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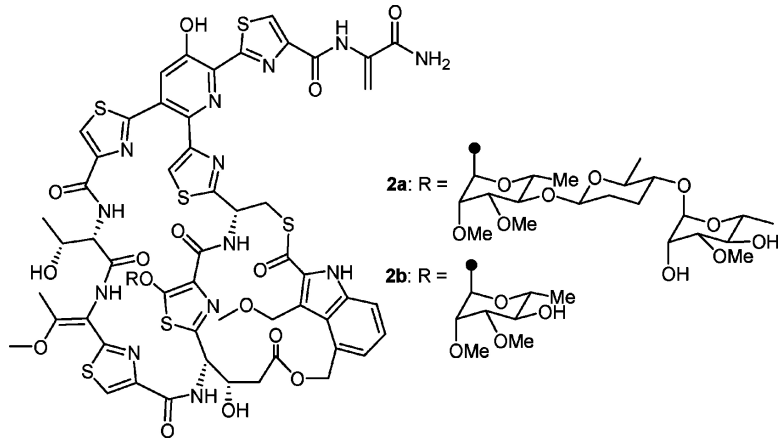

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Isolation, Structure, and Antibacterial Activity of Philipimycin, A Thiazolyl Peptide Discovered from *Actinoplanes philippinensis* MA7347

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Abstract: Bacterial resistance to antibiotics, particularly to multiple drug resistant antibiotics, is becoming cause for significant concern. The only really viable course of action is to discover new antibiotics with novel mode of actions. Thiazolyl peptides are a class of natural products that are architecturally complex potent antibiotics but generally suffer from poor solubility and pharmaceutical properties. To discover new thiazolyl peptides potentially with better desired properties, we designed a highly specific assay with a pair of thiazomycin sensitive and resistant strains of *Staphylococcus aureus*, which led to the discovery of philipimycin, a new thiazolyl peptide glycoside. It was isolated along with an acid-catalyzed degradation product by bioassay-guided fractionation. Structure of both compounds was elucidated by extensive application of 2D NMR, 1D TOCSY, and HRESIFT-MS/MS. Both compounds showed strong antibacterial activities against Gram-positive bacteria including MRSA and exhibited MIC values ranging from 0.015 to 1 μg/mL. Philipimycin was significantly more potent than the degradation product. Both compounds showed selective inhibition of protein synthesis, indicating that they targeted the ribosome. Philipimycin was effective *in vivo* in a mouse model of *S. aureus* infection exhibiting an ED_{50} value of 8.4 mg/kg. The docking studies of philipimycin suggested that a part of the molecule interacts with the ribosome and another part with Pro_{23}, Pro_{22}, and Pro_{26} of L11 protein, which helped in explaining the differential of activities between the sensitive and resistant strains. The design and execution of the bioassay, the isolation, structure, *in vitro* and *in vivo* antibacterial activity, and docking studies of philipimycin and its degradation product are described.

Introduction

The discovery of penicillin and other antibacterial agents of microbial origin to treat infections caused by pathogenic bacteria is arguably one of the greatest achievements of science and medicine in the mid twentieth century.[1,2] Incremental improvements to those discoveries led to development of new and improved antibiotics, which continue to serve humanity well. However, bacteria are adapting to these antibiotics, rendering them ineffective and leading to the emergence of resistance. While infections caused by Methicillin Resistant *Staphylococcus aureus* (MRSA) is rightfully well publicized and causing high degree of anxiety to all, concern over the emergence of antibiotic resistance by other bacteria such as *Pseudomonas aeriginosa* is perhaps more devastating.[3] Fortunately the frequency of resistance is quite variable among different bacterial types and the strains which are resistant to one antibiotic are currently susceptible to many others. However treatment options are continue to be limited. In order to treat drug resistant bacterial infections and avoid an epidemic, it is incumbent upon the scientific community to make new treatment options available by discovering new structural chemotypes those inhibit growth of bacteria by new modes of action (e.g., platensimycin[4,5] and platencin[6,7]).

Thiazolyl peptides are a class of naturally occurring antibiotics produced by filamentous prokaryotic organisms. The first of this class of compounds, micrococcin, was isolated in 1948 and the most studied member, thiostrepton, was first reported in 1954.[8]

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Many others include glycothiohexide α,[9,10] S54832A-I,[8] MJ347−81F4A and B[11] and nocathiacins.[12,13] These compounds are some of the most potent inhibitors of bacterial growth *in vitro* but they could not be advanced to clinic due to poor physicochemical properties, most notably low aqueous solubility. However, key attributes of this class of natural products—highly potent activity and new modes of action—remain very attractive and prompted us to revisit this area. Recent chemical advances enabled to make significant progress in structural modification of nocathiacins leading to the semisynthetic compounds with improved properties.[14−20] In order to find new thiazolyl peptides, we adopted two parallel approaches. In the first approach, we searched for the new compounds from the known producers of thiazolyl peptides using analytical methods, particularly LCMS after systematic manipulations of the seed and growth media. This led to the discovery of thiazomycin (**1**).[21] The second approach involved the use of a sensitive-resistant pair of strains of *S. aureus* grown on agar medium in two plates (two-plate assay). In this assay one plate was seeded with the wild type (sensitive) *S. aureus* strain and the second plate was seeded with well characterized thiazomycin resistant *S. aureus* strain. The resistance was mapped to the deletion of four amino acids (Pro_{23}-Val_{24}-Gly_{25}-Pro_{26}) in the *rplK* gene encoding L11 protein which resulted in greater than 500 fold increase in MIC of thiazomycin.[22] The two-plate assay allowed screening for inhibitors that exert their activity by binding at the thiazomycin binding site of L11 protein. The screening of microbial extracts, as part of our screening program, led to the identification of an extract that showed a large zone of clearance on the wild-type *S. aureus* plate and a very small zone of clearance on the plate containing the thiazomycin resistant *S. aureus* plate indicating the presence of inhibitors whose activity would be mechanistically similar to thiazomycin. The LCMS analysis indicated the absence of known thiazolyl peptides. This extract was produced by an *Actinoplanes philippinensis* strain and was selected for bioassay-guided isolation of the active compound that allowed the discovery of a new thiazolyl peptide, philipimycin (**2a**). The isolation, structure elucidation and antibacterial activity including *in vivo* activity of philipimycin and its acid degradation product are described.

Results and Discussion

The design of the bioassay consisted of seeding control *S. aureus* strain MB2865 on one agar plate and thiazomycin resistant *S. aureus* strain MB5832 on another. The actives that exert their activity by binding to thiazomycin binding site would be more active against the control strain and form larger zone of clearance compared to the plate containing the resistant strain. The compounds that inhibit bacterial growth by different mechanisms would show no differential in their zone of clearance between the two plates (see for example, Supporting Information Figure S1a). Aliquots of the acetone quenched aqueous extracts of the fermentation broths were tested in this assay and actives were scored by measuring zone differentials. The extract of *Actinoplanes philippinensis* MA7347 exhibited zone differential in a dose dependent manner starting from 0.25 to 1 mL of the whole broth. Spotting of 20 μL aliquot of these extracts at different dilutions showed 12−14 mm zone of clearance on the control plate and no zone of clearance on the plate containing the resistant strain.

Philipimycin (**2a**) producing strain MA7347 was isolated from a soil sample collected in Namaqualand, South Africa (see Supporting Information). The strain, initially identified as a member of the genus *Actinoplanes* on the basis of morphological and chemotaxonomic characteristics, was confirmed as a new strain of the species *Actinoplanes philippinensis* by 16S rDNA phylogenetic analysis (Supporting Information Figure S2). Two strains of this culture have been studied for secondary metabolite production and shown to produce unrelated compounds. Madumycin II (A 2315A) was reported[23] from *A. philippinensis* NRRL 5462 and isohematinic acid was reported from *A. philippinensis* SANK 61681.[24,25] Both of these compounds are shown to have modest antibacterial activities. The strain MA7347 was cultured in shake flasks in submerged conditions for 13 days and extracted with equal volume of acetone. Acetone was removed under *vacuo* after filtration and chromatographed on Amberchrome, a reversed-phase resin, and eluted with an aqueous methanol gradient followed by an acetone wash. The fractions were analyzed in the two-plate assay. The activity was concentrated in the fractions eluting in 100% methanol and acetone wash. These fractions were pooled and analyzed by reversed-phase HPLC which showed a major peak for philipimycin (**2a**). However, chromatography of this fraction on C_8 reversed-phase HPLC using aqueous CH_3CN containing of 0.05% TFA led to clean degradation of philipimycin to the truncated product **2b** which was further purified by a second reversed-phase HPLC to yield 1.5 mg from 1/4th of the original Amberchrome fraction. This acid catalyzed degradation was circumvented by performing chromatography under neutral conditions. Thus remaining 3/4th of the Amberchrome fraction

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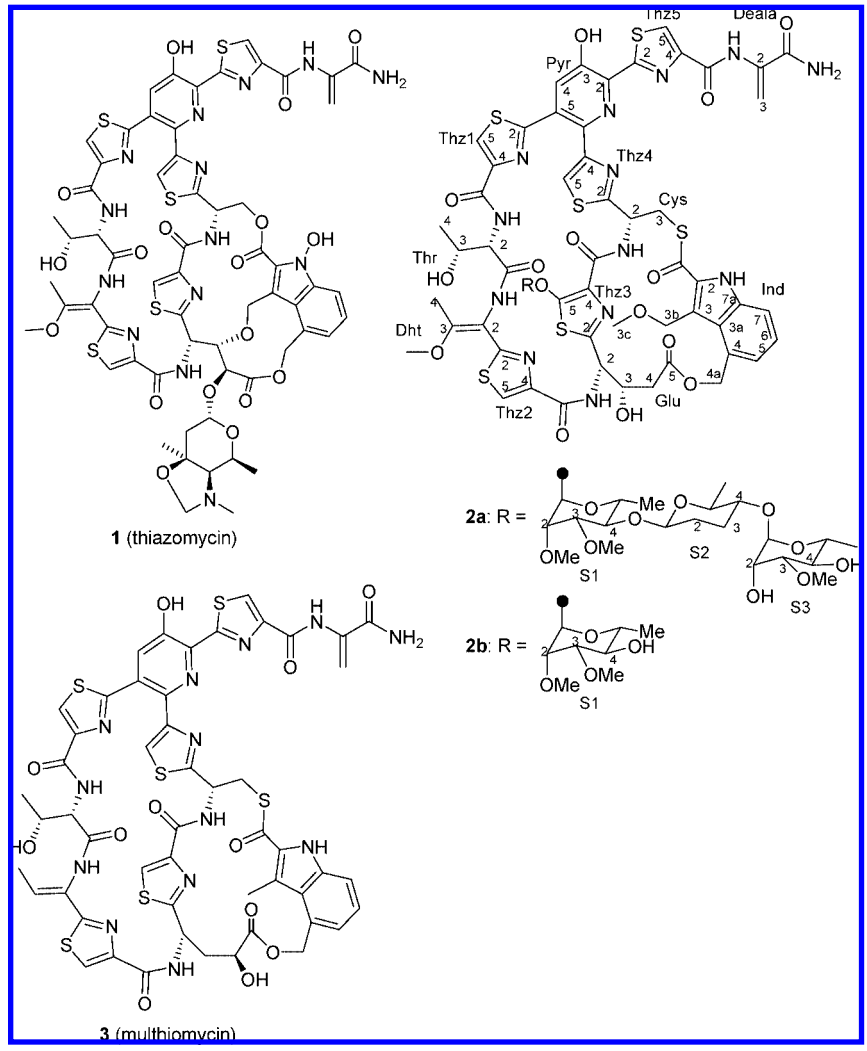

was chromatographed on C_8 reversed-phase HPLC under neutral condition to afford 9.4 mg of philipimycin (12.5 mg/L).

HRESIFTMS analysis of **2a** and **2b** afforded protonated molecular ions at *m*/*z* 1746.4058 and *m*/*z* 1472.2584 and suggested molecular formulas $C_{74}H_{83}N_{13}O_{25}S_6$+H and $C_{61}H_{61}N_{13}O_{19}S_6$+H, respectively. The molecular formula differential of **2a** and **2b** indicated a loss of $C_{13}H_{22}O_6$. The UV spectrum of both compounds was identical and displayed absorption maxima at λ_{max} 259, 306 and 350 nm analogous to the UV spectrum of thiazolyl peptides. The ^{13}C NMR spectra of **2a** and **2b** (Table 1) in $CDCl_3-CD_3OD$ (9:1) corroborated the molecular formula and also confirmed the presence of the thiazolyl peptide core. This mixture of solvents (other solvents used were DMSO-d_6 and C_5D_5N) turned out to be the best for the line shape and resolution of resonances both in ^1H and ^{13}C NMR spectra.

Analysis of ^1H and ^{13}C NMR spectra of smaller of the two compounds, **2b**, along with DEPT and HSQC indicated the presence of 61 carbons comprised of 10 sp^3 methines all substituted with hetero atoms, 8 sp^2 methines, 3 sp^3 methylenes all substituted with hetero atoms, a sp^2 methylene, 4 methoxy and 3 methyl groups with remaining carbons being quaternaries and resonating in the olefinic region of the ^{13}C spectrum. The spectrum showed the presence of resonances for three carboxy thiazoles, a fully substituted carboxy thiazole, a thiazole, a tetrasubstituted hydroxy pyridine (Pyr), a threonine (Thr), a methoxy dehydro-threonine (Dht), a dehydroalanine (Deala), a 2,3,4-trisubstituted indole (Ind), a dihydroxy-glutamic acid (Glu) and a modified cysteine (Cys) suggesting the presence of structural core of multhiomycin (**3**, nosiheptide) and not thiazomycin (**1**). The presence of the cysteine residue was confirmed by a $\Delta\delta$ 19.8 ppm downfield shift of the indole carboxyl carbonyl (δ_C 180.7) (ACD prediction δ_C 182.6) and a $\Delta\delta$ 34.9 ppm upfield shift of the methylene carbon (δ_C 29.4) (ACD prediction δ_C 29.5) compared to thiazomycin indicating the presence of a thiolactone ring similar to multhiomycin. The HMBC correlations of the Cys methylene protons to the indole carboxyl and C-2 of Thz4 confirmed the connectivity of Cys to Ind (via thiolactone) and Thz4 (Figure 1 and Table 1). The COSY correlation of the α-proton (δ_H 5.05) of the glutamic acid with the oxymethine proton (δ_H 3.95) placed the hydroxy group at the β-position (C-3) of the glutamic acid. The connectivity of the Glu moiety to Thz3 and indole was confirmed by the HMBC correlations of Glu methine proton to Thz3 C-2 (δ_C 154.9) and the methylene protons (H_2-4) of the indole to the lactone carbonyl C-5 of Glu. The HMBC correlations of the H_2-3b methylene protons (δ_H 4.86) to C-2, C-3 and C-3a of the indole moeity and the methoxy group resonating at δ_C 57.8 (δ_H 3.11) established a methoxy methyl substitution at C-3 of the indole and confirmed the lack of cyclization between C-3 indole and Glu. The COSY and 2D TOCSY correlations indicated the presence of a deoxy-hexose residue (S1) that was confirmed by corresponding HMBC correlations (Figure 1 and Table 1). Two methoxy protons

Table 1. ^{1}H (500 and 600 MHz) and ^{13}C (125 and 150 MHz) NMR Assignments of Philipimycin (**2a**) and Truncated Analog **2b** in $CDCl_3$–CD_3OD (9:1)

		2a			2b				
C#	type	δ_C	δ_H (mult, *J* in Hz)	gCOSY	gHMBC (H → C)	δ_C	δ_H (mult, *J* in Hz)	DQF-COSY	gHMBC (H → C)
Thz1-C2	C^o	164.5				164.6			
Thz1-C4	C^o	150.0				150.3			
Thz1-C5	CH	125.5	8.42, s		Thz1 (C4, C=O, C2)	125.5	8.44, s		Thz1 (C4, C=O, C2)
Thz1-CO	C=O	161.3				161.2			
Thz2-C2	C^o	162.1				162.2			
Thz2-C4	C^o	145.7				145.8			
Thz2-C5	CH	124.3	7.90, s		[a]Dht-C-2, Thz2 (C4, C2, C=O)	124.1	7.93, s		Thz2 (C4, C2)
Thz(2)-CO	C=O	160.6				160.5			
Thz3-C2	C^o	155.1				154.9			
Thz3-C4	C^o	131.3[b]				131.1[b]			
Thz3-C5	C^o	161.2				161.4			
Thz3-CO	C=O	160.3				ND[c]			
Thz4-C2	C^o	169.8				169.8			
Thz4-C4	C^o	154.5				154.6			
Thz4-C5	CH	120.3	7.66, s		Pyr-C6, Thz4 (C4, C2)	120.4	7.69, s		Pyr-C6, Thz4 (C4, C2)
Thz5-C2	C^o	169.4				169.5			
Thz5-C4	C^o	149.5				149.7			
Thz5-C5	CH	126.2	8.25, s		[a]Pyr-C2, Thz5 (C4, C=O, C2)	126.3	8.29, s		Thz5 (C4, C=O, C2)
Thz5-CO	C=O	158.4				159.0			
Pyr-C2	C^o	134.1				134.4			
Pyr-C3	C^o	151.2				151.3			
Pyr-C4	CH	127.0	7.60, s		Pyr (C2, C3, C6), Thz1-C2	127.1	7.64, s		Pyr (C2, C3, C6), Thz1-C2
Pyr-C5	C^o	130.4				130.4			
Pyr-C6	C^o	143.7				143.9			
Thr-CO	C=O	167.9				168.0			
Thr-NH	NH		7.90, d, 7.5	Thr-H2	Thr-C=O		8.00, d, 7.4	Thr-H2	
Thr-C2	CH	56.6	4.50, dd, 7.1, 4.0	Thr-H3, Thr-NH	Thr (C4, C3, C=O)	56.7	4.59, dd, 7.4, 3.9	Thr-H3, Thr-NH	Thr (C4, C3, C=O)
Thr-C3	CH	66.6	3.47, m	Thr-H4, Thr-H2	Thr-C=O	66.7	3.58, qd, 6.2, 3.8	Thr-H4, Thr-H2	
Thr-C4	CH_3	17.6	1.04, d, 6.3	Thr-H3	Thr (C2, C3)	17.6	1.09, d, 6.1	Thr-H3	Thr (C2, C3)
Dht-NH	NH	–			–				
Dht-C2	C^o	110.0				110.2			
Dht-C3	C^o	159.9				160.0			
Dht-C4	CH_3	13.4	1.95, s		Dht (C2, C3), [a]Thz2-C2	20.4	1.98, s		Dht (C2, C3)
Dht-OMe	CH_3	55.6	3.80, s		Dht-C3	55.8	3.84, s		Dht-C3
Glu-NH	NH		8.07, d, 9.6	Glu-H2	Thz2-C=O		8.16, d, 9.5	Glu-H2	
Glu-C2	CH	53.7	5.01, dd, 9.2,7.3	Glu-NH, Glu-H3	Glu-C3, Thz3-C2 Thz2-C=O	53.6	5.05, dd, 9.5, 6.5	Glu-NH, Glu-H3	Glu-C3, Thz3-C2 Thz2-C=O
Glu-C3	CH	71.1	3.95, m	Glu-H2, Glu-$H4_2$	Thz3-C2, Glu-C5	71.5	3.95, ddd, 2.1, 6.6, 11.6	Glu-H2, Glu-NH	Thz-C2
Glu-C4	CH_2	39.9	2.34, dd, 12.0, 13.0	Glu-H3	Glu (C3, C5)	40.0	2.39, dd, 11.5, 13.5	Glu-H3	Glu (C3, C5)
			2.52, dd, 13.0, 2.0				2.54, dd, 2.0, 13.5		
Glu-CO	C=O	170.2				170.4			
Ind-NH			10.41, s		Ind-(C2, C3, C3a)				
Ind-C2	C^o	131.3				131.4			
Ind-C3	C^o	117.6				117.7			
Ind-C3b	CH_2	64.3	4.83, d, 10.9 4.84, d, 10.9		Ind-(C2, C3, C3a)	64.3	4.86, brs		Ind-(C2, C3, C3a, C3c)
Ind-C3c	CH_3	57.4	3.10, s		Ind-C3b	57.8	3.11, s		Ind-C3b
Ind-C3a	C^o	124.7				124.7			
Ind-C4	C^o	129.4				129.6			
Ind-C4a	CH_2	66.6	5.41, d, 11.4		Ind-(C5, C3a, C4), Glu-C5	66.6	5.50, d, 11.5		Ind-(C5, C3a, C4), Glu-C5
			5.50, d, 11.4		Ind-(C5, C4), Glu-C5		5.53, d, 11.5		
Ind-C5	CH	124.0	7.13, d, 7.0	Ind-H6	Ind-(C4a, C7, C3a)	124.4	7.18, d, 7.0	Ind-H6	Ind-(C4a, C7, C3a)
Ind-C6	CH	125.3	7.32, dd, 7.2, 8.5	Ind-H5, Ind-H7	Ind-(C5, C4, C7a)	125.4	7.36, dd, 7.0, 8.4	Ind-H5, Ind-H7	Ind-(C5, C4, C7a)
Ind-C7	CH	114.4	7.86, d, 8.5	Ind-H6	Ind-(C5, C3a)	114.5	7.90, d, 8.4	Ind-H6	Ind-(C5, C3a)
Ind-C7a	C^o	137.3				137.4			
Ind-CO	C=O	180.7				180.9			
Cys-NH	NH		7.40, d, 10.4	Cys-H2	Cys-C2, Thz3-C=O		7.45, d, 10.4	Cys-H2	
Cys-C2	CH	49.5	5.90, ddd, 10.4, 4.2, 2.4	Cys-$H3_2$, Cys-NH		49.1	5.93, ddd, 10.4, 4.5, 2.0	Cys-$H3_2$, Cys-NH	Cys-C3
Cys-C3	CH_2	29.4	3.59, m	Cys-H2	Ind-C=O	29.4	3.64, dd, 14.3, 1.8	Cys-H2	Ind-C=O, Thz4-C2
			4.02, dd, 14.1, 4.7		Ind-C=O, Cys-C2, Thz4-C2		4.07, dd, 13.8, 4.4		
Deala-NH_2	NH_2								
Deala-CO	C=O	165.9				165.2			
Deala-C2	C	132.7				132.7			
Deala-C3	CH_2	104.0	5.59, brs		Deala-C=O	104.0	5.60, brs		Deala-(C2, C=O)
			6.53, brs		Deala-(C2, C=O)		6.59, brs		
Deala-NH	NH		9.70, s		Thz5-C=O, Deala-C3				
S1–C1	CH	101.8	5.41, d, 1.5	S1–H2	Thz3-C5, S1-(C5, C2, C3)	102.2	5.46, d, 1.6	S1–H2	Thz3-C5, S1-(C5, C2, C3)
S1–C2	CH	75.8	4.00, dd, 2.0,3.2	S1–H1, S1–H3	S1-(C2-OMe, C4, C3, C1)	75.4	4.05, dd, 1.7, 3.1	S1–H1, S1–H3	S1-(C2-OMe)

Table 1. Continued

	2a				2b			
C#	type	δ_C	δ_H (mult, J in Hz)	gCOSY	gHMBC (H → C)	δ_C	δ_H (mult, J in Hz)	DQF-COSY	gHMBC (H → C)
S1−C2−OMe	CH_3	59.1	3.50, s		S1−C2	59.4	3.54, s		S1−C2
S1−C3	CH	80.0	3.60, m	S1−H2, S1−H4		80.0	3.56, dd, 3.0, 9.0	S1−H2, S1−H4	
S1−C3−OMe	CH_3	57.6	3.43, s		S1−C3	57.4	3.49, s		S1−C3
S1−C4	CH	76.7	3.62, m	S1−H3, S1−H5		70.9	3.54, t, 9.0	S1−H3, S1−H5	
S1−C5	CH	69.8	3.63, m	S1−C5-Me, S1−H4		70.9	3.67, qd, 6.2, 9	S1−C5-Me, S1−H4	
S1−C5-Me	CH_3	17.3	1.26, d, 5.3	S1−H5	S1-(C5, C4)	17.2	1.30, d, 6.2	S1−H5	S1-(C5, C4)
S2−C1	CH	101.9	4.62, dd, 9.0, 1.8	S2−H2$_2$	S2−C2, S1−C4				
S2−C2	CH_2	30.4	1.42, m	S2−H1, S2−H3$_2$	S2-(C4, C1)				
			1.81, m						
S2−C3	CH_2	29.3	1.44, m	S2−H2$_2$, S2−H4	S2-(C4, C1,C5)				
			2.02, m						
S2−C4	CH	79.8	3.08, dt, 4.6, 9.4	S2−H3, S2−H5	S2−C5-Me				
S2−C5	CH	74.0	3.25, m	S2−H4, S2−C5-Me	S2-(C3, C4, C1)				
S2−C5-Me	CH_3	17.8	1.12, d, 6.0	S2−H5	S2-(C5, C4)				
S3−C1	CH	101.5	4.74, d, 1.5	S3−H2	S3-(C2, C5), S2−C4				
S3−C2	CH	66.8	3.91, dd, 3.5, 1.5	S3−H1, S3−H3	S3-(C4, C3)				
S3−C3	CH	80.6	3.22, dd, 3.4, 9.3	S3−H2, S3−H4	S3-(C3-OMe, C2, C4)				
S3−C3−OMe	CH_3	56.7	3.38, s		S3−C3				
S3−C4	CH	71.0	3.37, t, 9.6	S3−H3, S3−H5	S3-(C5-Me, C5)				
S3−C5	CH	68.3	3.61, m	S3−C5-Me, S3−H4					
S3−C5-Me	CH_3	17.0	1.18, d, 6.1	S3−H5	S3-(C5, C4)				

[a] Four bond correlation. [b] By integral only. [c] Not detected.

showed HMBC correlations to C-2 and C-3 of S1 confirming it as a 2,3-dimethoxy sugar. The configuration of the hexose was determined by measurement of scalar coupling. Since many spin systems showed significant overlap a 1D TOCSY experiment was used to isolate the spin systems that allowed precise measurement of scalar couplings. 1D TOCSY with selective excitation of the well-resolved methyl doublet (δ_H 1.26, J = 5.3 Hz) allowed isolation and coupling value measurement of H-5, H-4, and H-3 (see for example, Supporting Information). A 9 Hz coupling of H-4 with H-3 and H-5 suggested that all three protons were axial in pyranosyl conformation. H-2 showed small couplings both with H-3 (J = 3.1 Hz) and anomeric proton (J = 1.6 Hz) indicating that both H-1 and H-2 are equatorially oriented which was corroborated by NOESY correlations of H-1 to OMe at C-2. This configuration suggested that the sugar is a 2,3-dimethoxy-α -L-rhamnopyranoside. The anomeric proton showed an HMBC correlation to Thz3 C-5 (δ_C 161.4) establishing a glycosidic linkage to Thz3. The oxygen substitution at C-5 leads to an upfield shift of C-4 of Thz3 to a value of δ_C 131.1. The chemical shift values observed for C-4 and C-5 of Thz3 are consistent with the chemical shift values predicted by the ACD ^{13}C NMR shift prediction software. The predicted values were δ_C 159.1 for C-5 and δ_C 134.86 for C-4. The relative configuration of the asymmetric centers was determined by measurement of scalar couplings, NOESY, ROESY correlations, and comparison of the NMR spectra with nocathiacin I and thiazomycin and the data presented in Table 1.

HRESIFTMS analysis of **2b** showed a protonated moleular ion at *m*/*z* 1472 (**2b-1**) with likely protonation of the weakly basic nitrogen of indole (Figure 2). The molecule undergoes two distinct fragmentation pathways. In the first pathway, it loses a molecule of methanol from the indole unit and produces a significant fragment ion at *m*/*z* 1440 (**2b-2**). In the second, minor fragmentation pathway, it loses the sugar unit first producing a fragment ion at *m*/*z* 1298 (**2b-3**). A fragment ion at *m*/*z* 1266 (**2b-4**) was observed which could be derived from either of the ions *m*/*z* 1440 and *m*/*z* 1298. The *m*/*z* 1266 ion loses the Deala unit and produces a minor fragment ion at *m*/*z* 1197 (**2b-5**). No further discernible fragment ions were observed. The fragmentation pattern further helped in establishing the structure **2b**.

Comparison of the molecular formula of **2a** and **2b** indicated that **2a** contained an additional $C_{13}H_{22}O_6$ unit. The mass spectrum of **2a** produced fragment ions at *m*/*z* 1472 (**2b-1**) and *m*/*z* 1440 (**2b-2**), the same ions observed from **2b**, due to the loss of the additional unit present in **2a** suggesting that this unit is likely attached at the end. The ^{13}C NMR spectrum of **2a** displayed the presence of all the carbons observed in the ^{13}C spectrum of **2b** along with 13 additional carbons including 2 anomeric carbons, 6 oxy-methines, 2 aliphatic methylenes, 2 aliphatic methyls and a methoxy group (Table 1). This data together with COSY, 2D TOCSY and HSQC data suggested that the additional unit was a deoxy disaccharide leading to elucidation of a 2,3-dideoxy sugar (S2) and an additional unit of 3-methoxy rhamnose (S3). The anomeric proton of S2 (δ_H 4.62, dd, J = 9.0, 1.8 Hz) showed strong HMBC correlation to C-4 of S1 (δ_C 76.7) suggesting a 1,4- linkage. Similarly the anomeric proton of S3 (δ_H 4.74, d, J = 1.5 Hz) exhibited strong HMBC correlation to C-4 of S2 (δ_C 79.8) thus establishing a 1,4-linkage. The S2 anomeric proton appeared as a doublet of doublets with J = 9 and 1.8 Hz indicating that this proton was axially oriented and the deoxy sugar formed a β-linkage. S2−H4 appeared as a double-triplet with J = 4.6 and 9.4 Hz, respectively. The large vicinal couplings of S2−H4 with S2−H3

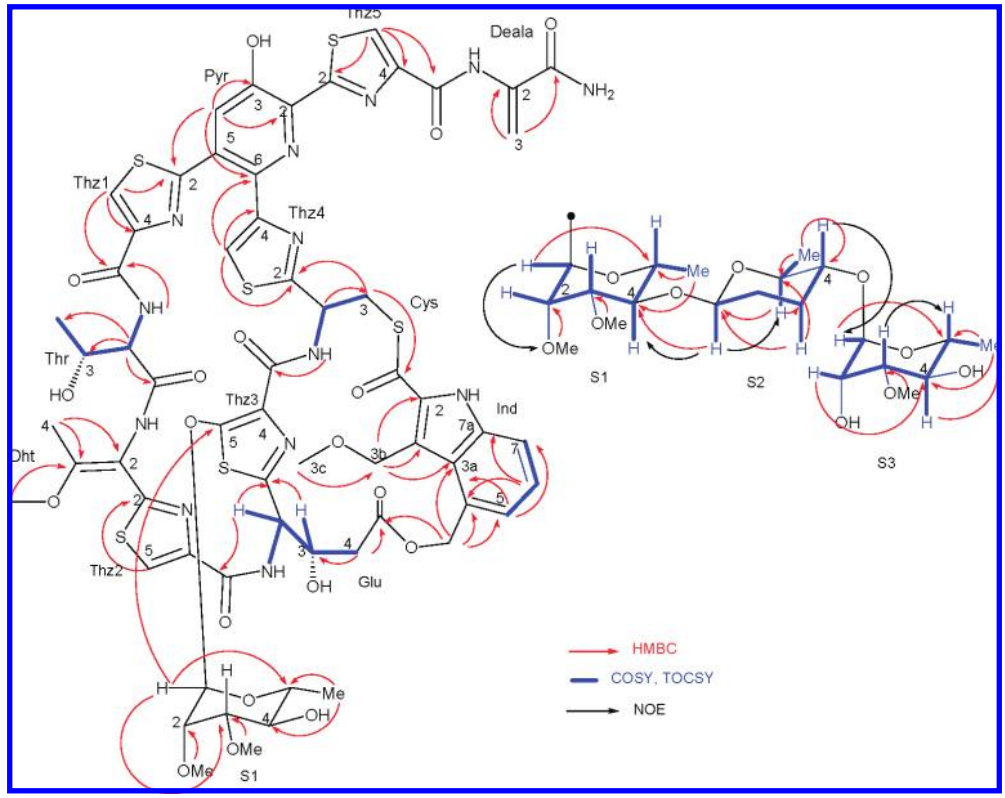

Figure 1. Selected HMBC, COSY, TOCSY, and NOESY correlations of **2a** and **2b**.

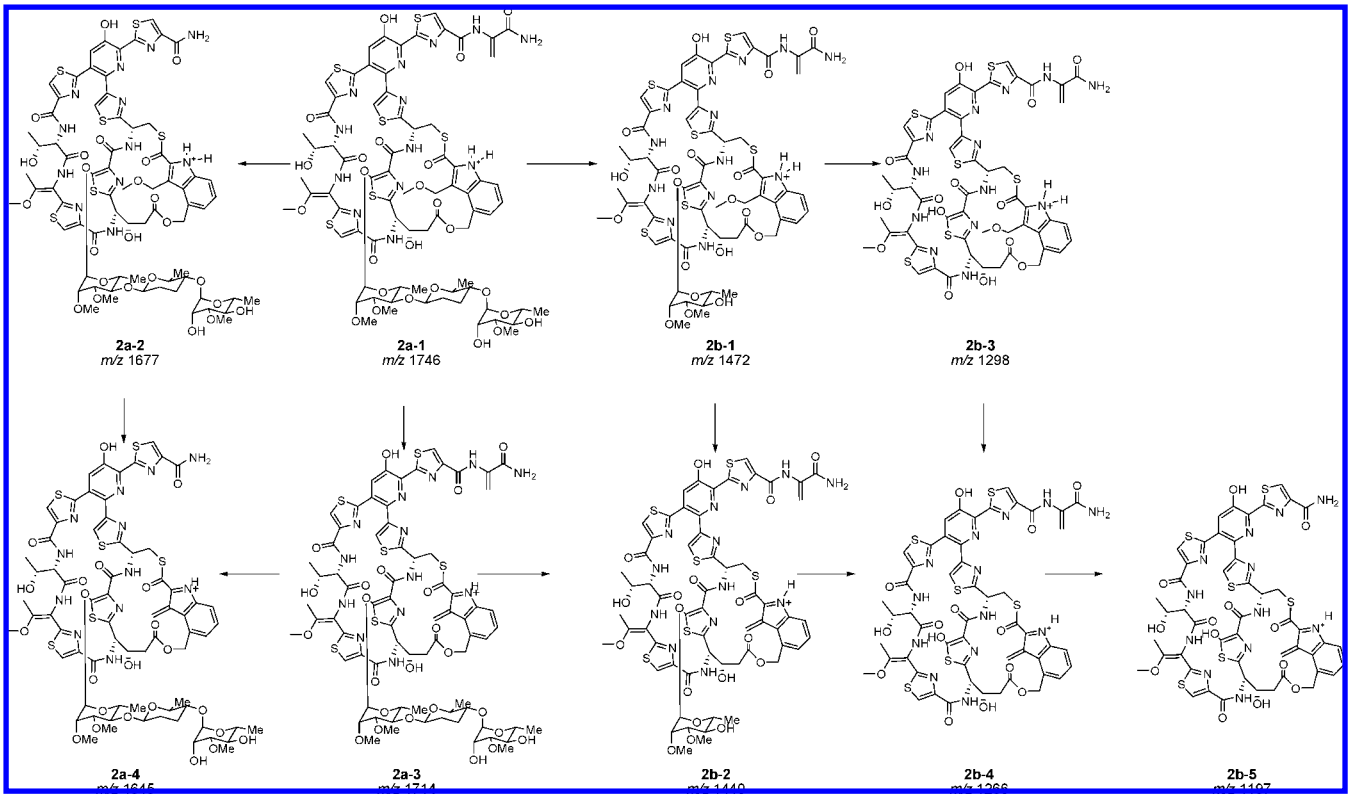

Figure 2. ESI mass spectral fragmentations of **2a** and **2b**.

and S2−H5 suggested that these three protons were axial. The S3 anomeric proton appeared as a doublet with small ($J = 1.5$ Hz) coupling indicating that S3 formed a α-linkage like S1. The vicinal coupling constants observed between S3 protons established this as a rhamnose similar to S1. Chair conformations of the sugar residues were confirmed by NOESY correlations of nonoverlapping 1,3-diaxial protons (S2−H1 and S2−H5 as well as S3−H3 and S3−H5) (Figure 1).

Table 2. Microbiological Profile (MIC) of Philipimycin (**2a**) and **2b**[a]

organisms	phenotype	strain #	2a	2b
S. aureus	$meth^S$	MB2865	0.015−0.03	1
S. aureus+50% human serum	$meth^S$	MB2865	0.5	>2
S. aureus (MRSA)	MRSA, mac^R, imp^R	MB5393	0.125	1
S. aureus	$Thiaz^R$	MB5832	>32	>32
S. pneumoniae	pen^S, $quin^S$, mac^S	CL2883[b]	≤0.015	0.06
S. pneumoniae	pen^S, $quin^S$, mac^S	CL2883[c]	≤0.015	1
S. pyogenes	Wt	CL10440	0.075	0.06
E. faecalis	van^S, mac^R	CL8516	≤0.015	0.5
E. faecium	van^R, lin^R, mac^R	CL5791	0.03	0.12
H. influenzae	Amp^S, $quin^S$, mac^S	MB4572	>16	>64
E. coli	Wt	MB2884	>16	>64
E. coli	envA1, tolC	MB5746	>16	>64
C. albicans	Wt	MY1055	>16	>64

[a] All strains were tested in Cation adjusted Mueller Hinton Broth (CAMHB) medium unless mentioned other wise. [b] Medium: CAMHB +2.5% Lysed Horse Blood. [c] Medium: Isosenitest.

The ESIFTMS analysis of **2a** produced three fragmentation pathways each with equal efficiency (Figure 2). First, the loss of the terminal disaccharide moiety produced ion **2b-1**(identical to the molecular ion of **2b**); second, the loss of methanol led to the fragment ion **2a-3** and the third, the loss of Deala residue produced the fragment ion **2a-2**. These ions went through further fragmentation to produce fragment ions shown in Figure 2. The ion at *m*/*z* 1266 (**2b-4**) was the most abundant ion observed in the mass spectrum of both compounds. Based on these data structure **2a** was assigned for philipimycin.

Biological Activities. Philipimycin (**2a**) showed a large zone of clearance in the thiazomycin sensitive-resistance pair two-plate assay displaying a 10−16 mm zone of clearance on the wild-type *S. aureus* plate and none to small (6−8 mm) zone of clearance on the thiazomycin resistant *S. aureus* plate at a concentration of 10−100 μg/mL and the activity was similar to thiazomycin (Supporting Information Figure S1b). Philipimycin (**2a**) exhibited potent growth inhibitory activities against Gram-positive bacterial strains. It inhibited the growth of *S. aureus* with minimum inhibitory concentration (MIC) value of 0.015−0.03 μg/mL (Table 2). The activity was somewhat affected by addition of 50% human serum in the media but the MIC value was maintained at the susceptible level (0.25−0.5 μg/mL). It was also effective against MRSA (MIC = 0.125 μg/mL). As expected it was not active (MIC > 16 μg/mL) against thiazomycin resistant *S. aureus* strain (MB5832) (also see Figure S1b, Supporting Information). This compound showed better potencies against *Streptococcus pneumoniae* and *Enterococcus faecalis*: each exhibited an MIC 0.015 μg/mL. The MIC against *S. pyogenes* was 0.075 μg/mL. Philipimycin exhibited very good activity against vancomycin, linezolid, and macrolide resistant *E. faecium* (MIC = 0.03 μg/mL). The truncated compound **2b** was less active across the board and exhibited MIC values of 1.0 μg/mL (*S. aureus*, MRSA), 0.06−1.0 μg/mL (*S. pneumoniae*), 0.06 μg/mL (*S. pyogenes*), 0.5 μg/mL (*E. faecalis*) and 0.12 μg/mL (*E. faecium*) and >16 μg/mL (thiazomycin resistant *S. aureus*). The *in vivo* efficacy of philipimycin was evaluated in a *S. aureus* infection (MB 2865, Smith strain) mouse model. In this model, **2a** showed modest efficacy exhibiting a 1.72 log reduction in colony forming units of kidney burden compared to control when administered at 10 mg/kg by subcutaneous route, resulting in an ED_{50} value of 8.4 mg/kg. Compound **2b** was not tested in the *in vivo* assay. To determine the mechanism of action at a broader level both compounds were tested for their ability to inhibit the synthesis of major macromolecules that are essential for bacterial growth in a whole cell assay. In this whole cell assay both **2a** and **2b** selectively inhibited protein synthesis in *S. aureus* EP167 with IC_{50} values of 0.063 and 0.25 μg/mL, respectively, and did not show inhibition of RNA, DNA, cell wall and phospholipids (Figure 3). Similarities of the IC_{50} values for protein synthesis and MIC values of the bacterial growth inhibition suggest that protein synthesis is likely the sole mechanism by which these compounds inhibit bacterial growth.

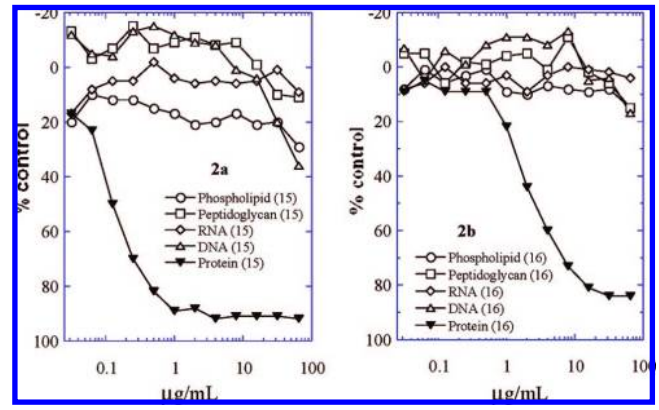

Figure 3. Whole cell labeling assay of **2a** and **2b** in *S. aureus*.

The lack of activity against thiazomycin resistant *S. aureus* (see Figure S1b, Supporting Information) indicates that these compounds bind at the same L11 and ribosome binding site where nocathiacins and thiazomycin bind. Most of the other thiazolyl peptides (e.g., thiostrepton and multhiomycin) showed equally strong cross resistance to thiazomycin resistant strain with MIC shift of 80 fold (thiostrepton; 0.06 vs 0.5 μg/mL) and >16000 fold (multhiomycin; 0.0019 vs >32 μg/mL). The extent of the MIC differences of thiazomycin and multhiomycin were very similar. This observation suggests that the mode of binding of thiazomycin, multhiomycin and philipimycin to L11 is much more similar to each other than to thiostrepton.

Molecular Modeling Studies. Docking of four compounds **1**, **2a**, **2b** and nocathiacin I (Supporting Information, Figure S3) was made into the L11/ribosome interface in reference to the NMR structure of the thiostrepton/L11/RNA complex (pdb2jq7 and pdb2ny0).[26] Because the structures of **1**, **2a**, **2b**, and just nocathiacin I are similar to the structure of thiostrepton, an assumption was made that the binding of compounds **1**, **2a**, **2b** and nocathiacin I to L11 and RNA would be similar to

(26) Jonker, H. R.; Ilin, S.; Grimm, S. K.; Wohnert, J.; Schwalbe, H. *Nucleic Acids Res.* **2007**, *35*, 441–454.

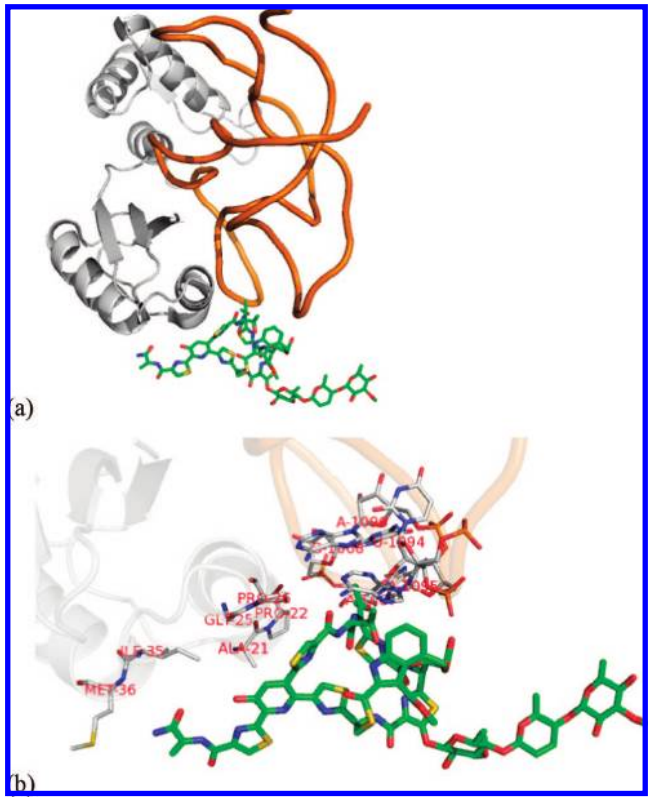

Figure 4. (a) Philipimycin (**2a**) in L11 and RNA complex. Gray backbone indicates the fold of L11 and yellow backbone RNA. (b) Close up view of **2a** in L11 and RNA complex. L11 protein (white backbone) and RNA (yellow), **2a** (green).

thiostrepton. The initial three-dimensional structures of the compounds **1**, **2a**, **2b** and nocathiacin I were constructed from c_view in MIX, the molecular modeling platform at Merck. For each compound, about 300 conformations were generated with the jg routine. The jg routine creates multiple conformations based on the distance geometry methods. Energy minimization of each conformer of four compounds was followed. Then each minimized conformer was overlaid with the crystal structure of thiostrepton and the best overlaying conformer was selected for each compound. The selected conformer replaced the thiostrepton within the L11 and RNA complex. Finally the selected conformer was refined further with minimization in the presence of L11 and RNA. During the final minimization, L11 and RNA were held fixed, while the selected conformer of each compound was allowed to be flexible. The complex of the compound **2a**/L11/RNA is shown in Figure 4a and 4b. While the pyridine core present in all three compounds makes a contact with L11, Thr and the indole ring interact with RNA (Figure 4). Thz1 interacts with A1095, Thr interacts with A1067 ribose and indole shows nucleic acid stacking with A1067 of the ribosome. Thz2, Thz3 and Thz4 show stacking interactions with Pro_{23}, Pro_{22} and Pro_{26}, respectively. Deletion of the four amino acids (Pro_{23}-Val_{24}-Gly_{25}-Pro_{26}) in the thiazomycin resistant strain MB5832 would eliminate two β-turns and remove these interactions, eliminating binding and resulting in loss of the activity of philipimycin and its class.

Structure activity relationship of this compound series indicates that the sugar chain connected to the thiazole ring (Thz3) reduces the activity. However, in this partial model the sugar chain does not interact with any part of L11 or RNA. It is possible that it may interact with L7 or EGF proteins, which may explain the differential activities.

In summary, we have described the isolation and structure elucidation of a highly complex thiazolyl peptide by using a method specifically designed to isolate newer members of this important class of compounds. Philipimycin is the first member of this class that has a highly unusual glycosidic substitution at one of the thiazole rings. It is also the rare member of the class that has exhibited *in vivo* activity.[8] Both philipimycin and its degradation product **2b** selectively inhibited protein synthesis. The lack of activity against the thiazomycin resistant strain could be easily explained by molecular modeling studies.

Experimental Section

General Procedure. All reagents were obtained from Sigma-Aldrich and were used without further purification. The NMR spectra were obtained on Varian Inova 500 or 600 MHz spectrometers operating at 500 or 600 MHz for ^{1}H and 125 or 150 MHz for ^{13}C nuclei. The chemical shifts were referenced to residual $CHCl_3$ (δ_H 7.27 ppm) and $CDCl_3$ (δ_C 77.0 ppm). Data were collected uniformly at 25 °C in 3 mm NMR tubes. A Nalorac 3 mm H{CN} indirect Z-gradient probe was used for all samples. Varian standard pulse sequences were used for all data collection. The 2D TOCSY data were collected with a 4900 Hz spin-lock field held for 80 ms, using the flopsy16 mixing scheme. The 1D TOCSY data were collected with a 6200 Hz spin-lock field using the MLEV17 mixing scheme. Mixing times were arrayed: 20, 40, 60, 80, and 90 ms. Peak selection was achieved using an IBURP waveform with a 50 Hz bandwidth. Proton homonuclear correlation data were obtained with the Varian gradient selected gCOSY (**2a**) or gDQF-COSY (**2b**) pulse sequences. ROESY data were collected using a 4500 Hz spin-lock field applied for 200 ms. Single and multiple bond heteronuclear connectivity data were observed using the gHSQC and gHMBC pulse sequences, respectively. The gHMBC data were collected using a mixing time optimized for a 7 Hz heteronuclear coupling constant. ^{13}C prediction software version 11 from ACD laboratories was used for ^{13}C NMR shift predictions. Optical rotations were obtained on a Perkin-Elmer 241 Polarimeter; IR spectral data were obtained on a Perkin-Elmer Spectrum One spectrometer. UV spectrum was recorded on a Perkin-Elmer Lambda 35 UV/vis spectrometer. High-resolution mass spectra were obtained on a Thermo Finnigan LTQ-FT using electrospray ionization using a Finnigan Ion Max source with source fragmentation on and equal to 18 V.

Production of Philipimycin by *A. philippinensis* MA 7347. The first seed culture of the strain MA 7347 (ATCC PTA 7551, for details of culture isolation and strain characterization, see Supporting Information) was prepared by inoculating 10 mL of seed medium (consisted of in g/L: soluble starch, 20; dextrose, 10; NZ amine type E, 5; beef extract, 3; yeast extract 5; peptone, 5; $CaCO_3$, 1, pH = 7.0) in a 50 mL tube with frozen agar plugs of the producing strain and incubating the tube at 28 °C while shaking at 220 rpm for about 96 h. A second seed culture was prepared by inoculating 50 mL of seed medium in a 250 mL flask with 2 mL of the first seed. Four mL of the second seed culture was transferred to each of the ten 500 mL flask containing 100 mL of the production medium (consisted of in g/L: glucose, 10; millet, 20; pharma media, 20; MOPS, 20) and the flasks were incubated at 28 °C at 220 rpm agitation for 13 days before harvesting.

Isolation of Philipimycin (2a) and 2b. One L acetone was added to the one L fermentation broth and shaken for 2 h at a platform shaker. The 50% aqueous acetone extract was filtered in a sintered glass funnel packed with celite. The filtrate was concentrated to 15% aqueous acetone under *vacuo* and was charged to a column packed with 50 mL Amberchrome. The column was eluted with a 100 min gradient of 5−100% aqueous MeOH at a flow rate of 10 mL/min. At the end, the column was washed with 100% MeOH for 25 min and finally with 200 mL acetone. The fractions eluting with 100% MeOH and

acetone wash possessed all of the biological activities assayed by two-plate assay. None of the other fractions showed any activities. These fractions were combined, concentrated under reduced pressure and lyophilized to give 201.8 mg active fraction.

A 50 mg portion of the Amberchrome active fraction was chromatographed on reversed-phase HPLC (Zorbax C_8, 21.2 × 250 mm, 10 mL/min, 40 min gradient of 10−95% aqueous CH_3CN each eluent containing 0.1% TFA). The fractions were tested in the two-plate assay. Two fractions that eluted at 16−17 min possessed all biological activity but overall extent of activity was reduced. They were pooled and lyophilized to give 5.0 mg material, which was rechromatographed by reversed-phase HPLC (Zorbax C_8, 21.2 × 250 mm, 10 mL/min, 38% aqueous CH_3CN + 0.1% TFA, for 38 min followed by a 5 min gradient of 38−95% aqueous CH_3CN + 0.1%TFA). Fraction eluting at 39 min was lyophilized to give **2b** (1.5 mg) as a colorless amorphous powder. It was quickly recognized by LC comparison that **2b** was different from the original compound present in the broth and was a acid catalyzed degradation product of **2a**. **2b** eluted faster (t_R 4.6 min, Zorbax SB phenyl, 4.6 × 75 mm, 1.0 mL/min, 7 min gradient of 38−60% aqueous CH_3CN + 0.1% TFA) than **2a** (t_R 5.2 min).

Remainder of the Amberchrome active fraction was chromatographed by reversed-phase HPLC without TFA (Zorbax C_8, 21.2 × 250 mm, 10 mL/min, 40 min gradient of 35−57% aqueous CH_3CN). Fraction eluting at 28 min was lyophilized to yield **2a** (9.4 mg, 12.5 mg/L) as a colorless amorphous powder.

2a: $[\alpha]_D^{23}$ −6 (*c* 0.5, CH_3OH-THF, 1:1); UV (CH_3OH+THF, 1:1) λ_{max} 259 (ϵ 38855), 306 (ϵ 26407), 350 (ϵ 13960); IR (ZnSe) ν_{max} 3328, 2937, 1650, 1530, 1482, 1424, 1247, 1192, 1140, 1119, 1068, 985, 926, 837 cm^{-1}; HRESIFTMS: 1746.4074 (calcd for $C_{74}H_{83}N_{13}O_{25}S_6$+H: 1746.4026), 1714.3840 (calcd for $C_{73}H_{79}N_{13}O_{24}S_6$+H: 1714.3758), 1677.3919 (calcd for $C_{71}H_{80}N_{12}O_{24}S_6$+H: 1677.3805), 1645.3673 (calcd for $C_{70}H_{76}N_{12}O_{23}S_6$+H: 1645.3543), 1472.2647 (calcd for $C_{61}H_{61}N_{13}O_{19}S_6$+H: 1472.2604), 1440.2399 (calcd for $C_{60}H_{57}N_{13}O_{18}S_6$+H: 1440.2342), 1298.1725 (calcd for $C_{53}H_{47}N_{13}O_{15}S_6$+H: 1298.1712), 1266.1437 (calcd for $C_{52}H_{43}N_{13}O_{14}S_6$+H: 1266.1449), 1197.1257 (calcd for $C_{49}H_{40}N_{12}O_{13}S_6$+H: 1197.1235); for ^1H and ^{13}C NMR, see Table 1.

2b: $[\alpha]_D^{23}$ +4 (c 0.5, CH_3OH-THF, 1:1); UV (CH_3OH+THF, 1:1) λ_{max} 245 (ϵ 17603), 298 (ϵ 11866), 350 (ϵ 6864), IR (ZnSe) ν_{max} 3257, 2959, 2890, 1673, 1536, 1440, 1368, 1344, 1191, 1121, 1061, 1036, 961, 924, 840 cm^{-1}, HRESIFTMS: 1472.2584 (calcd for $C_{61}H_{61}N_{13}O_{19}S_6$+H: 1472.2604), 1440.2332 (calcd for $C_{60}H_{57}N_{13}O_{18}S_6$+H: 1440.2342), 1298.1704 (calcd for $C_{53}H_{47}N_{13}O_{15}S_6$+H: 1298.1712), 1266.1427 (calcd for $C_{52}H_{43}N_{13}O_{14}S_6$+H: 1266.1449), 1197.1260 (calcd for $C_{49}H_{40}N_{12}O_{13}S_6$+H: 1197.1235); for ^1H and ^{13}C NMR, see Table 1.

Thiazomycin Sensitive Resistance Two-Plate Assay. The wild-type *S. aureus* Smith (MB2865) and thiazomycin-resistant *S. aureus* (MB5832) strains were grown in 5 mL LB-Miller (Invitrogen) media overnight. Growth was adjusted to 0.5 Optical Density (OD) in 5 mL saline (which showed a 0.08 reading with the Dade/Behring Turbidity Meter). One mL of the diluted culture was added to 100 mL molten LB agar and 20 mL was dispensed into Omni Plate. Pin lid was dropped immediately onto the agar before agar's solidification. Ten to 20 μL of sample solutions in DMSO or water was added to each plate at same location and incubated at 37 °C overnight. The activity was determined by measuring the differences of the zone of clearances observed between the wild type versus the resistant plate.

Minimum Inhibitory Concentration (MIC). The MIC against each of the strains was determined by National Committee for Clinical laboratory Standards (NCCLS)-now called the Clinical Laboratory Standards Institute-by 2-fold serial broth dilution method as previously described.[27] The culture was incubated at 37 °C for 20 h before activity was read. MIC is defined as the lowest concentration of antibiotic which inhibited visible growth.

Macromolecular Synthesis Inhibition in *S. aureus*. S. aureus Ep167 was incubated to log phase at 37 °C at 220 rpm in NB medium (1% NaCl, 34 μg/mL chloramphenicol). A 0.05 mL aliquot of this culture was mixed with 0.05 mL aliquots of NB (1% NaCl) that contained various concentrations of the test compounds and one of the following (erythromycin was included in the incubation to negate the effects of the stringent response): peptidoglycan and phospholipid syntheses: 20 μg/mL of erythromycin, 0.5 μCi/mL of [^{14}C-(U)]-glycine (PerkinElmer) and 0.5 μCi/mL of [2-^{3}H]-glycerol (Perkin-Elmer); DNA and RNA syntheses: 20 μg/mL of erythromycin, 0.5 μCi/mL of [2-^{14}C]-thymidine (PerkinElmer) and 3 μCi/mL of [5, 6-^{3}H]-uracil (PerkinElmer); protein synthesis: 5 μCi/mL of L-[4, 5-^{3}H]-leucine (PerkinElmer). The samples were incubated with aeration at 37 °C. After 30 min, incorporation was terminated by the addition of 0.025 mL of 25% TCA. The TCA-insoluble fraction was collected on a glass micro fiber filter mat (PerkinElmer 1405−421) with a Molecular Devices Micro96 Cell Harvester. The filter mat was dried under a stream of hot air for 5 min. It was then placed in sample bag (PerkinElmer 1450−432); 4 mL of Betaplate Scint scintillation fluid was added. The bag was heat sealed and placed in a cassette (PerkinElmer 1450−104). Radioactivity was measured in a PerkinElmer MicroBeta Plate 1450 scintillation counter and % inhibition was plotted against concentration (Figure 3).

***In vivo* Efficacy.** A frozen stock of *S. aureus* Smith (MB 2865) was reconstituted in a 10 mL tube of trypticase soy broth (TSB) and incubated at 35 °C with shaking at 250 rpm for 8 h. One mL of this 8 h culture was used to seed 49 mL TSB in a 250 mL flask and incubated at 35 °C with shaking at 250 rpm for 10 h. The culture was centrifuged at 5000 rpm for 20 min and cell pellet reconstituted in fresh TSB. This culture (>10^9 CFU/mL) was diluted 0.3:10 mL in TSB and the absorbance adjusted to 0.4 at 530 nm. This adjusted culture was further diluted 1:5000 (~4×10^4 CFU/mL) in TSB and then further 1:10 in 5% hog gastric mucin. Three CD-1 mice (weighing ~20 −22 g) per treatment group were challenged (0.5 mL, i.p., 2×10^3CFU/mouse) with *S. aureus* Smith inoculum prepared above. Therapy was initiated 10 min after infection and again 6 h post challenge with the total amount delivered in two subcutaneous (s.c.) doses of philipimycin. Mice were observed for general health and mortality for 24 h after challenge. After 24 h, 3 mice per group were euthanized, both kidneys aseptically removed, placed in sterile Whirl Pak bags, weighed and then homogenized in 5 mL of sterile saline. Tissue homogenates were then serially diluted 100-fold in sterile saline and plated on mannitol salt agar. Plates were incubated at 35 °C for 48 h. Colony forming units (CFU) per gram of tissue were determined for drug-treated and sham-treated control. The dosing samples were prepared as follows: 2.5 mg of philipimycin was dissolved in 6.25 mL sterile 5% dextrose (0.4 mg/mL). This solution was 2-fold serially diluted with 5% dextrose to yield 0.2 and 0.1 mg/mL. When 0.5 mL of each administered subcutaneously the final therapeutic doses = 10, 5, 2.5 mg/kg/dose twice-a-day (b. i. d., total of 2 doses) or 20, 10 and 5 mg/kg/day.

Supporting Information Available: ^1H, ^{13}C, gCOSY, gDQF-COSY, 2D TOCSY, NOESY, gHSQC, gHMBC of **2a** and **2b** and 1D TOCSY data of **2b**, thiazomycin sensitive-resistance two-plate assays, molecular models of thiazolyl peptides, experimental details of organism isolation and phylogenetic tree and complete refs 3, 5, 6, and 21. This material is available free of charge via the Internet at http://pubs.acs.org.

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