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Chimeric Streptogramin-Tyrocidine Antibiotics that Overcome Streptogramin Resistance

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Summary

Streptogramin antibiotics are comprised of two distinct chemical components: the type A polyketides and the type B cyclic depsipeptides. Clinical resistance to the type B streptogramins can occur via enzymatic degradation catalyzed by the lyase Vgb or by target modification through the action of Erm ribosomal RNA methyltransferases. We have prepared through chemical and chemo-enzymatic approaches a series of chimeric antibiotics composed of elements of type B streptogramins and the membrane-active antibiotic tyrocidine that evade these resistance mechanisms. These new compounds show broad antibiotic activity against gram-positive bacteria including a number of important pathogens, and chimeras appear to function by a mechanism that is distinct from their parent antibiotics. These results allow for the development of a brand new class of antibiotics with the ability to evade type B streptogramin-resistance mechanisms.

Introduction

Despite being discovered over 50 years ago, the streptogramin antibiotics have only recently seen significant clinical use [1]. The semisynthetic streptogramin formulation Synercid was approved by the FDA in 1999 for the treatment of serious life-threatening infections caused by antibiotic resistant gram-positive bacterial pathogens such as vancomycin-resistant enterococci [2, 3]. Streptogramins are produced by various soil bacteria of the genus Streptomyces and consist of two chemically distinct components: type A and type B. The type A streptogramins are cyclic hybrid peptide-polyketide macrolactones [4]. Type B streptogramins are cyclic depsipeptides consisting of 6–7 amino acids. These peptides are cyclized via an ester bond between the C-terminal carboxyl group and the secondary hydroxyl group of an invariant Thr residue at position 2. The mode of action of both type A and type B streptogramins is through inhibition of translation by binding to bacterial ribosomes [5]. The available evidence indicates that binding of the type A component facilitates binding of the type B streptogramins, a phenomenon that results in synergy of action and bacterial cell death [6]. Recent X-ray structure analysis of the 50S ribosomal subunit in complex with these antibiotics has revealed that type B streptogramins block the peptide exit tunnel while the type A streptogramins bind to the peptidyl transfer center [7].

The only clinically approved streptogramin in North America, Synercid, is a combination of dalfopristin (type A), and quinupristin (type B). Despite the relatively recent clinical introduction of this antibiotic, resistance to each component is well documented (reviewed in [8]). This may be the result of the fact that streptogramins have long been used in agriculture as animal growth promotion agents. In fact, studies have shown that commercial meat products can be contaminated with streptogramin-resistant organisms [9–11].

Resistance to the type B streptogramins can result from active efflux (Msr pumps), alteration of the target ribosome by methylation of the 23S rRNA (Erm methyltransferases), and by the inactivating enzyme Vgb. Vgb was originally found in streptogramin-resistant *Staphylococcus aureus* [12] but has now been identified in other gram-positive bacteria such as *Enterococcus faecium* [13]. The enzyme was thought to be a hydrolase but has now been determined to be a lyase that linearizes the cyclic depsipeptide through a novel elimination reaction [14]. This reaction results in cleavage of the ester bond between the C terminus of the peptide and a secondary hydroxyl group derived from the Thr residue found at position 2 of the type B streptogramin peptide.

This vulnerable and invariant ester bond arises as a result of the action of a cyclizing thioesterase (TE) during nonribosomal peptide biosynthesis of the antibiotic [15]. We reasoned that isosteric replacement of this ester bond with a more stable amide could result in streptogramin analogs that were not susceptible to the action of Vgb. We report here the preparation and biological evaluation of chimeric streptogramin-tyrocidine peptides that are cyclized via amide rather than ester bonds and are impervious to inactivation by Vgb or methylation of the ribosome by Erm.

Results and Discussion

Design and Antibacterial Activity of Streptogramin-Tyrocidine Chimeras

We reasoned that replacement of the ester linkage between the C-terminal carboxyl and side chain alcohol derived from Thr at position 2 with the more thermodynamically stable amide functionality could result in type B streptogramins that were not substrates for the Vgb lyase. We approached the synthesis of such peptides using two solid phase strategies: a biomimetic approach using on-bead enzyme catalyzed cyclization and a chemical method using base-assisted cyclization (Figure 1).

Macrocyclic antibiotics are biosynthesized by polyketide synthetases and nonribosomal peptide synthetases. Prior to cyclization, these antibiotics are tethered to carrier proteins and a cognate thioesterase catalyses their release and cyclization if appropriate. Kohli et al.

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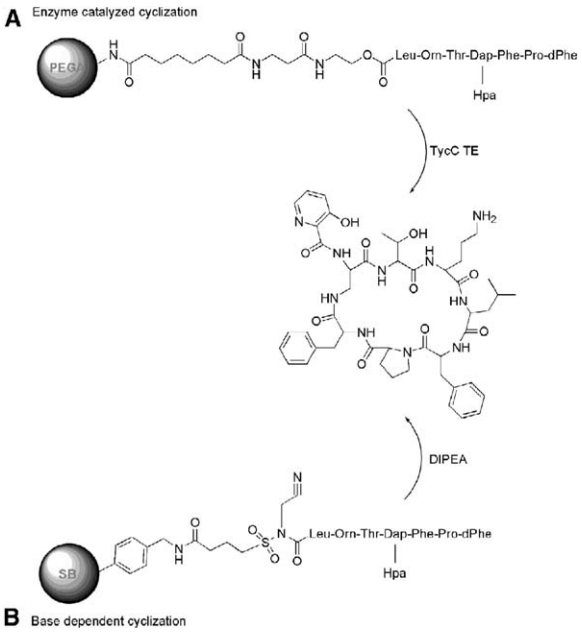

Figure 1. Solid-Phase Chemoenzymatic and Chemical Strategies Employed in Chimeric Peptide Synthesis

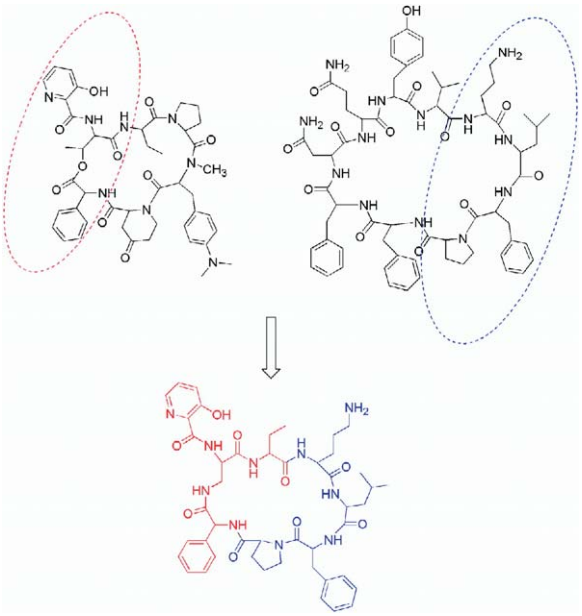

Figure 2. Dissection of Streptogramin-Tyrocidine Chimeric Antimicrobial Peptides

have shown that this in vivo process can be mimicked for tyrocidine using solid phase peptide synthesis on PEGA resin and recombinant tyrocidine thioesterase (TycC TE), providing an efficient route for the synthesis of cyclic peptide antibiotics [16, 17]. The TycC TE possesses the ability to cyclize a variety of tyrocidine analogs varying in length and functional groups [18, 19]. However, there are certain steric restrictions with respect to the amino acids incorporated into the peptide that allow the TE to function efficiently [20]. For example, it was determined that critical residues required for efficient cyclization via TycC TE were D-Phe^{1} and L-Orn^{9}. Changing L-Leu^{10} did not abolish cyclization; however a 4-fold decrease in k_{cat} was detected. Despite these restrictions, we felt that this approach could facilitate the synthesis of cyclic tyrocidine analogs with properties and constituents of type B streptogramin peptides (Figure 2).

We designed the chimeric peptides based on the diversity of type B streptogramin chemical structures (summarized in Table 1), knowledge of the important contact points of streptogramin B with the ribosome [7], and the steric constraints for TycC TE established in [20]. Since type B streptogramins occur both as cyclic hexa- and heptadepsipeptides (not counting the extracyclic 3-Hpa group), we synthesized both lengths in our chimeras. The hexadepsipeptides **1–4**, the heptapeptides **5** and **6**, and the hexapeptide **7** (Figure 3, Table 1) were initially prepared on PEGA resin and cyclized using the recombinant TycC TE.

Incubation of TycC TE with the resin-bound linear precursors resulted in cyclization of these peptides as determined by LC-MS. This indicated that TycC TE was not only capable of cyclizing tyrocidine analogs, but was also capable of cyclizing our designed tyrocidine-streptogramin chimeras. These results are consistent with previous observations that the active site of TycC TE can accommodate a wide variety of substrates [20]. While this enzymatic approach was successful as assessed by LC-MS analysis, the peptides were only obtained in extremely low yields (<1 mg). Partially purified material from these reactions was used to test their antibiotic properties in disk diffusion susceptibility assays using *E. coli* BAS901. This strain is deficient in the Imp/OstA outer membrane protein, which is involved in cell envelope biogenesis [21, 22] and as a result exhibits increased outer membrane permeability and susceptibility to antibiotics, and increased susceptibility toward organic solvents [23, 24]. This assay showed that compounds **5**, **6**, and **7** blocked cell growth, and importantly that these compounds retained antibacterial activity even in the presence of the streptogramin inactivating enzyme Vgb (Table 2).

In an effort to improve the yield of chimeric antibiotics, an alternative nonenzymatic method recently reported by Qin et al. [25] for the cyclization of tyrocidine was explored. This approach uses 20% DIPEA in THF on a solid phase support adopting a "safety-catch" sulfonamide linker [26] with activation by cyanomethylation to facilitate peptide cyclization [27]. Modifying this approach, 10 mg of purified cyclic compound **5** (Figure 1, path B) was obtained and its structure confirmed via ^{1}H, ^{13}C-NMR and mass spectrometry. For this study, we limited our efforts to study of compound **5** as initial results as reported in Table 2 indicated that **5**, **6**, and **7** were comparably active.

Antibacterial Properties of Compound 5

On-resin chemical cyclization of **5** provided sufficient material for more extensive analysis of antibacterial activity and investigation of its ability to inhibit the streptogramin inactivating enzyme Vgb. This enzyme how-

Table 1. Amino Acid Sequences of Antimicrobial Peptides

Antibiotic	AA1[a]	AA2	AA3	AA4	AA5	AA6	AA7	AA8	AA9	AA10
Pristinamycin IA	3-Hpa	Thr	dAbu	Pro	N-Me-DMAPhe	4-Oxo Pip	Phg			
Virginiamycin S1	3-Hpa	Thr	dAbu	Pro	N-MePhe	4-Oxo Pip	Phg			
Patricin A	3-Hpa	Thr	dAbu	Pro	N-MePhe	Pro	Phg			
Etamycin	3-Hpa	Thr	dLeu	dHyp	Sar	DMLeu	Ala	PheSar		
Compound **1**	Bz-3-Hpa	Thr	dAbu	Leu	dPhe	Pro	Phg			
Compound **2**	Bz-3-Hpa	Thr	Orn	Leu	dPhe	Pro	Phg			
Compound **3**	Pic	Thr	dAbu	Leu	dPhe	Pro	Phg			
Compound **4**	Pic	Thr	Orn	Leu	dPhe	Pro	Phg			
Compound **5**	3-Hpa	Dap	Thr	Orn	Leu	dPhe	Pro	Phe		
Compound **6**	3-Hpa	Dap	dAbu	Orn	Leu	dPhe	Pro	Phg		
Compound **7**	3-Hpa	Dap	Orn	Leu	dPhe	Pro	Phg			
Compound **8**	3-Hpa	Dap	dAbu	Pro	Phe	Pro	Phg			
Tyrocidine[b]	Gln	Tyr	Val	Orn	Leu	dPhe	Pro	Phe	dPhe	Asn

[a] Abbreviations: Abu, α-aminobutyric acid; Dap, diaminopropionic acid; DMLeu, N,β-dimethylleucine; 3-Hpa, 3-hydroxypicolonic acid; Hyp, hydroxyproline; N-MePhe, N-methylphenylalanine; N-Me-DMAPhe, N-methyl-p-dimethylaminophenylalanine; 4-Oxo Pip, 4-Oxopipecolic acid; Phg, phenylglycine; Pic, picolinic acid; Pip, pipecolic acid; Sar, sarcosine; PheSar, phenylsarcosine.
[b] Tyrocidine is a 10 amino acid macrocycle shown for comparison to the synthesized peptides.

ever was insensitive to **5** in concentrations of up to 0.4 mM. We had previously synthesized an isosteric amide analog of the minimal Vgb substrate Hpa-Thr(OPhg)-OMe [14]. This analog also was not an inhibitor of Vgb at concentration of up to 1 mM (unpublished). These observations suggest that the ester bond may be crucial for substrate binding within the active site of the enzyme, although this possibility awaits analysis of the 3D structure of the inactivating enzyme. Regardless of the actual mechanism, our design of these streptogramin-tyrocidine chimeras resulted in antibiotics that are not recognized by a streptogramin inactivation enzyme.

In vivo antibacterial activity of **5** was further explored using a variety of gram-positive and gram-negative bacteria (Table 2). Compound **5** demonstrated growth inhibition activity against the hyperpermeable gram-negative bacterium *E. coli* BAS901, but not wild-type *E. coli* 25922 or other gram-negative bacteria including *Salmonella enterica* serovar Typhimurium and *Haemophilus influenzae*. On the other hand, **5** was active against the gram-positive bacteria *Bacillus subtilis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, and *Micrococcus luteus*. This broad spectrum activity against important gram-positive human pathogens but not against gram-negative bacteria with wild-type outer membranes indicates that, like many other peptide and polyketide macrocyclic antibiotics including type B streptogramins, this barrier is impermeable to these chimeric antibiotics. The mechanism for the weak activity against *Staphylo-*

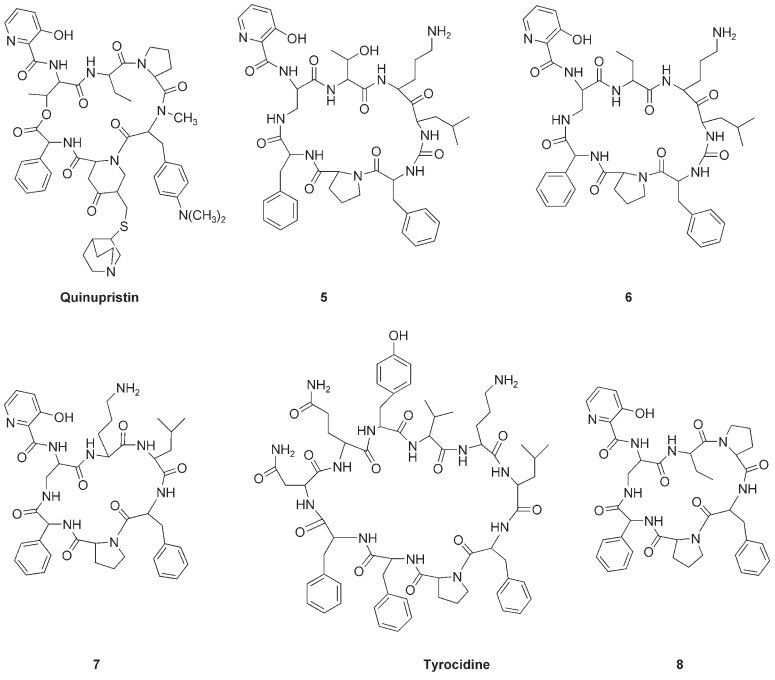

Figure 3. Structures of Antimicrobial Peptides Synthesized and Assayed in This Work

Table 2. Inhibition of Bacterial Growth by Chimeric Antibiotics

Bacterial Strain	Zone of Inhibition (cm)				
	Quinupristin	Compound **5**	Compound **6**[e]	Compound **7**[e]	Compound **8**
Escherichia coli BAS901	2.2	1.1			
E. coli BAS901/pUC19	2.2	1.1	1.2	1.2	n.i.
E. coli BAS901/pUCVgb	n.i.[a]	1.1	1.3	1.3	n.i.
Bacillus subtilis 1A752	1.8	1.1			
B. subtilis W23	2.1	0.9			
B. subtilis L5706	2.1	0.8			
B. subtilis 168	2.2	0.8			
Micrococcus luteus	2.7	1.0			
Enterococcus faecium CP54-32	1.8	0.8			
Enterococcus faecalis (ATCC 29212)	1.8	0.75			
Enterococcus faecalis (ATCC 51299)[b]	n.i.	n.i.			
S. saprophyticus (ATCC 15305)	2.3	0.8			
Staphylococcus epidermidis (ATCC 12228)	3.4	0.85			
Staphylococcus epidermidis (ATCC 14990)	3.0	0.75			
Staphylococcus aureus (ATCC 43300)[c]	n.i.	n.i.			
Staphylococcus aureus (ATCC 29213)	2.3	n.i.			n.i.
Staphylococcus aureus (ATCC 25923)	2.2	n.i.			
Staphylococcus aureus (ATCC 49476)	2.6	n.i.			
Streptococcus pneumoniae (ATCC 6305)	2.1	0.8			
Streptococcus pneumoniae (ATCC 49619)	2.0	n.i.			
E. coli (ATCC 25922)	n.i.	n.i.			
Salmonella enterica (ATCC 14028)[d]	n.i.	n.i.			
Haemophilus influenza	n.i.	n.i.			

[a] n.i., no inhibition of growth.
[b] Vancomycin, aminoglycoside resistant *Enterococcus faecalis*.
[c] Methicillin resistant, oxacillin resistant *Staphylococcus aureus*.
[d] serovar Typhimurium.
[e] **6** and **7** were analyzed following cyclization without further purification.

coccus aureus is unknown at this time, however **5** does inhibit the growth of this organism at 256 μg/ml (Table 3). The possibility that the elevated MIC could be the result of the ribitol-based wall teichoic acid polymer in *Staphylococcus aureus* was discounted as *B. subtilis* W23, which primarily synthesizes this polymer, was inhibited by **5** (Table 2).

Comparison of the MIC values for quinupristin and **5** against control *E. coli* (BAS901/pUC19), *E. coli* expressing the streptogramin inactivating enzyme Vgb (BAS901/pUC19VGB), and *E. coli* with the ribosome protecting enzyme Erm methyltransferase (BAS901/pUC19ermB) indicated that unlike quinupristin, the antibacterial effects of **5** are invariant in the presence of these resistance enzymes (Table 3). These results supported our in vitro experiments demonstrating that **5** was not a substrate for Vgb. Furthermore, **5** is not susceptible to the target modification resistance afforded through ErmB-mediated methylation of the 23S rRNA of ribosome.

Quinupristin and dalfopristin act in a synergistic manner; we therefore explored whether **5** also could synergize with dalfopristin. These experiments were performed against both *B. subtilis* 1A752 and *E. coli* BAS901 in microtiter plates using a standard checkerboard technique. The results revealed an additive, not synergistic, effect with dalfopristin (see Supplemental Data). Although we did not observe a synergistic antimicrobial effect, we could not rule out the possibility that **5** still bound the ribosome. It remained plausible that the structure of **5** precluded it from binding to the same site as quinupristin on the ribosome and thus synergy was not observed. This would also be consistent with the in vivo results observed with ErmB.

Table 3. MIC Values for Quinupristin and **5** against Susceptible Bacteria

Organism	MIC (μg/ml) Quinupristin	Compound **5**
E. coli BAS901/pUC19	4	32
E. coli BAS901/pUC19ermB	16	32
E. coli BAS901/pUCVgb	>256	32
Staphylococcus aureus RN4220/pJIM2246	2	256
Staphylococcus aureus RN4220/pJIM2246 Ω*ermB*	8[a]	256
Staphylococcus aureus RN4220/pJIM2246 Ω*vgb*	8	256
Staphylococcus aureus (ATCC 29213)	4	256
Staphylococcus epidermidis (ATCC 12228)	0.5	64
Staphylococcus epidermidis (ATCC 14990)	1	64
Staphylococcus saprophyticus (ATCC 15305)	8	64
Enterococcus faecalis (ATCC 29212)	8	128
Streptococcus pneumoniae (ATCC 6305)	1	32
Streptococcus pneumoniae (ATCC 49619)	0.5	128

[a] Indicates induction with quinupristin (1 μg/ml).

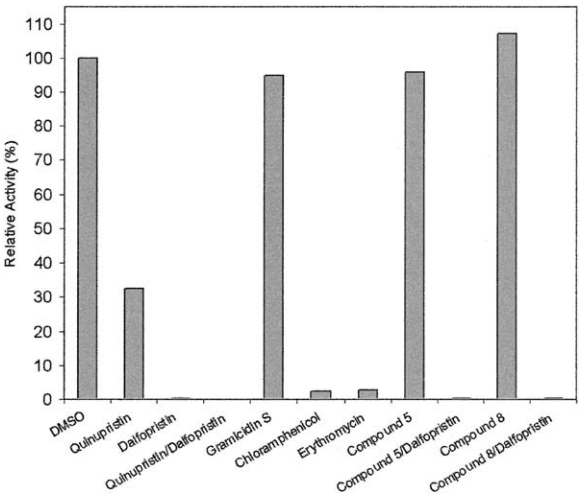

Figure 4. Impact of Antibiotics on In Vitro Translation

Antibiotics were added to a final concentration of 0.1 mg/ml and the amount of [^{35}S]-Met incorporated into in vitro translated chloramphenicol acetyltransferase quantified and normalized to the DMSO carrier control. The average of two experiments is presented. Duplicate values were within 6% of each other.

To establish if that antimicrobial activity of **5** was the result of inhibition of translation, we measured the impact of this compound on the efficiency of an in vitro translation system. While the known inhibitors of translation chloramphenicol, erythromycin, and the streptogramins dalfopristin and quinupristin did block in vitro translation, compound **5** did not at 250 μg/ml (Figure 4). Furthermore, fluorescence titration experiments with purified intact *E. coli* ribosomes that measures increase of the fluorescence associated with the Hpa moiety did support ribosome binding by quinupristin as previously shown [28], but compound **5** did not appear to interact with the ribosome in this study (see Supplemental Data).

Given that **5** is a chimera of tyrocidine, an antibiotic that acts by disrupting cellular membranes, we examined the hemolytic activity of **5** in comparison with the commercially available, channel-forming antibiotic gramicidin S (tyrocidine is not commercially available). Compound **5** showed no significant hemolytic properties even after a 9 hr exposure, while gramicidin lysed cells rapidly under identical conditions (Supplemental Data). Consistent with the lack of membrane damaging activity was our observation that **5** is bacteriostatic and not bactericidal against *E. coli* BAS901 and *B. subtilis* 1A752.

These results therefore indicate that the mode of action of these chimeric antibiotics, rather unexpectedly, do not parallel either type B streptogramins or channel forming peptides. In an effort to further explore the impact of altering the ester bond of type B streptogramins, we prepared an analog of the antibiotic patricin A replacing the Thr-phenylglycine cyclizing ester with the diaminopropionic acid-phenylglycine amide (compound **8**). This compound differs also from patricin A in the lack of N methylation between amino acid residues 4 and 5. Compound **8** showed no antibacterial activity nor did it inhibit in vitro translation (Table 2 and Figure 4). These results demonstrate strict structural requirements for streptogramin antibiotic activity. The chimeric antibiotics described in this work therefore serendipitously represent, to our knowledge, a new class of antimicrobial peptides of unknown function.

While a rigorous structure activity relationship was beyond the purpose of this study, we can make some general comments regarding the properties of these antibiotics. First, there is good tolerance of amino acid side chains in position 3. Second, the 3-Hpa is important in position 1. Cyclic depsipeptides **1** and **2**, where the hydroxyl of 3-Hpa is protected with a benzyl group and **3** and **4**, with a picolinic acid devoid of the hydroxyl at position 3, showed no antimicrobial activity. Admittedly these are depsipeptide and not peptides such as **5**, **6**, and **7**, therefore we cannot rule out that the cyclizing functionality may impact on activity as well. Further exploration of the additional details of structure versus activity will expand on our understanding of the biochemistry of these antibiotics.

Significance

The rampant increase in the number of antibiotic-resistant organisms has placed great pressure on the pharmaceutical, medical, and academic fields to gain a better understanding of the mechanism of resistance, and for developing new antimicrobials that can be used to combat these organisms. Our previous research had demonstrated that type B streptogramins are inactivated by Vgb lyase through elimination of the ester bond which joins the N and C termini of the peptide [14]. In this work, we have shown that streptogramin-tyrocidine chimeras possess encouraging antibacterial properties, and are simultaneously unaffected by the resistance afforded by Vgb lyase and by ErmB through methylation of the ribosome. This finding opens the possibility for further development of a class of chimeric antibiotics that evade resistance mechanisms preexisting in the clinic.

Experimental Procedures

Reagents and General Techniques

PEGA-NH_2 and sulfamylbutyryl resins, and all Fmoc and Boc-protected amino acids, except Fmoc-dAbu-OH, were purchased from Novabiochem. Recombinant Vgb from *Staphylococcus aureus* and truncated tyrocidine thioesterase (TycC TE) were expressed and purified as previously described [14, 20]. LC/MS was conducted on a Micromass Quatro LC by Dr. K. Green at the McMaster Regional Centre for Mass Spectrometry.

Solid-Phase Synthesis of Peptides

We used two solid-phase strategies to achieve the synthesis of cyclic peptides. The first was a recently described biomimetic approach on PEGA-NH_2 resin that uses a CoA-mimicking linker followed by sequential attachment of the Fmoc-protected amino acids and enzymatic cyclization [17]. In the second approach, standard Fmoc-amino acids were loaded on sulfamylbutyryl resin.

The general synthesis of the linear peptides followed the following procedure: to the resin loaded with the first amino acid (1 g) was added 10 ml of 20% piperidine in dimethylformamide (DMF) solution, followed by agitation for 20 min and wash cycles of DMF (3 × 10 ml), iso-propanol (3 × 10 ml), and DMF (2 × 10 ml). Subsequently, a freshly prepared mixture of the Fmoc-protected amino

acid (Fmoc-AA-OH) (5eq), 1-hydroxybenzotriazole (5 eq), PyBop (5 eq), and diisopropylethylamine (DiPEA) (10 eq) in DMF (5 ml) was added and the reaction mixture was agitated for 2 to 4 hr. The washing cycle was repeated and the resulting resin was subjected to Kaiser test [29] to assess the efficiency of coupling. If the test was positive, the coupling was repeated until a negative test result was obtained. Otherwise, the synthesis proceeded to the next round of deprotection and coupling. Following the coupling of the last amino acid to the resin, the protected peptide was treated with 10 ml of trifluoroacetic acid (TFA)/water/triisopropylsilane (TIPS) (9.5:0.25:0.25) for 2 hr at room temperature and washed sequentially with DMF, iso-propanol, and methanol and dried over KOH under vacuum for 4 hr.

TycC TE Catalyzed Peptide Cyclization

Purified recombinant TycC TE was added to 200–500 mg of deprotected peptide on PEGA resin as described in [17] for 30 min. The mixture was then filtered and the process repeated. The resin was subsequently washed twice with acetonitrile and the soluble fractions pooled, lyophilized, and analyzed by LC/MS.

Base-Promoted, On-Resin Peptide Cyclization

On-resin cyclization of the linear peptide precursor was carried out by immersing the resin in 2% DiPEA in THF (200 ml) for 16 hr at room temperature [25]. After filtration the solvent was removed in vacuo, the crude product was resuspended in 50% acetonitrile/water, and the solution lyophilized. The final product was characterized by LC-MS analysis and purified by preparative reverse phase HPLC (Vydac 218TP1022 column).

Synthesis of Peptides on Sulfamylbutyryl Resin

Peptide **5** was synthesized on sulfamylbutyryl resin (1 g) following the general procedure described above. 3-Hydroxypicolinic acid (3-Hpa) was the last amino acid to be incorporated in the peptide chain after trityl group deprotection of diaminopropionic acid with a mixture of 3% TFA, 5% TIPS, and 92% DCM (10 ml) for 2 hr at room temperature. At the end of the peptide synthesis, the resin was activated with iodoacetonitrile as already described [30]. Boc-deprotection was carried out using a mixture of TFA/phenol/TIPS/water (8.8:0.5:0.5:0.2) (10 ml) for 2 hr at room temperature. The cyclization reaction was carried out as described above and the peptide product purified by reverse phase HPLC in an overall yield of 10 mg (1.3%).

600 MHz, ^{1}H-NMR spectra data for **5** in 60:40 CD_3CN:D_2O, δ ppm, J in Hz: 0.78 (d, 3H, J 6.5); 0.83 (d, 3H, J 6.5); 1.12 (d, 3H, J 3.8); 1.21–1.27 (m, 1H); 1.33–1.39 (m, 1H); 1.41–1.49 (m, 2H); 1.63–1.77 (m, 8H); 1.81–1.83 (m, 1H); 2.78–2.82 (m, 4H); 2.84–2.98 (m, 1H), 3.06–3.10 (m, 4H); 3.46–3.50 (m, 2H); 3.57–3.61 (m, 2H); 3.64–3.68 (m, 2H); 4.15–4.17 (m, 1H); 4.21–4.23 (m, 1H); 4.25–4.30 (m, 1H); 4.33–4.41 (m, 1H); 4.64–4.68 (m, 1H); 4.73–4.75 (t, 1H, J_1 5.2, J_2 5.2); 7.06–7.29 (m, 10H); 7.37–7.39 (d, 1H, J 8.5); 7.47–7.50 (m, 1H); 7.57–7.61 (m, 1H); 7.83–7.85 (t, 1H, J_1 6.1, J_2 5.9); and 7.89–7.93 (m, 2H).

^{13}C NMR: 18.62, 19.88, 22.64, 22.69, 24.07-24.13, 25.38, 29.04, 29.89, 36.36, 37.32, 39.61, 41.04–41.17, 48.02, 59.91, 54.25–54.48, 56.29, 59.83, 61.15, 67.32, 127.78, 128.10, 129.43–129.51, 130.13–130.26, 136.92, 138.21, 158.40, 162.41, 168.75, 172.31–172.47, 173.30–173.41, 173.95, and 174.51.

TOF MS ES$^+$ m/z 927.4754 ([M+H]$^+$, calculated *m/z* for $C_{47}H_{62}N_{10}O_{10}$, 926.4650).

The patricin analog **8** was synthesized using the same solid phase strategy as for the synthesis of peptide **5**. The cyclized product was purified using reverse phase HPLC.

700 MHz, ^{1}H-NMR spectra data for **8** in 60:40 CD_3CN:D_2O, δ ppm, J in Hz:

0.86 (t, 3H, J_1 3.8, J_2 3.6); 1.66–1.72 (m, 2H); 1.85–1.95 (m, 4H); 2.05–2.15 (m, 4H); 2.80–2.91 (m, 2H); 3.06–3.21 (dd, 2H); 3.38–3.53 (m, 4H); 3.56–3.62 (m, 2H); 3.67–3.74 (m, 2H); 4.15–4.20 (m, 1H); 4.25–4.29 (m, 1H); 4.30–4.38 (m, 2H); 4.81–4.84 (m, 1H); 4.85 (d, 1H, J 7.1); 5.13–5.20 (m, 1H); 7.09–7.11 (m, 4H); 7.13–7.55 (m, 6H); 7.61–7.71 (m, 2H); 8.18–8.21; and 8.57–8.59 (m, 1H).

^{13}C NMR: 10.24, 23.87, 24.22, 26.25, 29.24, 30.20, 37.64, 48.47, 51.16, 53.16, 53.24, 55.63, 60.26, 61.60, 61.89, 119.31, 127.34, 127.88, 129.25, 129.53, 129.64, 129.95, 130.27, 130.48, 132.02, 137.10, 138.00, 141.64, 158.15, 170.33, 172.13, 172.26, 173.35, 173.87, and 174.59.

MS ES$^+$ m/z 767.8 [M+H]$^+$, calculated m/z for $C_{40}H_{46}N_8O_8$ 766.8.

Preparation of Streptogramin-Sensitive, *E. coli*-Expressing Streptogramin B Resistance Elements

A hyperpermeable *E. coli* strain, *E. coli* BAS901, harboring a plasmid constitutively expressing Vgb was created for in vivo studies. The plasmids pVGB, described previously in [14], and pUC19 were digested with XbaI and BamHI, to yield an approximately 1 kb fragment containing a ribosomal binding site upstream of *vgb*, and linearized pUC19 backbone, respectively. The *vgb* fragment was then subcloned into the linearized pUC19 backbone downstream of pLac yielding pUC19-VGB. Both pUC19-VGB and pUC19 were transformed into *E. coli* BAS901.

In order to test the effects of **5** against *E. coli* BAS901 harboring *ermB*, the 23S rRNA methyltransferase, the plasmid pJIM2246 Ω*ermB* [13] was purified from *Staphylococcus aureus* RN4220/pJIM2246 Ω*ermB*. *ermB* was subsequently amplified from pJIM2246 Ω*ermB* via polymerase chain reaction using primers 5′-GAATTCCATATGAACAAAAATATAAAATATTCTCAAAAC and 5′-GAATTCGGATCCTCATAGAATTAATTTCCTCCCG (engineered restriction enzyme sites are underlined). The amplified product was subcloned into pCR4Blunt-TOPO (Invitrogen) and transformed into *E. coli* XL10 GOLD, resulting in *ermB* being positioned in frame with the *lacZ* gene. This construct, designated pTOPOerm, was then used as template in the reamplification of *ermB* using a new forward primer containing a strong ribosomal binding consensus sequence (shown in italics); 5-ATCTAG*AGGAGGA*ATTCCATATG. The amplification product was again subcloned into pCR4Blunt-TOPO and transformed into XL10 GOLD, resulting in the construct pTOPOnewerm. pTOPOnewerm was digested with PstI and BamHI, and the resulting fragment contained *ermB* downstream of the ribosomal binding site. This fragment was ligated to pUC19 digested with the same restriction enzymes. The resulting construct, pUC19-ermB was subsequently transformed into *E. coli* BAS901.

Determination of Antibiotic Activity of Peptides

The antibiotic activity of the peptides synthesized in this study was initially assayed using a disk assay where samples were applied to sterile paper disks placed on a fresh lawn of bacterial culture on appropriate agar plates as required by the organism. These were then incubated for 16 hr at 37°C and the zone of growth inhibition surrounding the disks recorded. For quantitative assessment of antibiotic activity in liquid culture, the minimal inhibitory concentration (MIC) of peptides was determined by serial dilution studies. The peptides and standard antibiotics were dissolved in DMSO and the final concentration of DMSO in the liquid cultures was 5%. Both *S. aureus* RN4220 and *E. coli* BAS901 were able to grow efficiently at this level of DMSO.

Hemolysis Assay

Human red blood cells were freshly harvested, washed with 0.85% saline solution with 5 mM EDTA and resuspended in the same solution to an A_{412} of 0.6. The hemolysis assay was performed in 1.5 ml Eppendorf tubes with the addition of either **5** in DMSO, saline, gramicidin (final concentration 30 μg/ml), or DMSO (5% final), for both 3 and 9 hr at 37°C with occasion gentle mixing. The mixtures were then centrifuged and the A_{412} of the supernatant measured.

In Vitro Translation Assay

Inhibition of translation was monitored using an ActivePro In Vitro Translation Kit from Ambion. Briefly, a reaction mixture was prepared as per the manufacturer's instructions that contained all of the constituents necessary (including [^{35}S]-methionine) except for DNA template. These mixtures were then preincubated for 15 min at room temperature in the presence or absence of various antibiotics. Subsequent to the preincubation period, a control template containing a chloramphenicol acetyltransferase gene under control of a bacteriophage T7 promoter was added and the reaction carried out for 1 hr at 37°C. Due to the insoluble nature of some of the antibiotics, the final DMSO concentration in each reaction including

controls was maintained at 5% (v/v). Aliquots were taken after 1 hr, and separated on SDS polyacrylamide gels. The efficiency of translation of 35S-labeled chloramphenicol was analyzed and quantified using a Typhoon 9200 variable mode imager (ImageQuant 5.2 software).

Supplemental Data

Supplemental Data for this article is available online at http://www.chembiol.com/cgi/content/full/12/2/229/DC1/.

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References

1. Johnston, N.J., Mukhtar, T.A., and Wright, G.D. (2002). Streptogramin antibiotics: mode of action and resistance. Curr. Drug Targets *3*, 335–344.
2. Delgado, G., Jr., Neuhauser, M.M., Bearden, D.T., and Danziger, L.H. (2000). Quinupristin-dalfopristin: an overview. Pharmacotherapy *20*, 1469–1485.
3. Eliopoulos, G.M. (2003). Quinupristin-dalfopristin and linezolid: evidence and opinion. Clin. Infect. Dis. *36*, 473–481.
4. Cocito, C. (1979). Antibiotics of the virginiamycin family, inhibitors which contain synergistic components. Microbiol. Rev. *43*, 145–192.
5. Cocito, C., Di Giambattista, M., Nyssen, E., and Vannuffel, P. (1997). Inhibition of protein synthesis by streptogramins and related antibiotics. J. Antimicrob. Chemother. *39* (Suppl. A), 7–13.
6. Di Giambattista, M., Chinali, G., and Cocito, C. (1989). The molecular basis of the inhibitory activities of type A and type B synergimycins and related antibiotics on ribosomes. J. Antimicrob. Chemother. *4*, 485–507.
7. Harms, J.M., Schluenzen, F., Fucini, P., Bartels, H., and Yonath, A.E. (2004). Alterations at the peptidyl transferase centre of the ribosome induced by the synergistic action of the streptogramins dalfopristin and quinupristin. BMC Biol. *2*, 4.
8. Jones, R.N., Farrell, D.J., and Morrissey, I. (2003). Quinupristin-dalfopristin resistance in *Streptococcus pneumoniae*: novel L22 ribosomal protein mutation in two clinical isolates from the SENTRY antimicrobial surveillance program. Antimicrob. Agents Chemother. *47*, 2696–2698.
9. Simjee, S., White, D.G., Meng, J., Wagner, D.D., Qaiyumi, S., Zhao, S., Hayes, J.R., and McDermott, P.F. (2002). Prevalence of streptogramin resistance genes among *Enterococcus* isolates recovered from retail meats in the Greater Washington DC area. J. Antimicrob. Chemother. *50*, 877–882.
10. Simjee, S., White, D.G., Wagner, D.D., Meng, J., Qaiyumi, S., Zhao, S., and McDermott, P.F. (2002). Identification of *vat(E)* in *Enterococcus faecalis* isolates from retail poultry and its transferability to Enterococcus faecium. Antimicrob. Agents Chemother. *46*, 3823–3828.
11. Soltani, M., Beighton, D., Philpott-Howard, J., and Woodford, N. (2000). Mechanisms of resistance to quinupristin-dalfopristin among isolates of *Enterococcus faecium* from animals, raw meat, and hospital patients in Western Europe. Antimicrob. Agents Chemother. *44*, 433–436.
12. Allignet, J., Aubert, S., Morvan, A., and el Solh, N. (1996). Distribution of genes encoding resistance to streptogramin A and related compounds among staphylococci resistant to these antibiotics. Antimicrob. Agents Chemother. *40*, 2523–2528.
13. Bozdogan, B., and Leclercq, R. (1999). Effects of genes encoding resistance to streptogramins A and B on the activity of quinupristin-dalfopristin against *Enterococcus faecium*. Antimicrob. Agents Chemother. *43*, 2720–2725.
14. Mukhtar, T.A., Koteva, K.P., Hughes, D.W., and Wright, G.D. (2001). Vgb from *Staphylococcus aureus* inactivates streptogramin B antibiotics by an elimination mechanism not hydrolysis. Biochemistry *40*, 8877–8886.
15. de Crecy-Lagard, V., Saurin, W., Thibaut, D., Gil, P., Naudin, L., Crouzet, J., and Blanc, V. (1997). Streptogramin B biosynthesis in *Streptomyces pristinaespiralis* and *Streptomyces virginiae*: molecular characterization of the last structural peptide synthetase gene. Antimicrob. Agents Chemother. *41*, 1904–1909.
16. Kohli, R.M., Takagi, J., and Walsh, C.T. (2002). The thioesterase domain from a nonribosomal peptide synthetase as a cyclization catalyst for integrin binding peptides. Proc. Natl. Acad. Sci. USA *99*, 1247–1252.
17. Kohli, R.M., Walsh, C.T., and Burkart, M.D. (2002). Biomimetic synthesis and optimization of cyclic peptide antibiotics. Nature *418*, 658–661.
18. Kohli, R.M., Trauger, J.W., Schwarzer, D., Marahiel, M.A., and Walsh, C.T. (2001). Generality of peptide cyclization catalyzed by isolated thioesterase domains of nonribosomal peptide synthetases. Biochemistry *40*, 7099–7108.
19. Trauger, J.W., Kohli, R.M., and Walsh, C.T. (2001). Cyclization of backbone-substituted peptides catalyzed by the thioesterase domain from the tyrocidine nonribosomal peptide synthetase. Biochemistry *40*, 7092–7098.
20. Trauger, J.W., Kohli, R.M., Mootz, H.D., Marahiel, M.A., and Walsh, C.T. (2000). Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase. Nature *407*, 215–218.
21. Braun, M., and Silhavy, T.J. (2002). Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. Mol. Microbiol. *45*, 1289–1302.
22. Abe, S., Okutsu, T., Nakajima, H., Kakuda, N., Ohtsu, I., and Aono, R. (2003). n-Hexane sensitivity of *Escherichia coli* due to low expression of *imp/ostA* encoding an 87 kDa minor protein associated with the outer membrane. Microbiol. *149*, 1265–1273.
23. Sampson, B.A., Misra, R., and Benson, S.A. (1989). Identification and characterization of a new gene of *Escherichia coli* K-12 involved in outer membrane permeability. Genetics *122*, 491–501.
24. Aono, R., Negishi, T., and Nakajima, H. (1994). Cloning of organic solvent tolerance gene *ostA* that determines n-hexane tolerance level in *Escherichia coli*. Appl. Environ. Microbiol. *60*, 4624–4626.
25. Qin, C., Bu, X., Wu, X., and Guo, Z. (2003). A chemical approach to generate molecular diversity based on the scaffold of cyclic decapeptide antibiotic tyrocidine A. J. Comb. Chem. *5*, 353–355.
26. Backes, B.J., and Ellman, J.A. (1999). An alkanesulfonamide "safety-catch" linker for solid-phase synthesis. J. Org. Chem. *64*, 2322–2330.
27. Yang, L., and Morriello, G. (1999). Solid phase synthesis of 'head-to-tail' cyclic peptides using a sulfonamide 'safety-catch' linker: the cleavage by cyclization approach. Tetrahedron Lett. *40*, 8197–8200.
28. Parfait, R., de Bethune, M.P., and Cocito, C. (1978). A spectrofluorimetric study of the interaction between virginiamycin S and bacterial ribosomes. Mol. Gen. Genet. *166*, 45–51.
29. Kaiser, E., Colescott, R.L., Bossinger, C.D., and Cook, P.I. (1970). Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. *34*, 595–598.
30. Shin, Y., Winans, K.A., Backes, B.J., Kent, S.B.H., Ellman, J.A., and Bertozzi, C.R. (1999). Fmoc-based synthesis of peptide-thioesters: application to the total chemical synthesis of a glycoprotein by native chemical ligation. J. Am. Chem. Soc. *121*, 11684–11689.