), pooled, and concentrated to 4 mL using a 10K NMWL centrifugal filter device (Millipore). The solution was applied onto a Sephacryl S200 column (Pharmacia) (540 mL) equilibrated with 50 mM HEPES, pH 7.5. Fractions that were determined to contain pure Vgb were pooled and stored at 4 °C.

An N-terminal His6 fusion version of Vgb was also generated. The *vgb* gene was amplified in a polymerase chain reaction, using the primers listed in Table 1, with pVGB as template. The amplified product was digested with *Nhe*I and *Xho*I and subcloned into pET28a digested with the same enzymes generating pHVGB. A 1 L culture of *E. coli* BL21-(DE3) STAR/pHVGB was incubated at 37 °C and 250 rpm in the presence of 50 μg/mL kanamycin until the culture broth reached an optical density of 0.6 at 600 nm. The culture was then cooled to 14 °C on ice, and induction was initiated with isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. The culture was incubated for an additional 24 h at 14 °C, and the cells were harvested by centrifugation at 10000*g* and washed with 0.85% NaCl. The cell paste was resuspended in 10 mL of lysis buffer (50 mM HEPES, pH 7.5) and disrupted by three passes through a French pressure cell at 20 000 psi. The lysate was clarified by centrifugation at 12000*g* and applied to a 1.5 mL NTA−agarose (Qiagen) column. The protein was then recovered using a linear gradient of 0−250 mM imidazole in 50 mM HEPES, pH 7.5. The His6 fusion Vgb eluted at a final concentration of approximately 25 mM imidazole.

Expression and Purification of B. pertussis and S. coelicolor Vgb Orthologues. Genes orthologuous to *vgb* were identified by BLAST search of the unfinished genomes of *B. pertussis* (Tahoma I) and *S. coelicolor* A3(2). Genomic DNA from *S. coelicolor* A3(2) was isolated in-house by Dr. C. G. Marshall, and DNA from *B. pertussis* was the generous gift of Dr. Mark Peppler (University of Alberta, Edmonton). Amplification primers are listed in Table 1 and were designed on the basis of the available sequence data from the Sanger Centre (http://www.sanger.ac.uk/Projects/S_coelicolor/). Amplified genes were cloned into pET22b using *Nde*I and *Hin*dIII restriction sites as indicated above for *vgb*, as well as pET28a for expression as His6 fusions. The pET22b constructs did not contain His6 fusions and therefore were purified in a manner similar to that for *S. aureus* Vgb. Proteins that contained the N-terminal His6 fusion were purified through use of NTA−agarose (Qiagen).

Enzyme Assays. Quinupristin and pristinamycin IA were the gifts of Aventis. Linearization of quinupristin was routinely monitored by measuring the resulting decrease in absorbance at 344 nm using a Spectramax microtiter plate reader, with 96-well flat-bottom polystyrene microtiter plates. Reactions contained 1 mM $MgCl_2$, 50 mM HEPES, pH 7.5, and 0.25 μg of Vgb and were performed in a final volume of 250 μL. Under these buffer conditions, a molar extinction coefficient of 5322 M^{-1} cm^{-1} was determined for quinupristin at 344 nm. The pristinamycin IA assay was performed in the presence of 20% (v/v) dimethyl sulfoxide, which had no significant effect on enzyme activity. Data were analyzed using an extinction coefficient at 344 nm of 6375 M^{-1} cm^{-1}.

Initial rates were fit to the equation:

$$v = V_{max}S/(K_m + S)$$

using the Grafit 4 software package (*16*). Steady-state kinetic parameters are reported ± the standard error of the fit to the equation.

An alternative HPLC assay was also employed using a reverse-phase divinylbenzene column (Polysphere Technologies Inc.) equilibrated in 0.1% trifluoroacetic acid (TFA) (v/v) (solution A). Products were eluted using a linear gradient in acetonitrile/0.1% TFA (v/v) (solution B) at a flow rate of 1.0 mL/min. The method used included an isocratic step from 0 to 10 min at 95% solution A and 5% solution B, followed by an increasing gradient in solution B until at 40 min the solvent composition was 50% solution A and 50% solution B. Assay of model peptides was accomplished using a reverse-phase Vydac 218TP54 RP column equilibrated in 95% solution A (0.05% TFA). Products were eluted using a gradient in solution B (0.05% TFA in acetonitrile) at a flow rate of 1 mL/min. The method used included an isocratic step from 0 to 5 min at 95% solution A, followed by an increasing gradient in solution B until at 25 min the solvent composition was 5% solution A and 95% solution B.

Characterization of Products of Quinupristin Linearization by LiOH and Vgb. Quinupristin (1 mM) was hydrolyzed in a solution of 20 mM LiOH at 0 °C for 5 min and separated by reverse-phase HPLC as indicated above or was applied to a Superdex peptide HR10/30 column (Pharmacia) equilibrated with water at a flow rate was of 0.25 mL/min. Vgb-treated quinupristin was similarly purified. Linearization in

$H_2{}^{18}O$ (Isotech) was accomplished following evaporation of component solutions and resuspension in $H_2{}^{18}O$.

NMR Methods. NMR spectra of quinupristin and inactivated quinupristin were recorded on a Bruker Avance DRX-500 NMR spectrometer. Proton spectra were acquired at 500.13 MHz using a 5 mm broad-band inverse probe with triple axis gradient capability. Spectra were obtained in 48−100 scans in 32K data points over 4.735−6.775 kHz spectral widths (3.460−2.418 s acquisition times). The sample temperature was maintained at 30 °C by a Bruker Eurotherm variable temperature unit. The free induction decay (FID) was processed using either exponential multiplication (line broadening, 0.2 Hz) or Gaussian multiplication (line broadening, −1.5 Hz; Gaussian broadening, 0.2). The FID was also zero-filled to 64K before Fourier transformation.

Proton−proton NOE difference spectra were obtained by acquiring a control FID in which the decoupler irradiated at a frequency where there were no proton signals. An on-resonance FID was then recorded with the proton of interest being selectively saturated. In both cases the same decoupler power and duration of saturation (5.0 s) were used. The saturation period also served as the relaxation delay for both the control and on-resonance FID's. The decoupler was gated off during acquisition. A 90° ^{1}H pulse width of 6.7 μs was used. The cycle of alternate acquisition of control and on-resonance FID's was repeated two times for a total of 32 scans for each FID. The FID's were processed using exponential multiplication (line broadening, 4.0 Hz) and were zero-filled to 64K before Fourier transformation. The NOE difference spectrum was obtained by subtraction of the control spectrum from the on-resonance spectrum. The samples were not degassed.

Proton COSY 2-D NMR spectra were recorded in the absolute value mode using the pulse sequence 90°−t_1−45°−acq and included pulsed field gradients for coherence selection. The data were acquired in 2−16 scans for each of the 256 FID's that contained 2K data points in the F_2 dimension over a 5.123 kHz spectral width. The ^{1}H 90° pulse width was 6.7 μs. A 1.0 s relaxation delay was used between acquisitions. Zero-filling in the F_1 dimension produced a 1K × 1K data matrix with a digital resolution of 5.003 Hz/point in both dimensions. During 2-D Fourier transformation, a sine-bell squared window function was applied to both dimensions. The transformed data were then symmetrized.

Selective 1-D TOCSY ^{1}H NMR spectra were recorded over a 4.006 kHz spectral width in 32K data points (4.089 s acquisition time). Gaussian-shaped pulses were defined by 256 data points with the pulse being truncated at 1% of the maximum pulse amplitude. The 270° Gaussian pulses were with pulse widths of 78.0 ms. This pulse was followed by a 40 μs fixed delay and then by the standard TOCSY MLEV-17 spin-lock pulse sequence. The 90° spin-lock pulse width was 26.2 μs. A 1.0 s relaxation delay was used. The 100 ms spin-lock period was followed by a z-filter which contained 10 variable delay times ranging from 4 to 18 ms. The transmitter offset was adjusted to the frequency of the ^{1}H being selectively excited. Thirty-two scans were acquired for each of the delays in the z-filter, resulting in a total of 320 scans. The FID was processed using either exponential multiplication (line broadening, 0.2 Hz) or Gaussian multiplication (line broadening, −1.5 Hz; Gaussian broadening, 0.2) and was zero-filled to 64K before Fourier transformation.

The quinupristin peptides were dissolved in $CDCl_3$ (Isotec, Inc.) to a concentration of approximately 5.0−10.0 mg/mL. Chemical shifts are reported in ppm relative to tetramethylsilane using the residual solvent signal at 7.26 ppm as internal reference for the ^{1}H NMR spectra. The Thr model peptide (compound **6**, see below) was dissolved in DMSO-d_6 at an approximate concentration of 5.0 mg/mL. These spectra were referenced using the residual solvent signal at 2.50 ppm.

Synthesis of Depsipeptides. (a) Chemicals and General Procedures. Thin-layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminum sheets, silica gel 60 F_{254}) in the following solvent systems: (a) chloroform−methanol−acetic acid (95:5:3), (b) ethyl acetate−hexanes (1:1), and (c) ethyl acetate−hexanes (2:1). Routine ^{1}H and ^{13}C NMR spectra of compounds were recorded on a Bruker AV 200 NMR spectrometer, and chemical shifts are reported in parts per million (δ) relative to tetramethylsilane as an internal standard. ES mass spectra were recorded at the Mass Spectrometry Service Centre, McMaster University.

Pyridol (3-hydroxypiridine) was converted to 3-hydroxy-2-(hydroxymethyl)pyridine hydrochloride following a published method (*17*). The product was then benzylated and oxidized to 3-(benzyloxycarbonyl)picolinic acid hydrochloride sesquihydrate (*18*). Cbz-Phg-OH, Cbz-Thr-ONO_2Bzl, Boc-Thr-ONO_2Bzl, and Boc-Phg-OH were synthesized by routine methods (*19*).

*(b) Synthesis of O-(N-Benzyloxycarbonyl-*L*-phenylglycyl)-N-benzyloxycarbonyl-*L*-threonine p-Nitrobenzyl Ester [Cbz-Thr-(Cbz-Phg)-ONO_2Bzl] (**1**).* Isopropenyl chlorocarbonate (1.32 g, 0.011 mol) was added to a solution of Cbz-Phg-OH (3 g, 0.01 mol), Cbz-Thr-ONO_2Bzl (4.08 g, 0.01 mol), triethylamine (1.95 mL, 0.014 mol), and 4-(dimethylamino)-pyridine (0.48 g, 0.004 mol) in 40 mL of CH_2Cl_2 at 0 °C. The reaction mixture took a yellow cast after the addition of isopropenyl chlorocarbonate. After 1.5 h of stirring at 0−5 °C, the solution was washed with water (2 × 20 mL), 5% sodium bicarbonate solution (2 × 20 mL), and saturated brine. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. The compound was next purified by flash chromatography in chloroform.

Yield: 40%. $R_f{}^a$ 0.88; $R_f{}^b$ 0.56. ESMS ($C_{35}H_{33}N_3O_{10}$, MW 655.65): $(M + H)^+$ 656.3, $(M + Na)^+$ 678.3. δ_H ($CDCl_3$): 8.2 (2H, d, Bzl-NO_2),7.4 (2H, d, Bzl-NO_2), 7.2 (15H, m, Ar), 5.6 (1H, d), 5.4 (1H, q), 5.3 (6H, d, CH_2), 5.0 (1H, s, α H-Thr), 1.41 (3H, d, CH_3).

*(c) O-(N-Benzyloxycarbonyl-*L*-phenylglycyl)-N-tert-butyloxycarbonyl-*L*-threonine p-nitrobenzyl ester [Boc-Thr-(Cbz-Phg)-ONO_2Bzl] (**2**)* was similarly prepared.

Yield: 46%. $R_f{}^a$ 0.80; $R_f{}^b$ 0.68. ESMS ($C_{32}H_{35}N_3O_{10}$, MW 621.63): $(M + Na)^+$ 644.3; $(M - H)^-$ 620.3, $(M + Cl)^-$ 656.2. δ_H ($CDCl_3$): 8.2 (2H, d, Bzl-NO_2), 7.4 (2H, d, Bzl-NO_2), 7.2 (10H, m, Ar), 5.6 (1H, d, CH-Phg), 5.4 (1H, q, β H-Thr), 5.3 (6H, d, CH_2), 5.0 (1H, s, α H-Thr), 1.4 (9H, s, Boc), 1.2 (3H, d, CH_3). δ_C ($CDCl_3$): 169 (d), 129−127 (m), 123 (s), 80 (s), 72 (s), 67 (s), 66 (s), 65 (s), 58 (s), 57 (s), 28 (s), 17 (s).

*(d) Synthesis of O-(N-tert-Butyloxycarbonyl-*L*-phenylglycyl)-*L*-threonine p-Nitrobenzyl Ester [H-Thr-(Cbz-Phg)-ONO_2Bzl] (**3**).* This compound was obtained by treatment of **2** in 40% TFA/CH_2Cl_2 for 30 min at room temperature followed by ether precipitation.

Yield: 88%. R_f[a] 0.26; R_f[b] 0.18. ESMS ($C_{27}H_{27}N_3O_8$, MW 521.52): $(M + H)^+$ 522.1. δ_H ($CDCl_3$): 8.2 (2H, d, Bzl-NO_2), 7.4 (2H, d, Bzl-NO_2), 7.2 (10H, m, Ar), 5.6 (1H, d, CH-Phg), 5.4 (1H, q, β H-Thr), 5.3 (6H, d, CH_2), 5.0 (1H, s, α H-Thr), 1.2 (3H, d, CH_3). δ_C ($CDCl_3$): 169 (d), 129−127 (m), 123 (s), 80 (s), 72 (s), 67 (s), 66 (s), 65 (s), 58 (s), 57 (s), 17 (s).

*(e) O-(N-Benzyloxycarbonyl-*L*-phenylglycyl)-N-(3-benzyloxypicolinyl)-*L*-threonine p-Nitrobenzyl Ester [Pic(OBzl)-Thr-(Cbz-Phg)-ONO$_2$Bzl] (**4**)*. The synthesis was carried out following the method already described (*20*).

Yield: 88%. R_f[c] 0.81. ESMS ($C_{40}H_{36}N_4O_{10}$, MW 732.75): $(M + H)^+$ 733.1, $(M + Na)^+$ 755.2. δ_H ($CDCl_3$): 8.2 (2H, d, Bzl-NO_2), 7.4 (2H, d, Bzl-NO_2), 7.2 (10H, m, Ar), 5.6 (1H, d, CH-Phg), 5.4 (1H, q, β H-Thr), 5.3 (6H, d, CH_2), 5.0 (1H, s, α H-Thr), 1.2 (3H, d, CH_3).

*(f) O-(tert-Butyloxycarbonyl-*L*-phenylglycyl)-N-(3-benzyloxypicolinyl)-*L*-threonine Methyl Ester (**5**)* (*21*). The preparation of the protected tridepsipeptide was accomplished by coupling *N*-(3-benzyloxypicolinyl)-L-threonine methyl ester and *N*-(butyloxycarbonyl)phenylglycine.

*(g) N-(3-Benzyloxypicolinyl)-*L*-threonine Methyl Ester*. To a solution of L-threonine methyl ester (1.6 g, 0.01 mol) in ethyl acetate were added 3-(benzyloxycarbonyl)picolinic acid (2.2 g, 0.01 mol) and triethylamine (1.4 mL, 0.01 mol), followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.8 g, 0.01 mol). After being stirred at 0 °C for 1 h and at 24 °C for 16 h, the solution was evaporated and the residue partitioned between ethyl acetate and water. The ethyl acetate layer was further washed with water, 5% citric acid, water, saturated sodium bicarbonate, and water. Drying over sodium sulfate and evaporation gave an oil that crystallized after trituration with petroleum ether at 0 °C.

Yield: 50%. R_f[a] 0.47; R_f[b] 0.1. ESMS ($C_{18}H_{20}N_2O_5$, MW 344.14): $(M + H)^+$ 345.2, $(M + Na)^+$ 367.2. δ_H ($CDCl_3$): 8.45 (1H, d, Pic), 8.26 (1H, d, Pic), 7.2 (5H, m, Ar), 5.2 (2H, s, CH_2), 4.8 (1H, d, β H-Thr), 4.4 (1H, m, α H-Thr), 3.7 (3H, s, OCH_3), 1.21 (3H, d, CH_3).

N-(3-Benzyloxypicolinyl)-L-threonine methyl ester (1 g, 0.003 mol) was dissolved in 20 mL of dry tetrahydrofuran. The solution was then cooled to −10 °C under an N_2 atmosphere. Then *N-t*-Boc-phenylglycine (2.18 g, 0.006 mol) and dicyclohexylcarbodiimide (1.8 g, 0.006mol) were added. After 0.5 h 4-(dimethylamino)pyridine (0.088 g, 0.0007mol) was added. The solution was stirred for 24 h, allowing the temperature to rise to room temperature, and the tetrahydrofuran solvent was removed under reduced pressure (temperature below 30 °C). The remaining solid was dissolved in chloroform (30 mL). The chloroform layer was further washed with 10% sodium bicarbonate solution (2 × 20 mL), 10% citric acid solution (2 × 20 mL), and brine (2 × 20 mL). After drying over anhydrous sodium sulfate and evaporation of the chloroform under reduced pressure, the solid was redissolved in ethyl acetate and the formed dicyclohexylurea filtered out under cooling. The crude product was purified by flash chromatography.

Yield: 90%. R_f[a] 0.61; R_f[b] 0.57. EIMS ($C_{31}H_{35}N_3O_8$, MW 577.24): $(M + H)^+$ 578.2, $(M + Na)^+$ 601.2. δ_H ($CDCl_3$): 8.45 (1H, d, Pic), 8.26 (1H, d, Pic), 7.2 (10H, m, Ar), 5.5 (1H, d, β H-Thr), 5.2 (2H, s, CH_2), 5.1 (1H, m, α H-Thr), 3.7 (3H, s, OCH_3), 1.4 (9H, s, Boc), 1.21 (3H, d, CH_3). δ_C ($CDCl_3$): 170 (s), 169 (d), 158 (s), 139 (s), 136 (s), 130 (s), 129 (m), 128 (s), 127 (s), 126 (s), 80 (s), 77 (s), 72 (s), 58 (s), 55 (s), 28, (s), 16 (s).

Table 2: Purification of Vgb

purification step	total activity (μmol/min)	total protein (mg)	specific activity (μmol min^{-1} mg^{-1})	% recovery	purification *x*-fold
cell lysate	741	239	3.10	100	1.00
Q Sepharose[a]	257	85.8	3.00	35	0.97
Sephacryl S200	253	53.3	4.76	34	1.53

[a] The apparent low activity of this fraction is the result of dependent inhibition of activity originating from the presence of NaCl.

*(h) N-Picolinyl-O-(tert-butyloxycarbonyl-*L*-phenylglycyl)-*L*-threonine Methyl Ester (**6**)*. To a solution of **5** (0.9 g, 0.0015 mol) in 30 mL of 4.4% HCOOH in MeOH was added 10% Pd/C (0.9 g) (*22*). The solution was hydrogenated at atmospheric pressure for 16 h followed by filtration over Celite and evaporation of the filtrate.

Yield: 80%. R_f[a] 0.89; R_f[c] 0.87. EIMS ($C_{24}H_{29}N_3O_8$, MW 487.2): $(M + H)^+$ 488.2. δ_H ($CDCl_3$): 11.7 (1H, br s, OH, Pic), 8.45 (1H, d, Pic), 8.26 (1H, d, Pic), 7.2 (5H, m, Ar), 5.5 (1H, d, β H-Thr), 5.1 (1H, m, α H-Thr), 3.7 (3H, s, OCH_3), 1.4 (9H, s, Boc), 1.21 (3H, d, CH_3). δ_C ($CDCl_3$): 170 (s), 169 (d), 158 (s), 139 (s), 136 (s), 130 (s), 129 (t), 128 (s), 127 (s), 126 (s), 80 (s), 77 (t), 72 (s), 58 (s), 55 (s), 28 (s), 16 (s).

*(i) Isolation and Characterization of the Products of Treatment of Compound **6** with Vgb*. Compound **6** (0.006 mmol) was incubated with purified Vgb at 37 °C for 1 h, and the solution was evaporated to dryness by lyophilization. Vgb was then ether precipitated, the products (**7** + **8**; see Scheme 2) were extracted with chloroform, and the solvent was evaporated under reduced pressure.

Compound **7**: δ_H ($CDCl_3$): 11.7 (1H, br s, OH, Pic), 8.45 (1H, d, Pic), 8.26 (1H, d, Pic), 7.2 (5H, m, Ar), 6.8 (1H, q, CH═), 3.7 (3H, s, OCH_3), 1.8 (9H, s, Boc), 1.7 (3H, d, CH_3).

Spin decoupling at 6.8: δ_H ($CDCl_3$): 11.7 (1H, br s, OH, Pic), 8.45 (1H, d, Pic), 8.26 (1H, d, Pic), 7.2 (5H, m, Ar), 3.7 (3H, s, OCH_3), 1.8 (9H, s, Boc), 1.7 (3H, s, CH_3).

The signal for the 3H protons from the CH_3 group changes from doublet to singlet following enzyme treatment.

RESULTS

Characterization of Recombinant S. aureus Vgb. Vgb expressed well in both His6-tagged and nontagged forms in *E. coli* BL21(DE3), typically yielding over 50 mg of pure enzyme from a 1 L cell culture (Table 2, Figure 3). The purified enzyme behaved as a monomer by calibrated analytical gel filtration.

The enzymatic properties of the enzyme were assessed by monitoring the change in absorbance of the quinupristin or pristinamycin IA substrate at 344 nm upon ring opening (Figure 4). Steady-state kinetic parameters are summarized in Table 3.

Metal Dependence of Vgb. Vgb required the presence of a divalent metal for optimal activity. The metal requirements of the enzyme were probed after demetalation of the enzyme solution and buffer using Chelex 20 resin (Bio-Rad) followed by assay of quinupristin inactivation activity in the presence of 5 mM metal ions followed by HPLC assay. Mg^{2+} was

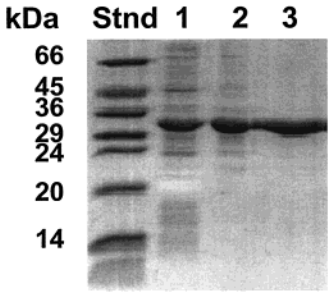


FIGURE 3: Purification of Vgb. The SDS−polyacrylamide gel (15%) was stained with Coomassie blue. Lanes: Stnd, molecular mass standards; 1, cell lysate; 2, pooled fractions from the Q Sepharose anion-exchange column; 3, pooled fractions from the Sephacryl S200 gel filtration column.

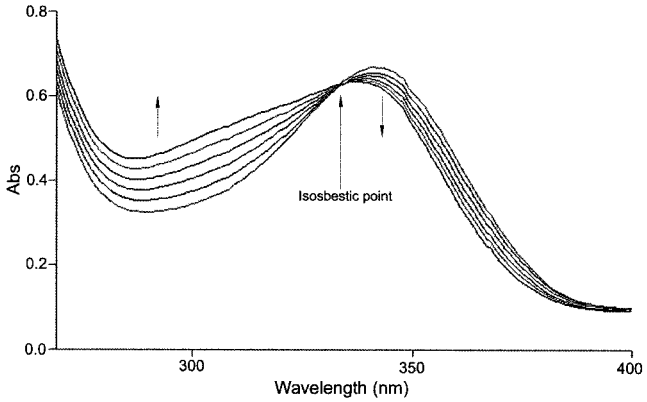


FIGURE 4: Change in quinupristin UV spectrum over time upon treatment with Vgb.

Table 3: Steady-State Kinetic Parameters of Vgb and Orthologues

enzyme	substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
S. aureus Vgb	quinupristin	8 ± 1	2.1 ± 0.3	2.6×10^5
S. aureus His6 Vgb	quinupristin	18 ± 3	1.8 ± 0.3	1.0×10^5
S. aureus Vgb	pristinamycin IA	19 ± 3	2.9 ± 0.5	1.5×10^5
S. coelicolor	quinupristin	27 ± 5	2.8 ± 0.5	1.0×10^5
B. pertussis	quinupristin	31 ± 5	0.3 ± 0.0	9.6×10^3
B. pertussis His6	quinupristin	33 ± 12	0.2 ± 0.1	5.7×10^3

Table 4: Metal Dependence of Vgb

metal	% activity	metal	% activity
Mg^{2+}	100	Zn^{2+}	3.3
Co^{2+}	59	Ca^{2+}	2.9
Mn^{2+}	36	Cu^{2+}	1.8
Ni^{2+}	22	no metal control	1.5

determined to be the optimal metal for catalyzing the inactivation of quinupristin (Table 4). The order of efficacy for each divalent ion in facilitating Vgb catalysis was $Mg^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+} \gg Zn^{2+} > Ca^{2+} \gg Cu^{2+}$.

Mg^{2+} titration data plotted as either free or total Mg^{2+} resulted in a substrate inhibition curve with an optimal activity at 1 mM Mg^{2+} and $K_{m(Mg^{2+})}$ of 0.19 mM.

Characterization of the Products of Quinupristin Inactivation. Inactivation of quinupristin could, in principle, be the result of any one of several mechanisms; however, precedent suggested that inactivation was the result of linearization of the cyclic depsipeptide, which can be accomplished in several ways (Scheme 1). The infrared spectrum of quinupristin

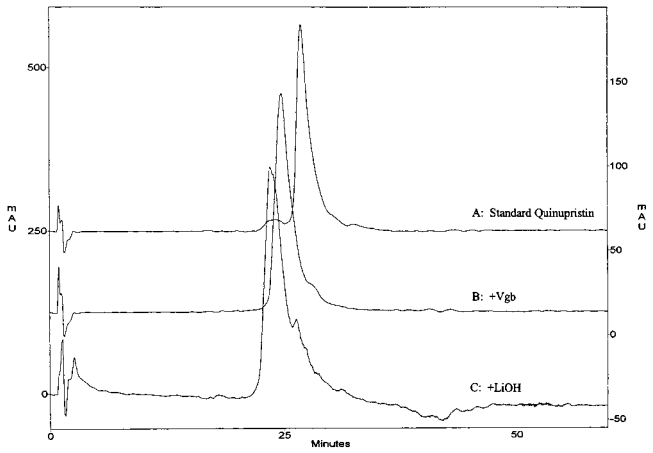


FIGURE 5: HPLC of products of quinupristin linearization. Products were separated on a reverse-phase divinylbenzene column and monitored by the absorption at 220 nm.

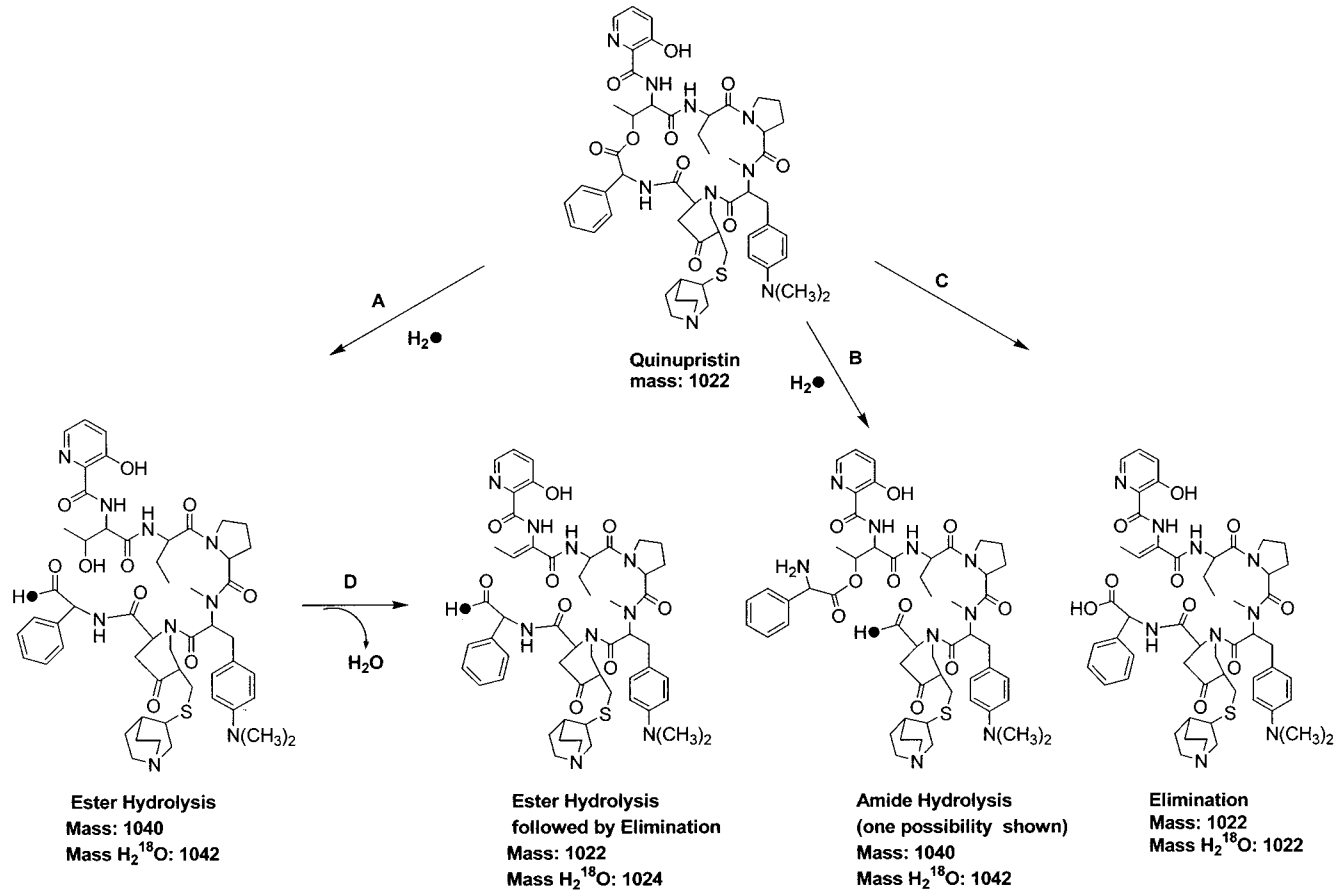
Table 5: Mass Spectral Analysis of Quinupristin Linearization Products

sample	*m/z*	difference from quinupristin mass
quinupristin	1022.5	
quinupristin + LiOH	1040.3	17.8
quinupristin + Vgb	1022.6	0.1
quinupristin + LiOH in $H_2{}^{18}O$	1042.6	20.1
quinupristin + Vgb in $H_2{}^{18}O$	1022.6	0.1

revealed the expected 1740 cm^{-1} ester absorption, but the Vgb-treated compound lacked this signature stretch, demonstrating that cleavage of the ester bond was occurring eliminating peptide hydrolysis (path B in Scheme 1) as a mechanism of inactivation and pointing to the ester bond as the site of linearization. Quinupristin was treated with Vgb or dilute LiOH, and the products were separated by reverse-phase HPLC (Figure 5) to establish the precise mechanism of linearization. Exposure of quinupristin to Vgb resulted in a change of retention time on the divinylbenzene column from 26.8 to 24.6 min (compare trace A to trace B in Figure 5). Base hydrolysis of the quinupristin lactone, however, generated a new peak at 23.5 min (trace C, Figure 5), which did not coelute with Vgb-treated antibiotic. Mass spectral analysis confirmed that LiOH treatment resulted in hydrolysis of the lactone (mass +18, path A in Scheme 1) but that there was no mass change upon Vgb-catalyzed reaction (Table 5). These results suggest that either (1) a direct elimination reaction was occurring (path C in Scheme 1) or (2) hydrolysis is followed by an elimination reaction (path D in Scheme 1). In an effort to address the latter possibility, quinupristin cleavage reactions were then performed in $H_2{}^{18}O$, and as predicted the mass of the base-hydrolyzed product increased by 2, but there was no change in the Vgb-treated product (Table 5), demonstrating that hydrolysis followed by elimination was not occurring and implicating a direct elimination reaction (path C in Scheme 1).

NMR Characterization of the Product of Vgb-Catalyzed Quinupristin Inactivation. Confirmation of an elimination reaction was established by one- and two-dimensional NMR experiments. Complete details of the assignments of quinupristin and the product of the Vgb-catalyzed reaction are found in the Supporting Information. Comparison of the

Scheme 1



proton NMR spectrum of quinupristin and the product clearly demonstrated that the action of the enzyme resulted in the generation of a compound with many isomers, consistent with ring opening (compare spectra a and b in Figure 6). Selective excitation of the Thr γ-CH_3 doublet in a one-dimensional TOCSY experiment failed to detect the Thr α proton and established that the Thr β proton had undergone a 0.652 ppm shift to the higher frequency to 6.522 ppm, conclusively demonstrating that lactone ring opening generated a dehydrobutyrine residue in place of Thr.

Model Depsipeptide Substrates of Vgb. In an effort to establish a minimal Vgb substrate, several depsipeptides were synthesized and their potential to be cleaved by the action of Vgb was assessed by reverse-phase HPLC.

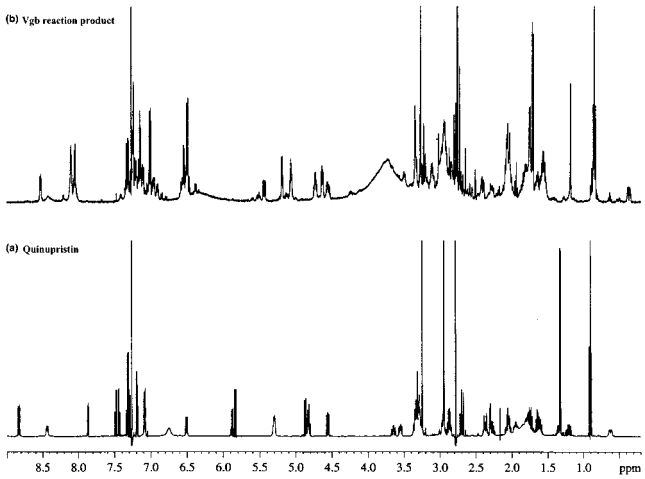


FIGURE 6: Comparison of 1-D ^{1}H NMR spectra of standard quinupristin (a) and the product of Vgb-catalyzed inactivation of the antibiotic (b).

All compounds (**1**−**6**, Table 6) were tested as Vgb substrates, but only compound **6**, possessing the deprotected picolinyl moiety in its structure, was a substrate. Saturation of the enzyme was not achieved at the limits of solubility of the compound; however, an estimate of k_{cat}/K_m was made from the linear rate vs depsipeptide substrate concentration plot where the slope is equivalent to (k_{cat}/K_m)[Vgb]. The value obtained from this analysis was 150 M^{-1} s^{-1}.

The products of LiOH-hydrolyzed and Vgb-catalyzed cleavage of model depsipeptide **6** were purified by HPLC and analyzed by mass spectrometry (Scheme 2, Table 7). With this minimal substrate the enzymatic reaction yielded products with masses consistent with elimination product **7** and Boc-phenylglycine (**8**), paralleling the reaction with intact quinupristin. On the other hand, base hydrolysis generated two major products, hydroxypicolynyl-Thr (**9**) and Boc-Phg-OH (**8**). The structure of compound **7** was also confirmed by ^{1}H NMR (assignment in Materials and Methods).

Stereochemical Analysis of Vgb Reaction. Streptogramin B ring opening by Vgb generates the olefinic dehydrobutyrine in the N-terminal position. The methyl group, therefore, can in principle be in either the *Z* or *E* conformations (Scheme 3). To determine which isomer is generated during the reaction, we performed a large-scale cleavage with depsipeptide **6**, purified **7** by HPLC, and analyzed it by NMR. In the model peptide, the NH was observed as a sharp signal at 10.055 ppm. A series of NOE difference experiments were performed in which saturation of the Thr γ-CH_3

Scheme 2

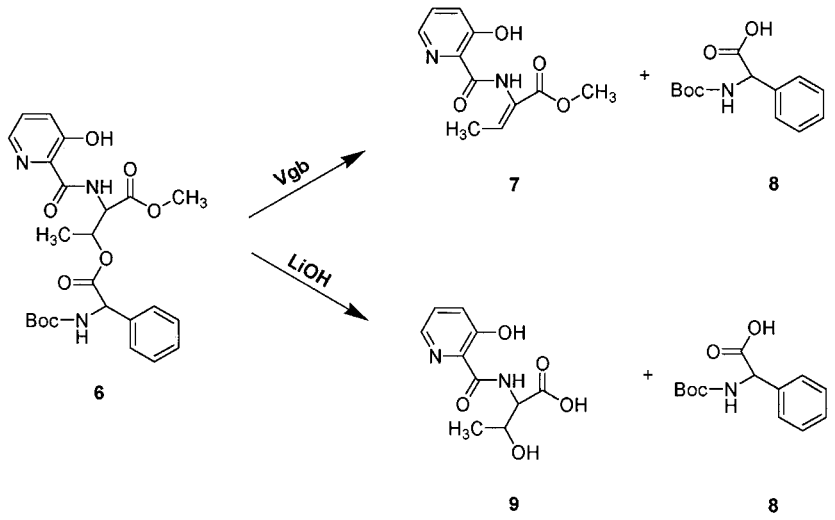


Table 6: Structures of Model Depsipeptides and Vgb Substrate Outcomes

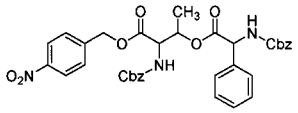
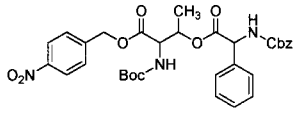
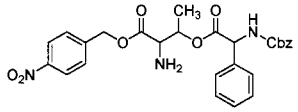
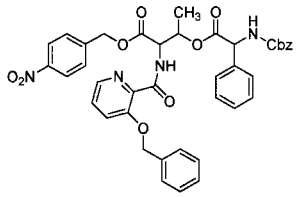
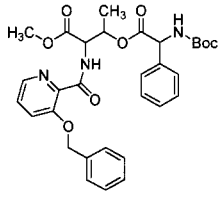
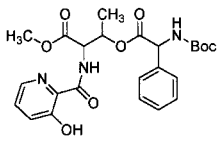
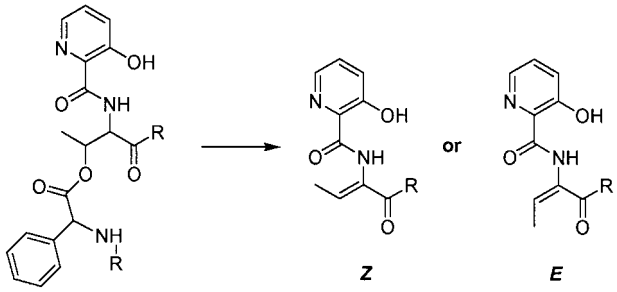
	1	Not a substrate
	2	Not a substrate
	3	Not a substrate
	4	Not a substrate
	5	Not a substrate
	6	Substrate k_{cat}/K_m = 150 $M^{-1}sec^{-1}$

Table 7: Analysis of Products of Vgb Action on Depsipeptide Products[a]

compd	predicted mass	*m/z*[a]	retention time (min)
6	487.2	486.2	23.11
7[b]	236.1	236.1	17.40
8	251.1	250.0	18.50
9[c]	240.1	239.0	12.94

[a] Electrospray mass spectra were recorded in the negative ion mode. [b] A 200 μM solution of compound **6** was treated with 1 μg of Vgb for 1 h at 37 °C. [c] Compound **6** (2 mg) was incubated in 1 mL of 20 mM LiOH for 10 min at 0 °C.

Scheme 3



resulted in a strong enhancement of the olefinic β proton as well as a moderate enhancement of the NH. Saturation of the Thr β proton resulted in no detectable enhancement of the NH signal. These results were consistent with the enzyme reaction product being the *Z* isomer.

Vgb Homologues from B. pertussis and S. coelicolor. Sequencing of bacterial genomes has revealed several orthologuous *vgb* genes within the chromosomes of several bacteria. Two of these putative streptogramin B inactivating genes were selected for characterization of the corresponding proteins: one from the Gram-positive filamentous soil bacterium *S. coelicolor* and the second from the Gram-negative bacillus *B. pertussis*, which is the causative agent of whooping cough. Both proteins were expressed in *E. coli*, purified (Figure 7), and assessed for quinupristin inactivation by the UV assay (Table 3) and by HPLC. HPLC analysis revealed that the products of quinupristin inactivation eluted simultaneously with the Vgb-cleaved product and distinct from the base-hydrolyzed compound (not shown).

DISCUSSION

The dramatic rise of multidrug- resistant bacteria over the past several years has prompted a renewed interest in the development and identification of new antimicrobial agents and the reexamination of "old" antibiotics as sources of new drugs. The streptogramin antibiotics were originally discovered in the 1950s and used primarily in agriculture over the

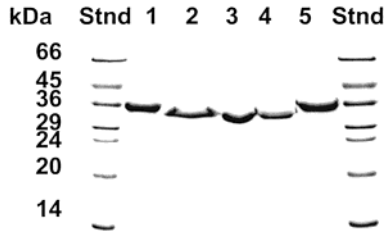


FIGURE 7: Purity of Vgb orthologues. The 15% SDS−polyacrylamide gel was stained with Coomassie blue. Lanes: Stnd, molecular mass standards; 1, His6-tagged Vgb from *S. aureus*; 2, Vgb from *S. aureus*; 3, His6-tagged Vgb orthologue from *B. pertussis*; 4, Vgb orthologue from *B. pertussis*; 5, Vgb orthologue from *S. coelicolor*.

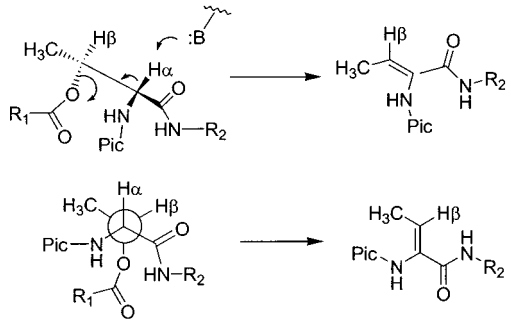


FIGURE 8: Possible mechanism of Vgb-catalyzed streptogramin linearization.

past 40 years. Recently, semisynthetic, water-soluble derivatives of the natural products pristinamycins I and II, quinupristin, and dalfopristin have been introduced for the treatment of serious infections caused primarily by multidrug-resistant Gram-positive bacterial pathogens (*8*).

Despite the fact that these antibiotics have not seen widespread clinical use since their discovery, bacterial resistance to the streptogramin antibiotics has been recognized for several decades. The mechanism of resistance to the type B streptogramins, which are hexadepsipeptides circularized through an ester bond between the secondary alcohol of the N-terminal Thr and the C-terminal carboxylate, has largely been accepted to occur through hydrolysis of the lactone to generate the linear peptide. Consequently, enzymes such as Vgb identified in streptogramin-resistant *S. aureus* clinical isolates have been termed lactonases or hydrolases (*12*, *13*). More recently, Bateman et al. performed careful analysis of the products of inactivation of the type B streptogramin etamycin in cell extracts of the soil bacterium *S. lividans* and concluded that cleavage of the lactone occurred through an elimination mechanism not hydrolysis (*15*). In view of this ambiguity in the literature, we undertook to establish the precise mechanism of streptogramin B antibiotic inactivation by the clinically important resistance determinant Vgb by purifying the enzyme and analyzing the products of antibiotic inactivation by a series of methods.

Purified Vgb linearized both quinupristin and the parent antibiotic pristinamycin Ia with virtually identical k_{cat}/K_m, demonstrating that addition of a bulky alkylthiomethylene group at position 5 of the 4-oxopipecolic acid residue had no effect on substrate specificity. A series of model depsipeptides incorporating the ester linkage between the N-terminal Thr and the C-terminal phenylglycine established that the a hydroxypicolynyl group linked to the N-terminal amine of Thr was essential for enzyme recognition, though the linear didepsipeptide had a significantly diminished capacity to be productively captured by the enzyme as evidenced by the over 10^3-fold drop in k_{cat}/K_m. Therefore, while the hydroxypicolynyl group is an essential component of enzyme recognition, other elements in the remaining cyclic peptide also play a role in maximizing antibiotic inactivation.

Inactivation of streptogramin B antibiotics could occur through a number of mechanisms including chemical modification or linearization of the cyclic depsipeptide by (1) hydrolysis of one of the five constituent amide bonds, (2) hydrolysis of the ester bond, or (3) an elimination reaction. Vgb inactivated streptogramins in the absence of any additional cofactors or substrates such as ATP or acetyl-CoA, and mass spectral analysis of the reaction products ruled out a chemical modification mechanism. The loss of the ester signature absorbance at 1740 cm^{-1} in the infrared spectrum upon incubation of quinupristin with Vgb indicated that the ester bond and not one of the amides was the site of inactivation. Furthermore, the mass spectra of quinupristin treated with Vgb in the presence of both $H_2{}^{16}O$ and $H_2{}^{18}O$ demonstrated that water did not directly participate as a nucleophile in inactivation of streptogramins; therefore, Vgb is not a hydrolase. NMR and mass spectral analysis of the reaction products, on the other hand, confirmed that the ester bond had been cleaved by an elimination reaction, generating an N-terminal dehydrobutyrine residue. Vgb from *S. aureus* is therefore a lyase with activity similar to that of the enzyme activity detected in cell extracts of the soil bacterium *S. lividans* by Bateman et al. (*15*).

Elimination reactions can occur through either concerted (E2) or stepwise mechanisms generating carbocation (E1) or carbanion (E1cB) intermediates, and the stereochemistry of the products can have either *E* or *Z* geometries (*23*, *24*). NMR analysis of the products of Vgb-catalyzed cleavage of the model depsipeptide **6** demonstrated that the *Z* isomer was generated, consistent with anti elimination with abstraction of the α proton (Figure 8). The pK_a of this proton is not known, but studies of model systems such as ethyl acetate and glycine suggest a value of greater than 25 (*25*, *26*), which makes generation of a carbanion intermediate (E1cB mechanism) daunting for biologically available bases. Gerlt and colleagues have shown that, for enzymes that catalyze β eliminations such as mandelate racemase, a stepwise mechanism comprising a concerted general acid and general base step to stabilize an enol intermediate, followed by an E2 elimination of the β constituent, effectively provides a mechanism for lowering the pK_a of the α proton to values accessible to active site bases (*27*−*29*). The active site residues that would participate in such a mechanism in Vgb are unknown at present. The requirement for Mg^{2+} for type B streptogramin inactivation provides a possible electrophilic catalyst that could stabilize developing negative charges in the transition state; however, confirmation of the mechanism requires further research.

The accumulating genome sequence data from a number of bacteria have surprisingly shown that genes with the potential to encode Vgb-like enzymes are quite prevalent in the *chromosomes* of several bacteria including the Gram-negative bacteria *Pseudomonas aeruginosa*, various *Bordetella* species including *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*, the cyanobacterium *Synechocystis* PCC6803,

and the Gram-positive soil bacterium *S. coelicolor* [which is closely related to *S. lividans*, the bacterium shown to harbor a Vgb-like activity in cell extracts (*15*)]. In an effort to establish whether these genes encode bona fide streptogramin B inactivating enzymes, we expressed and purified orthologues from the Gram-negative bacterium *B. pertussis* and the Gram-positive bacterium *S. coelicolor*. These were shown to be effective catalysts with k_{cat}/K_m of 10^3-10^5 M^{-1} s^{-1} for quinupristin and were demonstrated by HPLC analysis of the reaction products to be lyases and not hydrolases. This result speaks to a deep reservoir of antibiotic resistance genes not just in the clinics but also in the environment. The presence of these genes within the chromosomes of environmental bacterial isolates is intriguing. These resistance elements may be a line of defense against streptogramin-producing organisms that share similar ecological niches. Alternatively, streptogramins may not be the preferred substrates for these enzymes; rather, this activity may be secondary to a primary metabolic role. There is precedent for this possibility in the chromosomal aminoglycoside antibiotic acetyltransferase of *Providencia stuartii* where the primary activity is in peptidoglycan acetylation and reaction with aminoglycosides is hypothesized to be a fortuitous secondary activity (*30*).

CONCLUSIONS

Vgb, the enzyme responsible for inactivation of type B streptogramins in clinical isolates of Gram-positive pathogens such as *S. aureus*, has unequivocally been demonstrated not to be a hydrolase but rather is a lyase that linearizes the hexadepsipeptide by an elimination reaction. Orthologues of Vgb from both Gram-negative and Gram-positive bacteria were shown to catalyze the identical reaction. These resistance elements are therefore widely distributed among bacteria and have the potential to impact the use of streptogramins in clinical therapy. These studies on enzyme mechanism therefore provide the opportunity to design selective Vgb inhibitors that could rescue antibiotic activity against resistant bacteria.

ACKNOWLEDGMENT

We thank Kari-ann Draker and Christina Capone for assistance in preparing the pVGB expression vector.

SUPPORTING INFORMATION AVAILABLE

Complete details of the assignments of quinupristin and the product of the Vgb-catalyzed reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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