Signal peptidase (SPase) I is responsible for the cleavage of signal peptides of many secreted proteins in bacteria. Because of its unique physiological and biochemical properties, it serves as a potential target for development of novel antibacterial agents. In this study, we report the production, isolation, and structure determination of a family of structurally related novel lipoglycopeptides from a *Streptomyces sp.* as inhibitors of SPase I. Detailed spectroscopic analyses, including MS and NMR, revealed that these lipoglycopeptides share a common 14-membered cyclic peptide core, an acyclic tripeptide chain, and a deoxy-α-mannose sugar, but differ in the degree of oxidation of the N-methylphenylglycine residue and the length and branching of the fatty acyl chain. Biochemical analysis demonstrated that these peptides are potent and competitive inhibitors of SPase I with *K*<sub>i</sub> of 50 to 158 nM. In addition, they showed modest antibacterial activity against a panel of pathogenic Gram-positive and Gram-negative bacteria with minimal inhibitory concentration of 8–64 μg against *Streptococcus pneumoniae* and 4–8 μg against *Escherichia coli*. Notably, they mechanistically blocked the protein secretion in whole cells as demonstrated by inhibiting β-lactamase release from *Staphylococcus aureus*. Taken together, the present discovery of a family of novel lipoglycopeptides as potent inhibitors of bacterial SPase I may lead to the development of a novel class of broad-spectrum antibiotics.

Proteins destined for secretion in both prokaryotic and eukaryotic organisms are initially synthesized as precursors with an amino-terminal extension known as signal (or leader) peptide. The signal sequence is removed by a signal peptidase (SPase)<sup>1</sup> that is localized in the cytoplasmic membrane in bacteria. Cleavage of precursors by SPase leads to the release of secreted proteins from the outer surface of cytoplasmic membrane. In bacteria, two major SPases, SPase I and SPase II with different cleavage specificities, have been identified. SPase I is responsible for processing the majority of secreted proteins (1–3), and SPase II is exclusively involved in processing glyceride-modified lipoproteins (4).

SPase I is an attractive target for development of antibacterial agents because of its unique biochemical and physiological properties. It is essential for bacterial viability and growth as demonstrated by gene knockout and other genetic experiments (5–8). It is widely distributed in both Gram-positive and Gram-negative bacteria, as well as in *Chlamydia*. Genes encoding SPase I have been cloned and sequenced from different bacterial species, including many of clinically relevant bacteria (8, 9). The active domain of bacterial SPase I is exposed to the surface of cytoplasmic membrane as revealed by sequence and topological analysis (10–12), and thus is accessible to potential inhibitors. In addition, SPases from bacteria and eukaryotic cells are different in composition, location, and possibly catalytic mechanism (13–17). These differences make it possible to identify selective bacterial SPase I inhibitors without toxicity to mammalian cells.

SPase I belongs to a novel class of serine protease that utilize a serine and a lysine to form a unique catalytic dyad for peptide hydrolysis (14–16). Because of this unique catalytic mechanism, they are not sensitive to the classic protease inhibitors (2, 3, 18). The first effective inhibitor of bacterial SPase I was described in 1994 by Kuo and colleagues (19), who reported that β-lactams could inhibit *Escherichia coli* SPase I in a pH- and time-dependent manner (19). Certain β-lactams have also been shown to be effective irreversible inhibitors of a number of serine proteases and hydrolases, such as elastase (20, 21), phospholipase A<sub>2</sub> (22), and β-lactamase (23). Researchers at Smith-Kline Beecham have extensively studied β-lactam (or penem)-type inhibitors against *E. coli* SPase I (24, 25). To our knowledge, allyl (5S,6S)-6-((R)-acetoxyethyl)-penem-3-carboxylate (5S,6S-penem) is one of the most potent inhibitors of *E. coli* SPase I reported thus far.

In previous studies, we cloned the gene encoding SPase I of *Streptococcus pneumoniae* and identified the precursor of streptokinase as a native substrate of the enzyme (9). Consequently, we developed a fluorescent peptide substrate, KLTGTVKAbz-FQVQAIAQYG/NO2/EWL and a continuous fluorimetric assay for this enzyme (26). In this report we identify a family of novel lipoglycopeptides from a microbial source, which are potent inhibitors of bacterial SPase I and are able to inhibit the growth of clinically relevant bacteria.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**— *S. pneumoniae* R6 was an unencapsulated D39 derivative (27, 28). *Staphylococcus aureus* ATCC 33592 was a gentam-
BL21(DE3) was transformed with pET15b-EcoliSPase, grown, and in-
Because overexpressed
E. coli (40 mg) mostly containing lipoglycopeptides
fraction D over a Waters Symmetry C18 column (7.8
Amberchrom CG161m column equilibrated with 3:1 (v/v) H2O/CH3OH.
determined by HPLC using a Hewlett Packard Series 1100 system
enzyme, and did not influence the enzymatic activity (data not shown).
Previously described peptide substrates, KLTFGTVK—
found that 1M urea in the elution buffer increased the recovery of active
and 1-ml fractions were collected and analyzed by SDS-PAGE. We
/NaCl, and 8 M urea. After centrifugation at 50,000
doing in the inclusion
body, the purification went through a denaturing and refolding process.
Typically, 1 liter of isopropyl-1-thio-β-galactosidase-induced E. coli
BL21(DE3) cells harboring pET15b-EcoliSPase were resus-
/CH3OH, 4
incubated at 30
°
Fermentation
was extracted 2
was diluted with 600 ml of H2O and applied onto a 500 ml of TosoHaas
39 cm, Amersham Biosciences) with CH3OH as the solvent. The effluent containing the
lipoglycopeptides was evaporated (960 mg) and further chromato-
FIG.1 . Structures of lipoglycopeptides 1–8 and glycopeptides
mg of lipoglycopeptide 2. Repeated chromatography of fraction E twice as
described above yielded 0.3 mg of lipoglycopeptide 3, 0.5 mg of
lipoglycopeptide 4, and 3.2 mg of lipoglycopeptide 5. Fraction A was
chromatographed over a PolyLC polyhydroxyethyl aspartamide column
(95–65% CH3CN gradient over 48 min and holding at 65% CH3CN for
24 min) to furnish fraction F (90 mg) containing lipoglycopeptides 6 and
7, and fraction G (51 mg) containing lipoglycopeptide 8. Rechromatog-
ography of fraction F over a Waters Symmetry Prep C18 column (19 × 300
mm, 7 μm, flow rate 17 ml/min, 25–40% CH3CN gradient buffered with
0.05% NH4OAc over 48 min and holding at 40% CH3CN for 24 min)
yielded 9 mg of enriched lipoglycopeptide 6 and 11 mg of enriched
lipoglycopeptide 7. Each sample was re-purified as detailed above to
yield 3.7 mg of lipoglycopeptide 6 and 2.3 mg of lipoglycopeptide 7.
Similar chromatography of fraction G gave 6.4 mg of lipoglycopeptide 8.
The purity of isolated peptides was determined by analytical chroma-
tography over a Waters Symmetry C18 column (4.6 × 150 mm, 3.5 μm,
flow rate 1 ml/min, 35–70% CH3CN gradient over 15 min).

Amino Acid Analysis—Amino acid analysis of lipoglycopeptide 1 was
performed as described (32).

Decaylization of Lipoglycopeptides 7 and 8—A solution containing
mostly lipoglycopeptide 7 and 8 (200 mg) in 20 ml of CH3CN/H2O/ trifluoroacetic acid (6:3:1) was stirred for 90 h at room temperature.
The mixture was purified over a PolyLC polyhydroxyethyl aspartamide column
(50.8 × 250 mm, 12 μm, flow rate 45 ml/min, 90–50% CH3CN gradient buffered with 0.05%
NH3OAc over 72 min) to yield two fractions.
These fractions were further purified independently over a CG161 column (85 ml) to yield 10.7 and 14 mg of lipoglycopeptides 9 and 10, respectively.

LC-MS Study—LC-MS analysis was carried out on a Waters Alliance
2690 Separations Module coupled with a Platform LCZ mass spectrom-
eter. ESI spectra were collected with capillary and sample cone poten-
tials set at 3000 and 50 V, respectively. Accurate mass determination
was performed using a Micromass Q-TOF 1 quadrupole/orthogonal
time-of-flight mass spectrometer. Desvansacamine ion of vancomycin
(mlz 1305,3434) was used as the lock mass in all accurate mass determinations.
TABLE I

$^1$H and $^{13}$C NMR chemical shift assignments of lipoglycopeptide 1 in CD$_3$OD

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<td></td>
<td>4'</td>
<td>0.86</td>
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</table>

$^a$ Chemical shifts derived from HSQC and HMBC data.

NMR Study—NMR experiments were carried out on a Varian Inova spectrometer equipped with a pulse-field gradient module and a Nobelrad Z-SPEC microdual 3-mm probe, operating at 500 MHz for $^1$H and 125.7 MHz for $^{13}$C. Proton and carbon chemical shifts were referenced to the residual solvent signal (CD$_3$OD or CD$_2$OD) at 3.30 and 49 ppm, respectively. Two-dimensional experiments including TOCSY, DQCOSY, HSQC, and ROESY were performed using Varian standard pulse sequences.

IC$_{50}$ Determination—IC$_{50}$ was determined by fluorometric assay as described (26). Standard reaction (50 μl) contained 50 nM of either E. coli or S. pneumoniae SPase I, 50 μM substrate and different concentrations of an inhibitor. Reactions were incubated at 37 °C for 1 h for E. coli SPase I and 2 h for S. pneumoniae SPase I. IC$_{50}$ was calculated using nonlinear regression method with GraphPad Prism Software.

Kinetic Analysis—Kinetic analysis was performed with E. coli SPase I and the non-fluorescent peptide substrate, KLTFGTVKPVQA-

FIG. 2. MS/MS spectrum and diagnostic ESI mass spectral fragmentation pattern of lipoglycopeptide. A, MS/MS spectrum of lipoglycopeptide 1. B, diagnostic ESI mass spectral fragmentation pattern of lipoglycopeptide.
brain heart infusion medium and resuspended in same volume of fresh brain heart infusion medium. After 3 cycles of freeze-thaw, the mixture was used for measurement of retained \( \beta \)-lactamase. For determination of \( \beta \)-lactamase activity, typically, a 100-\( \mu \)l reaction containing 30 \( \mu \)l of supernatant or lysed cells was incubated with 250 \( \mu \)M nitrocefin in phosphate-buffered saline buffer, pH 6.8, at 37 °C for 30–60 min. The absorbance at 482 nm (\( A_{482} \)) was measured at the beginning and end of the reaction. The secreted and the retained \( \beta \)-lactamase activities were calculated based upon \( A_{482} \).

RESULTS

Production and Purification of Lipoglycopeptides—To identify novel inhibitors of bacterial SPase I, 50,000 pre-fractionated natural product samples were screened in a high-throughput screen using the fluorimetric assay (26). This led to the identification of a single sample derived from a microbial source with reproducible activity. Further fractionation of limited amounts of this sample resulted in the identification of two isomeric components responsible for the activity. UV-visible and MS-based literature searches revealed that the active components might be novel. To fully characterize and study their antibiotic properties, we undertook a large scale fermentation. Fermentation followed by chromatographic purification resulted in the isolation of eight closely related lipoglycopeptides 1–8. The purity of compounds, except lipoglycopeptide 3 (92%), used for structural and biochemical studies exceeded 98% as determined by HPLC. The purity was estimated based on evaporative light scattering detector response (data not shown).

Structure Determination of Lipoglycopeptides—The two most abundant new isomeric lipoglycopeptides 1 and 2 showed identical molecular composition (C\(_{52}\)H\(_{78}\)N\(_{6}\)O\(_{16}\)) as determined by high resolution ESIMS (calculated 1043.5553 (M+H), observed 1043.5531 and 1043.5551, respectively). The structures were primarily deduced by NMR spectroscopy (Fig. 1). After trial and error, the optimum resolution of the amide proton signals of lipoglycopeptide 1 was observed at 10 °C in \( \mathrm{CD}_{3} \)OH solution. The NMR results of lipoglycopeptide 1 reported in Table I were obtained after a detailed analysis of 1H, 13C, DQCOSY, TOCSY, HSQC, and HMBC, which revealed the presence of four common amino acid residues, i.e. glycine, two alanines, and \( N \)-methylserine, in addition to two uncommon aromatic amino acid residues, i.e. a 3-substituted tyrosine and a 3,4,5-trisubstituted \( N \)-methylphenylglycine. Consistent with this observation, the amino acid analysis revealed the presence of glycine and alanine in a ratio of 1:2.

The other prominent features in the NMR spectra of lipoglycopeptide 1 included resonances for a 6-deoxy sugar and a fatty acyl chain. In accordance with the proposed molecular formula and MS/MS, the number of carbons in the acyl chain was determined to be 16 (Figs. 1 and 2). Furthermore, the NMR spectrum suggested that the acyl chain terminated with an isopropyl group in lipoglycopeptide 1 (\( \delta_{\mathrm{H}} \) 0.86, d, \( J = 6 \) Hz, 6

\[ \text{FIG. 3. Spectroscopic analysis of lipoglycopeptide. A, selected } 1H-\text{-}{ }^{13}C \text{ HMBC correlations of lipoglycopeptide 1. HMBC data were acquired as } 2048 \times 512 \text{ data points with } 52 \text{ transients per } t_1 \text{ increment. The } J \text{-filter was optimized for } 1J_{\text{C-H}} = 140 \text{ Hz and } \text{N bond delay was set to } 0.063 \text{ s corresponding to } 2J_{\text{C-H}} = 8 \text{ Hz. B, selected ROESY correlations of lipoglycopeptide 1. ROESY data were acquired as } 2048 \times 128 \text{ data points with } 32 \text{ transients per } t_1 \text{ increment. A mixing time of } 0.2 \text{ s was used. C, proton coupling constants and ROESY correlations of deoxy-} \alpha\text{-mannose. ROESY data were acquired as } 2048 \times 128 \text{ data points with } 32 \text{ transients per } t_1 \text{ increment. A mixing time of } 0.2 \text{ s was used. Proton coupling constants } 3J_{1,2} = 2 \text{ Hz, } 3J_{2,3} = 3 \text{ Hz, } 3J_{3,4} = 9 \text{ Hz, } 3J_{4,5} = 9 \text{ Hz, and } 3J_{5,6} = 6 \text{ Hz were measured directly from the one-dimensional spectra of lipoglycopeptides 1, 5, 9, and 10.} \]
protons) and with a normal methyl group in lipoglycopeptide 2 (δH 0.89, t, J = 7 Hz, 3 protons), which, in fact, is the only structural difference between 1 and 2. The HMBC cross-peaks observed from the amide carbonyl carbon (δ 176.9) of N-methylserine to the CH₂ protons (δ 2.44) of the acyl chain, N-CH₃ protons (δ 3.09) of N-methylserine, and α proton (δ 4.97) of the N-methylserine strongly suggested attachment of the long fatty acyl chain on the N terminus of N-methylserine (Fig. 3A). Likewise, the HMBC correlations observed from the sugar anomeric proton (δ 5.36) and the mutually coupled phenylglycine protons (δ 6.47 and 6.77) to the same carbon (δ 143.8, C-4 of N-methylphenylglycine) clearly indicated that the 6-deoxy-sugar unit was attached to the 4 position of the phenylglycine through a glycosidic linkage (anomeric carbon resonance at δ 103.9). The HMBC data (1³C–¹H correlations between the carbonyl and the adjacent amino acid amide proton and/or proton attached to the α carbon) established the linear amino acid sequence as N-methylserine-alanine-phenylglycine- (3,4,5-trisubstituted N-methylphenylglycine)-alanine- (3-substituted tyrosine) (Fig. 3A) and was confirmed by ROESY (correlations from the amide proton to the adjacent amino acid α-proton) data (Fig. 3B).

The molecular formula of lipoglycopeptides 1 and 2, C₅₂H₇₈N₆O₁₆, requires 17 degrees of unsaturation. The six amino acid carbonyls, one acyl group, one sugar unit, and two aromatic rings account for 16 of the 17 degrees of unsaturation. This suggested that the peptide is monocyclic. The HMBC correlations observed from H-2 of tyrosine to C-5 of N-methylphenylglycine and H-6 of N-methylphenylglycine to C-3 of tyrosine indicated that the two aromatic amino acids were linked via a carbon-carbon bond resulting in the 14-membered cyclic structure as shown in Fig. 1. This was further confirmed by a strong ROESY correlation observed between the H-2 of tyrosine and H-6 of N-methylphenylglycine (Fig. 3B). Significant fragments observed in the quadrupole/orthogonal time-of-flight mass spectrometry corroborated the gross structure assignment of lipoglycopeptide 1 by NMR spectroscopy (Fig. 2).

In regard to stereochemistry, amino acid analysis of lipoglycopeptide 1 revealed L- and D-configuration for the two alanines (34). The coupling constant analysis of the sugar protons and ROESY data established the identity of the deoxy sugar as deoxy-a-mannose (Table I, Fig. 3C). Thus, small coupling constants observed between H-1 and H-2, and H-2 and H-3 were consistent with equatorial/axial (H-1), equatorial (H-2), and axial (H-3) orientations for these protons. The large coupling constants observed between H-3 and H-4, and H-4 and H-5 were consistent with axial orientations for H-3, H-4, and H-5. Accordingly, in the ROESY spectrum, a strong correlation was observed between the 1,3-diaxially oriented H-3 and H-5 and no correlation was observed between H-1 (equatorial) and H-3 (axial). The absence of ROESY correlation between H-3 and H-1 clearly demonstrated equatorial orientation for H-1.

The related lipoglycopeptide 5 had the molecular formula C₅₀H₇₄N₆O₁₅ as deduced by the high resolution ESIIMS (calculated for 1027.5603 (M + H), observed 1027.5637), which differed from lipoglycopeptide 1 by one less oxygen. The ¹H NMR spectrum of lipoglycopeptide 5 overall contained resonances reminiscent of a lipoglycopeptide and was very similar to lipoglycopeptide 1, except that the aromatic ring of the N-methylphenylglycine residue was tri-substituted rather than tetra-substituted, indicating that lipoglycopeptide 5 is a dehydroxy analog of lipoglycopeptide 1 (Table II). Notably, the lack of the phenolic group ortho to the sugar substituent in lipoglycopeptide 5 resulted in the restoration of the sugar methyl to its normal frequency (δH 0.64 in 1 versus 1.12 ppm in 5).

The structure of the remaining lipoglycopeptides 3, 4, 6, 7, and 8 was suggested by the high resolution ESIIMS data shown in Table III and confirmed by ¹H NMR data (data not shown). Thus, the family of lipoglycopeptides obtained from the Strep-tomyces sp. can, in general, be classified into two major cores differing only in the oxidation state of the N-methylphenylgly-
They also inhibited S. pneumoniae (Fig. 1). The presence of two different peptide cores and the acyl chain that differs in chain length and degree of branching residue. The rest of the diversity stems from the nature of the acyl chain that differs in chain length and degree of branching. The presence of two different peptide cores and the attachment of the fatty acyl chain on the N terminus of N-methylserine were further confirmed by the trifluoroacetic acid-mediated hydrolysis of a mixture of lipoglycopeptides 7 and 8 to yield glycopeptides 9 and 10 (Fig. 1). The structures of glycopeptides 9 and 10 were fully supported by high-resolution mass measurement (Table III) and NMR data (Table IV). Specifically, in NMR spectra of glycopeptides 9 and 10, the N-methyl resonance of N-methylserine exhibited a significant up-field shift (−0.7 ppm) when compared with their acylated counterparts.

At the time of the completion of this work, a literature search revealed that this family of lipoglycopeptides is novel. Subsequently, we learned that structures of a similar family of lipopeptides, called arylomycins, have been disclosed in a conference proceeding (35) followed by a publication (36). The major structural difference is the absence of the sugar unit in arylomycins.

**Determination of Bacterial SPases I Activity**—As illustrated in Table V, all 8 compounds showed potent inhibitory activity against E. coli SPase I with IC$_{50}$ ranging from 0.11 to 0.19 µM. They also inhibited S. pneumoniae SPase I with IC$_{50}$ of 2.4–24.9 µM. The activity was further confirmed by the HPLC assay. As revealed by HPLC profiles, the intact peptide substrate has one peak with retention time of 5.45 min (Fig. 4A). After incubation with SPase I, the substrate was specifically cleaved, two products with retention times of 4.1 and 4.9 min, respectively, were generated (Fig. 4B). When a lipoglycopeptide was included in the reaction mixture, the cleavage of the peptide substrate was inhibited (Fig. 4C), thus confirming the inhibitory activity.

**Determination of Mechanism of Inhibition**—Kinetic analysis with lipoglycopeptides 2, 5, 7, and 8 by HPLC assay revealed competitive inhibition with $K_i$ ranging from 50 to 158 nM (Table V). Fig. 5 is an example of the Lineweaver-Burk plot for lipoglycopeptide 5. Analysis demonstrated that increasing substrate concentration proportionally reduced the inhibition of SPase I activity by lipoglycopeptide 5 as determined by velocity changes. Therefore, the mechanism of action of this compound is competitive with respect to the substrate. Similar kinetic analysis was also performed for compounds 2, 7, and 8, which also showed competitive mechanism (data not shown).

**Determination of Antibacterial Activity**—Table VI summarizes the antibacterial activity of these novel lipoglycopeptides against a panel of major human bacterial pathogens, including...
Gram-positive S. pneumoniae and S. aureus, and Gram-negative H. influenzae and E. coli. They exhibited moderate antibacterial activity with MIC of 4–8 μM against E. coli strain EL683, 8–32 μM against E. coli strain EL744, 8–64 μM against S. pneumoniae, 32–64 μM against S. aureus, and 64–64 μM against H. influenzae.

Inhibition of β-Lactamase Secretion in S. aureus—SPase I is responsible for processing many secreted proteins in both Gram-positive and Gram-negative bacteria and is essential for effective protein secretion. β-Lactamase is a well-characterized secretory protein that is processed by SPase I. The uncleaved leader sequence of β-lactamase acts as a membrane anchor and prevents release of the β-lactamase from membrane. After cleavage by SPase I, the mature β-lactamase is released and secreted to the growth medium. Before performing the β-lactamase secretion experiment, we screened a few S. aureus strains for β-lactamase production. We found that S. aureus ATCC33592 produced maximum β-lactamase and was most suitable for protein secretion study (data not shown). To test if these lipoglycopeptides mechanistically inhibit protein secretion within bacterial cells, lipoglycopeptides 1, 2, and 5 were tested in S. aureus ATCC33592. The results demonstrated that the cells treated with lipoglycopeptides secreted less β-lactamase in the medium in a dose-dependent manner when compared with the control cells, and conversely, the activity of β-lactamase retained within the cells treated with lipopeptide was slightly higher than the control (Fig. 6), suggesting that these SPase I inhibitors indeed inhibit protein secretion within the bacterial cells.

**DISCUSSION**

Proteases in general are divided into four classes according to their mechanism of action, and they are serine, cysteine, metallo-, and aspartyl proteases. However, recent investigations have unambiguously demonstrated that SPase I is not a member of any of these four traditional classes. For example, it is not sensitive to any of the standard protease inhibitors (2, 3). The catalytic mechanism of the bacterial SPase I has been studied by site-directed mutagenesis using E. coli enzyme (15, 16), Bacillus subtilis SipS (37), and S. pneumoniae enzyme (9). In all these cases, a conserved serine and a conserved lysine were identified to be critical for enzymatic activity. These results suggest that SPases belong to a novel class of serine proteases that utilize a serine and a lysine to form a catalytic dyad. This serine/lysine catalytic dyad structure has been recently confirmed by the structure of E. coli SPase I in complex with 5,6R-penem (38). This unique catalytic mechanism may explain the difficulty to identify effective SPase inhibitors and thus, to date, only β-lactams with moderate potency have been reported (19, 24, 25). In the present study, we introduce a novel class of lipoglycopeptides as potent inhibitors of SPase I. These peptides have shown competitive inhibition of SPase I, with Kᵢ ranging from 50 to 158 nM. Mechanistically, they blocked the bacterial protein secretion and inhibited the growth of both Gram-positive and Gram-negative bacteria. These favorable biochemical and physiological properties place this class of compounds as potential candidates for further development as effective novel antibiotics.

Our work has shown that these lipoglycopeptides inhibit SPase I from both E. coli and S. pneumoniae, but with different potency. In general, they are more potent against Gram-negative E. coli SPase I than Gram-positive S. pneumoniae enzyme (Table V). A similar difference in potency was also observed with a known β-lactam inhibitor, 5S,6S-penem, which had IC₅₀ of 4.67 and 733.4 μM against E. coli and S. pneumoniae enzymes, respectively, in our assay conditions. This difference in sensitivity of SPases to inhibitors may reflect some subtle structural variations between them. In fact, it is documented...
 Novel Lipoglycopeptides as Signal Peptidase Inhibitors

**TABLE VI**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. pneumoniae R6</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>8, 16</td>
</tr>
<tr>
<td>5</td>
<td>16, 8</td>
</tr>
<tr>
<td>6</td>
<td>&gt;64</td>
</tr>
<tr>
<td>7</td>
<td>&gt;64</td>
</tr>
<tr>
<td>8</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>12.5</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.04</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.33</td>
</tr>
</tbody>
</table>

* ND, not determined.

**FIG. 6. Effects of lipoglycopeptide 5 on β-lactamase secretion from S. aureus.** The selected inhibitor with the indicated concentrations was added to S. aureus cells, and incubated at 37 °C for 2 h. The A_{420} was measured at the end of the incubation (connected line). The cells were then centrifuged, and the supernatant was saved for determination of the secreted β-lactamase activities (white bars). The pellet was used for determination of retained β-lactamase activities (black bars) as described under “Experimental Procedures.”

that SPases from Gram-positive bacteria are generally one-third smaller than those from Gram-negative species (1). In future, detailed structural studies of SPases from Gram-positive bacteria may offer a better explanation why these enzymes are more difficult to inhibit.

Gene knock-out and other genetic experiments have shown that SPase I is an essential component for bacterial viability and growth in both Gram-positive and Gram-negative bacteria (5–8). Hence, inhibitors of SPase I should have antibacterial activity. Overall, the MIC values observed for the lipoglycopeptides against Gram-positive species, *S. pneumoniae* and *S. aureus* (8–64 µM), are in good correlation with the inhibition of *S. pneumoniae* SPase I (IC_{50} 2.4–24.9 µM). However, the MIC values observed against Gram-negative species, *E. coli* and *H. influenzae* (4–64 µM), are significantly higher than the enzyme inhibition of *E. coli* SPase I (IC_{50} 0.11–0.19 µM). The possible reason for this difference in potency is likely because of the outer membrane barrier that exists in Gram-negative bacteria. As we know, SPase I is located in the outside of the inner membrane of Gram-negative bacteria, and any potential SPase I inhibitor has to cross the outer membrane to access the active site of the SPase I. In our studies, we have demonstrated that modifying the permeability of *E. coli* outer membranes could indeed result in the improvement of antibacterial activity of these novel lipoglycopeptides. *E. coli* strain EL683 utilized in this study is an isogenic envA mutation of wild type *E. coli* K12. envA is a component involved in the biosynthesis of lipid A, the hydrophobic anchor of lipopolysaccharide, which makes up the outer monolayer of outer membrane. envA mutation lowers the lipopolysaccharide content of the outer membrane by 25–30%, and allows passage of large hydrophobic and hydrophilic molecules through the outer membrane (39). As shown in Table VI, the lipoglycopeptides showed MIC of 4–8 µM against *E. coli* EL683 strain, whereas these compounds had MIC >64 µM against the wild type *E. coli* K12 strain. Apparently, changing the outer membrane permeability of Gram-negative *E. coli* improved the antibacterial activity of these lipoglycopeptides. Therefore, the structure of these lipoglycopeptides needs to be optimized for better permeability to inhibit the growth of Gram-negative bacteria. In addition, these lipoglycopeptides also showed MIC of 8–32 µM against another *E. coli* strain, EL744. EL744 is an *E. coli* K12 isogenic strain in which tolC, a component of an outer membrane efflux pump, was deleted (40). This implies that an intrinsic resistance to these lipoglycopeptides may exist via a multidrug efflux system in *E. coli*. Further investigation to confirm this mechanism is needed.

From mechanism of action standpoint, the currently marketed antibiotics inhibit either bacterial cell wall formation or synthesis of essential macromolecules, such as DNA, RNA, proteins, or lipids. Use of these antibiotics over the past three decades has resulted in bacterial resistance and limited the effectiveness of these drugs to treat bacterial infections. Hence, there is an urgent need for new antibiotics with novel mechanisms of action to combat bacterial resistance and infection. In this study, we have demonstrated that these novel lipoglycopeptides are able to inhibit activity of bacterial SPase I, a key enzyme essential for bacterial growth. They also mechanistically block bacterial protein secretion in *vivo* and cause bacterial death. This consequence suggests that the killing mechanism of these lipoglycopeptides is highly likely by inhibiting SPase I activity and blocking protein secretion within bacterial cells. SPase I has long been considered as an attractive target for antibiotic development, and the data presented in this study has proved this concept by a SPase I specific inhibitor. Therefore, inhibition of bacterial SPase I and the protein secretion machinery indeed represents a new approach to develop novel antibacterial agents with previously unexploited mechanisms of action.

Several naturally occurring glycopeptides and lipopeptides and their analogs have been successfully marketed or are in late phase clinical development. Among them, vancomycin, teicoplanin, ramoplanin, and oritavancin act by inhibiting bac-
terial cell wall biosynthesis (41–43). The recently marketed daptomycin and related cyclic peptides are promising new antibiotics that act on the cytoplasmic membrane by inhibiting lipoteichoic acid biosynthesis (44, 45). Polymyxin, octapeptin, and related peptides are also membrane-acting antibiotics by interacting with lipopolysaccharide on the outer membrane of Gram-negative bacteria (46, 47). The present research encountered yet another class of lipoglycopeptides with distinct structural features and unique mechanisms of action from any of the previously known peptide antibiotics. Considering the success of peptides in antibiotic development, we feel that the present discovery offers hope for the development of a new class of antibiotics to combat bacterial resistance and infections.

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Novel Lipoglycopeptides as Inhibitors of Bacterial Signal Peptidase I

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