Isolation, Structure, and Antibacterial Activities of Lucensimycins D−G, Discovered from *Streptomyces lucensis* MA7349 Using an Antisense Strategy[⊥]

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Bacterial resistance to existing antibiotics continues to grow, necessitating the discovery of new compounds of this type. Antisense-based whole-cell target-based screening is a new and highly sensitive antibiotic discovery approach that has led to a number of new natural product antibiotics. Screening with a *rps*D-sensitized strain led to the discovery of a number of natural product polyketides from *Streptomyces lucensis*. Complete workup of the fermentation extract of this strain allowed for the isolation of seven new compounds, lucensimycins A−G (**1**−**3**, **4a**, **5**−**7**), with varying degrees of antibacterial activities. Lucensimycin E (**5**) exhibited the best activity and showed MIC values of 32 μg/mL against *Staphylococcus aureus* and 8 μg/mL against *Streptococcus pneumoniae.* The isolation, structure elucidation, and antibacterial activities of four new members, lucensimycins D−G, are described. Lucensimycins D (**4a**) and E (**5**) are *N*-acetyl-L-cysteine adducts of lucensimycin A (**1**). Semisynthesis of lucensimycins D and E from lucensimycin A has also been described. Lucensimycins F and G are *myo*-inositolyl-α-2-amino-2-deoxy-L-idosyl amide derivatives of lucensimycins D and E, respectively. The relative configuration of these compounds was determined, in part, by molecular dynamics simulations.

The emergence of drug-resistant bacteria continues to grow and remains a serious threat to human life.[1] Protein synthesis is a highly effective and well-validated antibacterial drug target. A number of clinically useful and FDA-approved antibiotics are inhibitors of bacterial protein synthesis.[2,3] These include chloramphenicol, macrolides, aminoglycosides, tetracyclines, streptogramins, lincosamides, and oxazolidinones. Protein synthesis is catalyzed by the ribosome that is composed of two asymmetric macromolecular components, the large (50S) and small (30S) subunits. The large subunit consists of two rRNAs and 34 unique ribosomal proteins (r-proteins), L1−L34.[4−6] The small subunit is composed of 16S rRNA and 21 r-proteins, S1−S21.[4−6] The ribosome is a catalyst of protein synthesis, and its quaternary structure is maintained by r-proteins. Most of the clinically used protein synthesis inhibitors not only bind to rRNA but also bind to one or more r-proteins. Disruption of the rRNA binding to r-proteins inactivates the protein synthesis function; hence any agent that can selectively alter the conformation of a particular r-protein, or inhibit the synthesis of an r-protein, would lead to the loss of function of protein synthesis. Small ribosomal protein S4 is one of the essential proteins and is encoded by the *rps*D gene. It is conserved across Gram-positive and Gram-negative bacterial species and essential for bacterial growth.[7,8]

We recently reported an antisense-based two-plate whole-cell differential sensitivity screening assay for the discovery of new antibiotics. In the first version of this assay, we used an antisense-sensitized *S. aureus* strain with reduced expression of the *fab*F/*fab*H gene leading to reduction of the FabF/FabH target proteins and hypersensitivity for FabF/FabH inhibitors.[9,10] This led to the discovery of platensimycin and platencin, two novel and potent inhibitors of FabF and FabF/H with in vivo antibiotic properties.[11−14] Subsequently, a similar two-plate assay with a reduced expression of the *rpsD* gene by antisense was developed and employed for screening of natural product extracts. This screening strategy followed by bioassay-guided fractionation led to the isolation of lucensimycins A (**1**), B (**2**),[15] and C (**3**)[16] from *Streptomyces lucensis* and coniothyrione,[17] pleosporone,[18] and phaeosphenone.[19] Bioassay-directed isolation of the extract of *S. lucensis* led to the isolation of the four new lucensimycins D−G (**4a**−**7**). These new compounds are structurally more complex and biologically more potent than lucensimycins A−C (**1**−**3**). Complete workup of the extract with details of isolation, structure elucidation, and the biological activity of lucensimycins is described herein.

Results and Discussion

The producing strain MA7349 was isolated from a soil sample collected in Martinique Island, West Indies. It was classified as *S. lucensis* by 16S rDNA analysis and comparison with known organisms using phylogenetic analysis (see Supporting Information, Figure S1). A strain of *S. lucensis* is known to a produce polyene antibiotic, etruscomycin (lucensomycin, lucimycin), with antifungal activity.[20−24] The strain MA7349 was grown in a liquid production medium for seven days. Originally these compounds were isolated from a 1 L fermentation broth using bioassay-guided three-step fractionation (Amberchrome-Sephadex LH 20 and reversed-phase HPLC) in which each fraction was evaluated against an antisense *rps*D two-plate assay. However, this report describes a simplified procedure for reisolation of these compounds from a scaled up 3 L fermentation batch. The fermentation broth was extracted with an equal volume of acetone and chromatographed on Amberchrome resin. Fractions containing lucensimycins were rechromatographed by reversed-phase C_8 HPLC to afford lucensimycins A (**1**, 19.2 mg, 6.4 mg/L), C (**3**, 17.8 mg, 5.9 mg/L), D (**4a**, 82.2 mg, 27.4 mg/L), E (**5**, 161.3 mg, 53.7 mg/L), F (**6**, 23.9 mg, 8 mg/L), and G (**7**, 15 mg, 5 mg/L), as colorless, amorphous powders. Lucensimycin B (**2**) was not isolated from this batch. Whether the absence of **2** in the current batch was due to the lack of production during fermentation or lucensimycin B was produced as an artifact of isolation during the first bioassay-guided isolation, which took about 2−3 weeks, is not clear and was not investigated further.

Lucensimycins D and E (4a and 5). High-resolution ESIMS analysis of **4a** showed a protonated molecular ion at *m*/*z* 688.2435, which analyzed for a molecular formula of $C_{34}H_{41}NO_{12}S$+H. The

[⊥] Dedicated to Dr. David G. I. Kingston of Virginia Polytechnic Institute and State University for his pioneering work on bioactive natural products.

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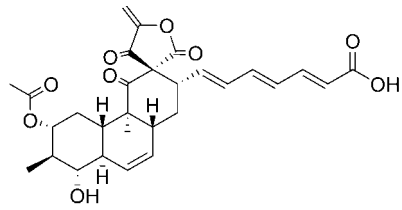
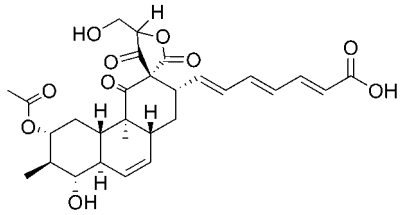


1:Lucensimycin A



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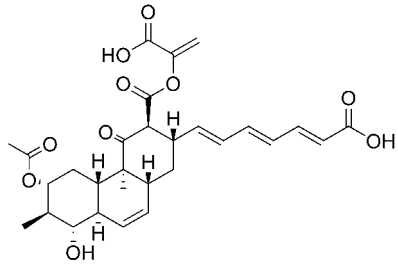
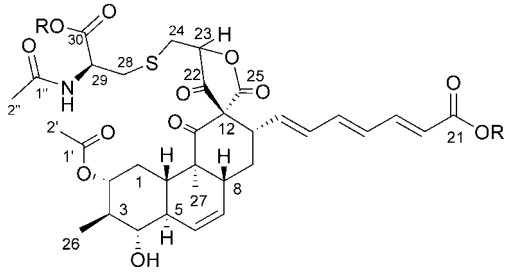
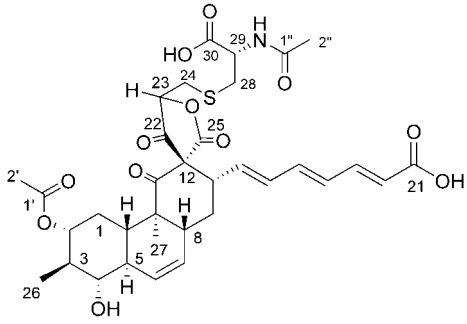


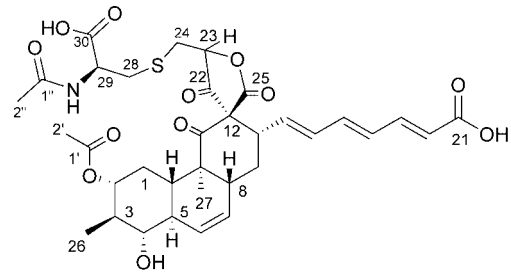
3: Lucensimycin C



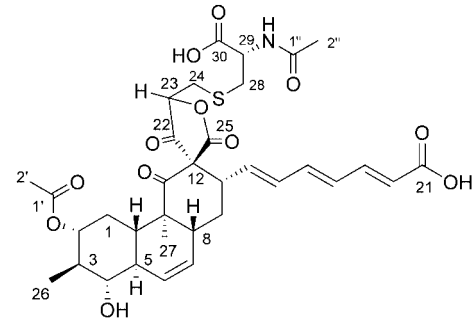
4a: R = H, Lucencimycin D
4b: R = Me



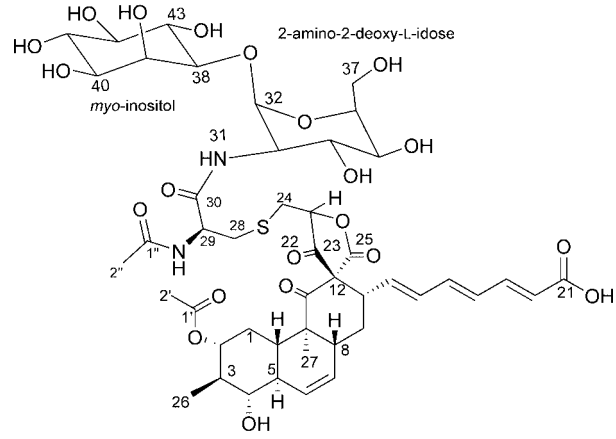
5: Lucencimycin E



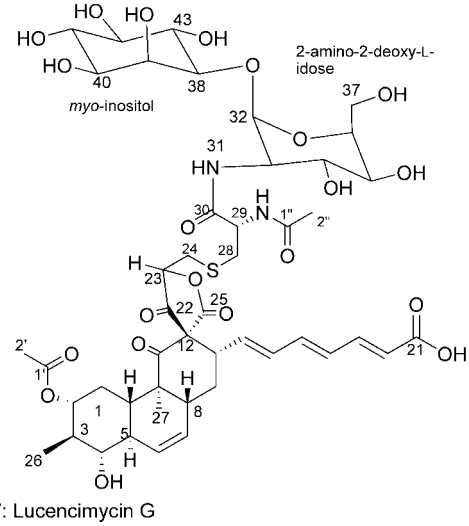
4a'



5'



6: Lucencimycin F



7: Lucencimycin G

UV spectrum of **4a** was identical to the UV spectra of lucensimycins A−C (**1**−**3**) and showed an absorption band at λ_{max} 285 nm, suggesting that these compounds possess a common trienoic acid chromophore. The IR spectrum of **4a** exhibited prominent absorption bands for a hydroxy group (ν_{max} 3333 cm^{-1}) and an ester/lactone (ν_{max} 1747 cm^{-1}). The ^{13}C NMR spectrum of **4a** in CD_3OD and DMSO-d_6 showed the presence of 34 resonances dispersed throughout the ^{13}C spectral range, which when examined in combination with the DEPT spectrum suggested the presence of eight olefinic methines, nine methines (including three oxymethines), four methylenes, two ketones, five acid/ester/lactone/amide ketones, four methyls, and two quaternary carbons (Table 1). Comparison of the ^{13}C NMR spectrum of **4a** with those of lucensimycins A−C (**1**−**3**) indicated that the spectrum of **4a** closely resembled the spectrum of **2** with the presence of five additional resonances (a methyl, two amide/acid carbonyls, a methylene, and a methine) and a significantly upfield shifted C-24 methylene

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignment of Lucensimycins D (**4a**) and E (**5**)[a]

position #	δ_C	**4a** (CD_3OD) δ_H (J in Hz)	**4a** (CD_3OD) HMBC	δ_C	**4a** (DMSO-d_6) δ_H	**4a** (DMSO-d_6) HMBC	**5** δ_C	**5** (CD_3OD) δ_H (J in Hz)
1	33.1	1.23, m		31.5	1.05, m		32.5	1.23, m
		1.14, m			1.10, m			1.14, m
2	76.7	4.43, dt, 4.5, 10	C-1, 3, 23, 1′	74.3	4.29, dt, 5, 10	C-1, 3, 23, 1′	77.6	4.03, dt, 4.5, 10
3	46.1	1.55, qd, 6.5, 10	C-1, 2, 4, 23	43.2	1.45, qt, 6.5, 10	C-1, 2, 4, 23	45.8	1.56, qt, 6.5, 10
4	77.3	2.95, t, 10	C-5, 6, 10	75.4	2.81, t, 10	C-5, 6, 10	77.5	2.90, t, 10
5	45.3	1.81, brt, 2, 10		43.5	1.69, brt, 10		45.6	1.80, brt, 10
6	127.4	6.07, brd, 10	C-4, 8, 10	126.3	5.99, brd, 10	C-4, 8, 10	127.2	6.04, brd, 10
7	130.1	5.81, ddd, 10, 5, 2.5	C-5, 9	128.5	5.73, ddd, 10, 5, 2.5	C-5, 9	130.4	5.83, ddd, 10, 5, 2.5
8	44.9	2.42, md, 12.5	C-6, 9, 10, 13, 14	42.5	2.45, md, 12	C-6, 9, 10, 13, 14	44.7	2.42, brd, 10
9	52.9			51.7			54.0	
10	32.3	2.30, dt, 3, 12.5	C-1, 2, 5, 9, 24	30.5	2.11, m	C-1, 2, 5, 9, 24	33.5	2.00, m*
11	202.6			201.3			201.5	
12	71.9			71.8			73.8	
13	47.8	3.23, dt, 3.5, 9	C-8, 12, 14, 16, 22	42.8	3.22, ddd, 12, 8.5, 3.5	C-8, 12, 14, 16, 22	45.1	3.23, dt, 3.5, 9
14	30.9	2.27, q, 13	C-7, 8, 9, 12, 13, 15	29.5	2.11, m	C-7, 8, 9, 12, 13, 15	31.3	2.47, q, 13
		1.94, td, 3.5, 13	C-7, 8, 9, 12, 13, 15, 22 (4-b)		1.89, m	C-7, 8, 9, 12, 13, 15, 22 (4-b)		1.96, m*
15	135.4	5.69, dd, 15, 9	C-12, 13, 14, 16, 17	132.9	5.63, dd, 15, 9	C-12, 13, 14, 16, 17	135.9	5.82, dd, 15, 9
16	133.9	6.36, dd, 15, 11	C-12 (4-bond), 13, 17, 18	133.5	6.28, dd, 15, 11	C-12 (4-bond), 13, 17, 18	134.2	6.32, dd, 15, 11
17	140.3	6.58, dd, 15, 11	C-15, 16, 18, 19	139.2	6.60, dd, 15, 11	C-15, 16, 18, 19	140.9	6.56, dd, 15, 11
18	132.7	6.45, dd, 14.5, 11	C-16, 17, 19, 20	131.2	6.47, dd, 15, 11	C-16, 17, 19, 20	132.2	6.40, dd, 15, 11
19	145.8	7.29, dd, 15.5, 11	C-17, 18, 20, 21	143.5	7.16, dd, 15, 11	C-17, 18, 20, 21	146.0	7.29, dd, 15, 11
20	123.2	5.93, d, 15.5	C-17, 18, 21	122.9	5.92, d, 15.5	C-17, 18, 21	122.8	5.90, d, 15
21	170.3			167.4			170.4	
22	204.0			200.2			203.0	
23	86.8	4.85, (HDO overlap)	C-12, 22, 24, 25	84.2	5.10, dd, 8, 4	C-12, 22, 24, 25	84.6	4.96, dd, 4.5, 3.5
24	34.1	3.17, dd, 15, 3		32.5	3.03, dd, 15, 4		32.3	3.21, dd, 15, 3.5
		2.84, dd, 15, 7.5	C-22, 23, 28		2.68, dd, 15, 8	C-22, 23, 28		3.06, dd, 15, 4.5
25	172.8			169.3			173.2	
26	14.7	1.07, d, 6.5	C-2, 3, 4	14.3	0.96, d, 6.5	C-2, 3, 4	14.7	1.07, d, 6.5
27	17.1	1.12, s	C-8, 9, 10, 11	15.8	0.98, s	C-8, 9, 10, 11	16.5	1.12, s
28	35.2	3.21, dd, 14, 4.5	C-30, 29, 24	33.5	3.02, dd, 14, 5	C-30, 29, 24	36.3	3.07, dd, 14, 5
		3.00, dd, 14, 8.5	C-30, 29, 24		2.78, dd, 14, 9	C-30, 29, 24		2.92, dd, 14, 8
29	53.8	4.65, dd, 8.5, 4.5	C-30, 28,1″	51.9	4.39, dt, 5, 9	C-30, 28,1″	53.9	4.59, dd, 8, 5
30	173.4			171.9			173.7	
1′	172.5			169.3			172.7	
2′	21.1	2.04, s	C-1′	20.8	1.96, s	C-1′	21.0	2.04, s
1″	173.3			169.8			171.4	
2″	22.5	2.05, s	C-1″	22.3	1.85, s	C-1″	22.5	2.05, s
	NH			NH	8.22, d, 9	C-1″		

[a] Both compounds showed common NOESY correlations and were consistent with the NOESY correlations of lucensimycins A (**1**) and C (**3**).

resonance (δ_C 34.1 vs δ_C 60.2). These findings suggested that the changes were at C-24. The new methylene protons (C-28) appeared as a pair of doublets of doublets (δ_H 3.21 and δ_H 3.00) and showed COSY correlations to the new methine proton (C-29) resonating at δ_H 4.65 (dd, J = 8.5 and 4.5 Hz). In DMSO-d_6, the methine proton resonated as a doublet of a triplet and displayed an additional COSY correlation to an exchangeable proton doublet resonating at δ_H 8.22, which was assigned to an amide NH group. The NH protons showed TOCSY correlations to the methylene protons at C-28. H-29 showed HMBC correlations to the acid carbonyls C-30 (δ_C 173.4), C-28 (δ_C 35.2) and the *N*-acetyl carbonyl C-1″ (δ_C 173.3). The acetate methyl protons C-2″ (δ_H 2.05) showed a HMBC correlation to C-1″, which also showed a correlation to the NH protons in DMSO-d_6. Both of the methylene protons at C-28 gave HMBC correlations to C-29, C-30, and C-24 (δ_C 34.1) in both solvents. Taken together, including the presence of a sulfur atom in the molecular formula and the presence of an upfield shifted methylene resonance at δ_C 26.1, this suggested an oxygen to sulfur substitution at C-24 and hence the presence of *N*-acetylcysteine as an additional unit that would account for the additional carbons in the molecular formula.

Lucensimycin E (**5**) produced the same molecular weight and isomeric formula as that of **4a**. The ^{1}H NMR spectrum of **4a** in DMSO-d_6 showed evidence of isomerization to **5** and vice versa at 25 °C. The isomerization was slow, and in five days a pure sample of **4a** turned into a 1:1 mixture of **4a** and **5** (see Supporting Information). The interconversion was highly retarded in CD_3OD. The reaction of *N*-acetyl-L-cysteine with lucensimycin A (**1**) in CH_3CN at room temperature after 170 h (Figure 1) produced a 2:1 mixture of **5** and **4a**, the same ratio as the two compounds isolated from the fermentation broth. This confirmed the structure of **4a**

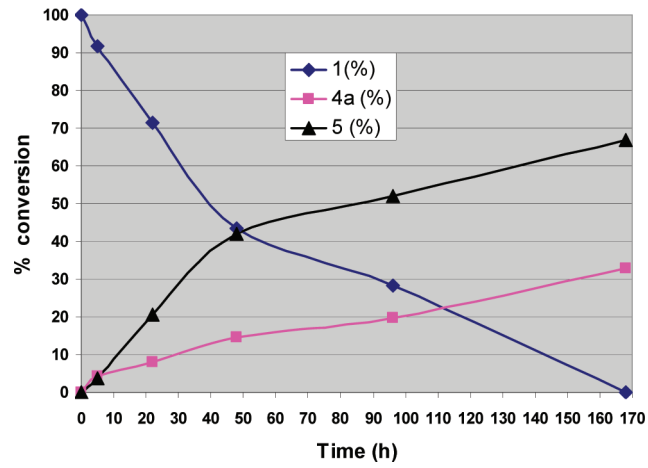
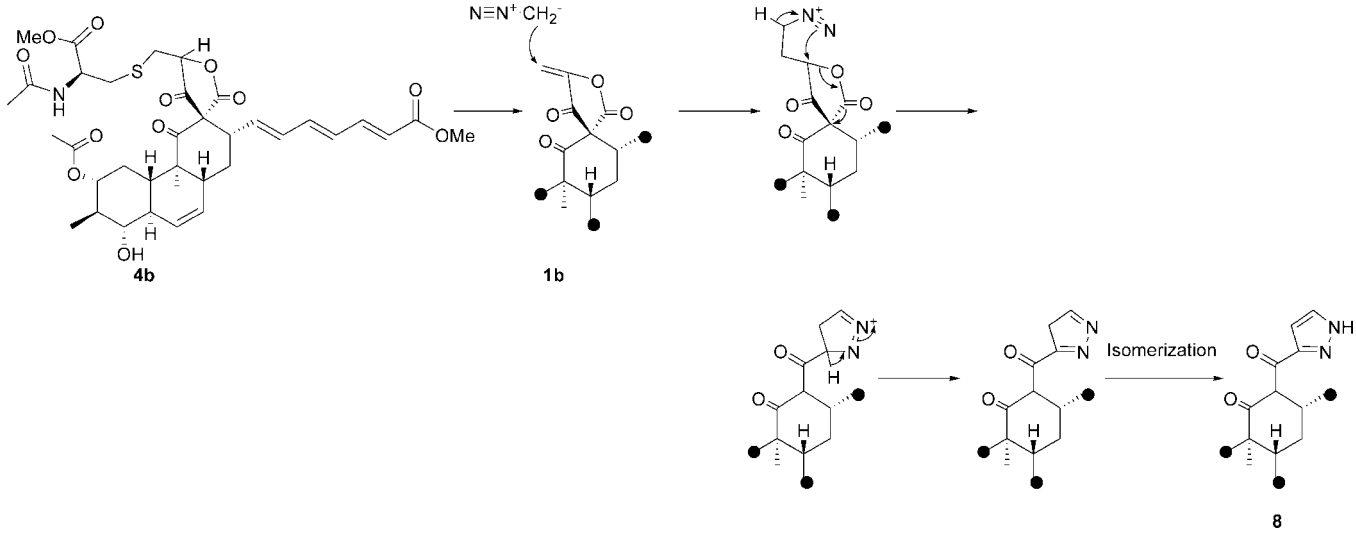


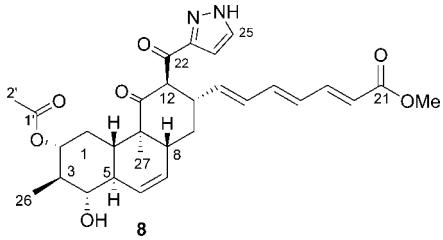
Figure 1. Time course of the reaction of lucensimycin A (**1**) with *N*-acetyl-L-cysteine.

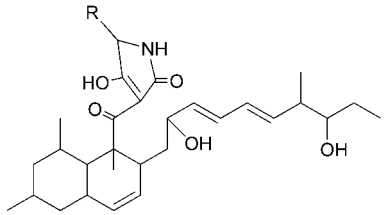
and **5** as *N*-acetyl-L-cysteine adducts of lucensimycin A (**1**) and confirmed the configuration of cysteine in **4a** and **5** as *S*. Reaction of **4a** with diazomethane produced a small amount of a dimethyl ester (**4b**) and a pyrazole (**8**). The formation of pyrazole **8** was highly facile and was often formed exclusively. For example, only pyrazole **8** could be isolated from the diazomethane reaction of **5**. The pyrazole is likely formed by 1,3-dipolar addition of diazomethane with concomitant decarboxylation (Scheme 1). The reaction of **1** with diazomethane also produced **8** and thus validated the proposed mechanism. It is clear that reaction of **4a** and **5** with

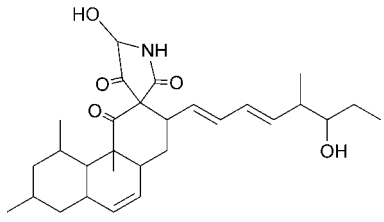
Scheme 1. Plausible Mechanism of Pyrazole Formation from Lucensimycins



diazomethane proceeds through a retro-Michael reaction, producing **1** that then undergoes 1,3-dipolar cycloaddition to produce pyrazole **8**.







NOESY and ROESY data were not useful for the configurational assignments at C-12 and C-23 of **4a** and **5**. The configuration was assigned on the basis of the following arguments, which became the foundation for assignments of these centers for lucensimycins A−G (**1**−**3**, **4a**, **5**−**7**). Comparison of the ^{1}H NMR shifts of compounds **4a** and **5** indicated that most of the proton chemical shifts of both compounds were virtually identical, with a few clear and significant differences (Table 1). H-2 (+$\Delta\delta$ 0.4 ppm) and H-10 (+$\Delta\delta$ 0.3 ppm) were shifted downfield in **4a** compared to **5**, and H-14 (+$\Delta\delta$ 0.2 ppm) and H-15 (+$\Delta\delta$ 0.13 ppm) were shifted downfield in **5** compared to **4a**. The proximal conformation of the side chain could be forced by potential hydrogen bonds of NH with the C-11 ketone and the carboxyl group with the C-2 acetate. The downfield shifts of H-2 and H-10 could be attributed to the shielding effect of the *N*-acetyl-L-cysteine group present in the *exo* and *endo* positions at C-23 in **4a** and **5**, respectively. The comparison of the Dreiding model suggests that the flanking of the *N*-acetyl-L-cysteine group from the top face will fit better when the configuration at C-12 is as drawn. In these C-12 and C-23 configurations a double-bond-induced shielding effect can be envisioned for H-15 for compound **5**.

These configurational assignments of **4a** and **5** were supported by molecular dynamics (MD) simulations. MD simulations were carried out for four possible stereoisomers (**4a**, **4′**, **5**, and **5′**). The temperature was raised from 0 to 300 K during 900 femptoseconds (fs). Five picoseconds (ps) of equilibration were followed by one nanosecond simulation. During simulation, 1000 conformations were generated in each 1 ps interval. For each conformation the distances of oxygen at C-11, the NH at C-29, the hydroxy group of a carboxylic acid at C-30, and the oxygen atom of the acetate at C-2, for both *exo* conformers **4a** and **4a′** as well as the NH-acetate and C-15 in the *endo* conformers **5** and **5′**, were measured. The average distances of the selected atoms in **4a**, **4a′**, **5**, and **5′** are tabulated in Table 2. When conformational changes took place during the simulation, the average distances were calculated separately. Major conformers for each isomer during the simulations are shown in Figure 2. The nitrogen (NH at C-29) in the side chain from the dioxolane ring is making a good hydrogen bond contact with the ketone oxygen at C-11 in **4a**, as shown in Figure 2. The side chain is close to the tricyclic ring, therefore, influencing the chemical shifts of H-2 and H-10. The analysis of the *endo* structures **5** and **5′** was not as clear-cut due to flexibility. Generally longer distances were observed between NH-acetate and C-15 in both structures except for one conformer of **5′**, which showed a distance of 5.3 Å. However, epimerization of **4a** to lucensimycin E would rule out the stereochemical change at C-12 and hence favor the structure **5** for lucensimycin E. The chemical shift differences of H-15 and H-14 could be explained from the eclipsing of the *N*-acetyl-L-cysteine side chain with the trienoic acid chain. Thus isomeric structures **4a** and **5** were assigned to lucensimycins D and

Table 2. Distance History during 1 ns Molecular Dynamics Simulations

	distance in Å			
atoms	**4a**	**4a′**	**5**	**5′**
NH, O at C-11	2.9, 3.9	4.2	6.2	6.0, 9.3
NH, C-15	6.2, 8.7	6.6	6.2	5.3, 6.9
O at C-1′, OH at C-30	3.6, 6.2	5.8	9.7	14.5, 11.3

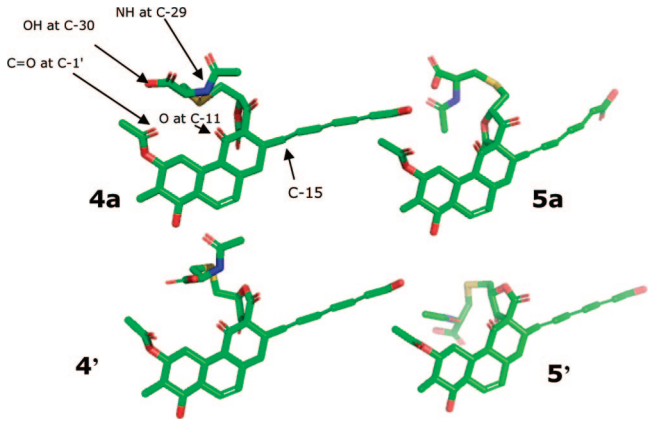


Figure 2. Major conformers of **4a**, **4′**, **5**, and **5′** during one 1 ns MD simulations.

E, respectively. Interestingly, the *endo*−*exo* substitution of the side chain has a significant impact on the specific rotation of these compounds, including a change in sign.

Lucensimycins F (6) and G (7). Mass spectrometric analyses of **6** and **7** gave molecular weights of 1010 and molecular formulas of $C_{46}H_{62}N_2O_{21}S$. The UV spectra of these two compounds were similar to the UV spectra of other lucensimycins, suggesting the retention of the trienoic acid chromophore. The IR spectra showed stronger absorption bands for hydroxy groups in addition to acetyl and ketone groups. The ^{13}C NMR spectroscopic analysis of the two compounds suggested the presence of all resonances that were present in lucensimycins D and E with an additional 12 resonances (Table 3). DEPT spectra suggested that nine of these new resonances were an oxymethine, an oxymethylene, and an anomeric and a nitrogen-containing methine, which were supported by the HSQC data. The COSY and TOCSY spectra of the two compounds suggested the presence of a hexahydroxy cyclohexane and an amino-hexose. These two moieties were identified as *myo*-inositol[25,26] and 2-amino-2-deoxy-L-idose[27] based on the scalar couplings. The latter amino-sugar is known to be present in some of the aminoglycoside antibiotics, e.g., the paromomycin class (e.g., compound X_1).[28] The ESIMS produced a fragment ion at *m*/*z* 831 due to the loss of the *myo*-inositol, suggesting that this moiety was present at the terminal position. The anomeric proton H-32 (δ_H 5.11, δ_C 100.1) showed an HMBC correlation to C-38 (δ_C 80.4) of the *myo*-inositol, thus establishing the glycosidic linkage. H-33 (δ_H 3.92) showed a strong HMBC correlation with the carboxyl carbonyl of the cysteine at C-30 (δ_C 172.6), providing evidence for an amide bond between the amino-sugar and the *N*-acetyl-L-cysteine. The smaller *J* value (4 Hz) between H-1 and H-2 of the amino-L-idosopyranoside indicated an α-glycosidic linkage. Methylation of **7** with diazomethane produced pyrazole **8** just like compounds **4a** and **5**. The **6** and **7** pair displayed similar chemical shift differences of H-2, H-10, H-14, and H-15 to those observed for **4a** and **5**, indicating similar configurations at C-12 and C-23. On the basis of these data isomeric structures **6** and **7** were assigned for lucensimycins F and G, respectively.

Biological Activity. All lucensimycins were tested against the *S. aureus rps*D antisense two-plate assay. In this assay, lucensimycin D (**4a**) showed somewhat better activity and the best selectivity of the series. It showed a zone of clearance of 12 mm in the antisense *rps*D plate vs 8 mm on the control *S. aureus* EP167 plate at 0.5 mg/mL (10 μg spotted). Lucensimycin E (**5**) showed similar activity and displayed a 12 mm zone of clearance in the antisense *rps*D plate and 10 mm against the control plate at 0.5 mg/mL. At the same concentration, lucensimycin A (**1**) showed a zone of clearance of 12 and 10.8 mm against antisense and control plates, respectively. Lucensimycins F (**6**) and G (**7**) were isolated later and not tested in this assay. All of the compounds were then tested for their effect in inhibiting bacterial growth by standard NLSI protocol. Lucensimycin E (**5**) showed the best activity and inhibited the growth of *S. aureus* Smith strain with a MIC value of 32 μg/mL. It showed better sensitivity for *Streptococcus pneumoniae* CL 2883 and inhibited with a MIC value of 8 μg/mL. Lucensimycin D (**4a**) was also most sensitive for inhibition of the growth of *S. pneumoniae* (MIC 250 μg/mL) and did not inhibit *S. aureus* growth at 250 μg/mL. None of the other lucensimycins showed any antibacterial activity against these two bacterial strains at 250 μg/mL or inhibited growth of other Gram-positive bacteria (*Enterococcus faecalis*, *Bacillus subtilis*) and Gram-negative bacteria (*Haemophilus influenzae* and *Escherichia coli*) at 250 μg/mL. They also did not inhibit growth of *Candida albicans* at 250 μg/mL. The activity of these compounds was also tested at higher concentrations on agar plates impregnated with *S. aureus* Ep167. In this assay lucensimycins A (**1**), F (**6**), and G (**7**) showed better activities than lucensimycins D (**4a**) and E (**5**). Lucensimycin C (**3**) was the least active (see Supporting Information, Figure S2). However, none of these compounds appeared potent enough to warrant any further studies.

Lucensimycins show a structural resemblance to delaminomycins A−C (**9a**−**c**) reported from *Streptomyces albulus* MJ202-72F3.[29−32] Delaminomycins have shown antibacterial activity (MIC 25 μg/mL against *S. aureus*) and have also shown various activities such as inhibition of cell adhesion to extracellular matrix receptor, immunomodulator activity, and cytotoxicity.[30] It has been reported that acid treatment of delaminomycin A produces compound **10**. Unlike compound **10**, lucensimycins appear to be true natural products and are found to be present by HPLC in the EtOAc extract of the broth (pH 5.0).

In summary, we have described here a complete workup of extracts of *S. lucensis*, which was selected for further follow-up on the basis of differential activity in a *rps*D-sensitized antisense assay. This led to the discovery of seven new compounds, three with polyketide origin and the other four involving mixed polyketide and NRPS origin. These compounds show modest to poor antibacterial activity and would have not been discovered without enhanced sensitivity of the antisense assay.

Experimental Section

General Experimental Procedures. All reagents were obtained from Sigma-Aldrich and were used without further purification. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter. IR data were obtained on a Perkin-Elmer Spectrum One spectrometer. UV spectra were recorded on a Perkin-Elmer Lambda 35 UV/vis spectrometer. The NMR spectra were obtained on a Varian Inova 500 or 600 MHz spectrometer operating at 500 or 600 MHz for ^1H and 125 or 150 MHz for ^{13}C nuclei. The chemical shifts were referenced to residual solvent DMSO-d_6 (δ_H 2.49 ppm and δ_C 39.5 ppm) and CD_3OD (δ_H 3.30 ppm and δ_C 49.0 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. Proton homonuclear correlation data were obtained with the Varian gCOSY or DQF-COSY pulse sequences. Single- and multiple-bond heteronuclear connectivity data were observed using the gHSQC or HMQC and gHMBC or HMBC pulse sequences, respectively. The gHMBC and HMBC data were collected using a mixing time optimized for a 7 Hz heteronuclear coupling constant. High-resolution mass spectra were obtained on a Thermo Finnigan LTQ-FT using electrospray ionization from a Finnigan Ion Max source with source fragmentation on and equal to 18 V.

Isolation Conditions for Producing Strain. Strain MA 7349 was isolated from a soil sample collected at Martinique Island, West Indies, in the Montagne du Vauclin (180 m altitude), in a zone of dense humid vegetation and underneath a decomposing dead tree. This soil was air-dried, pretreated with dry heat at 100 °C for 1 h, and suspended in sterile water. This soil suspension was serially diluted, plated on selective isolation medium, and incubated at 28 °C for at least six weeks. Strain MA 7349 was isolated from a NZ-amine-based agar medium containing nalidixic acid (20 μg/mL). The colony was purified on yeast

Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignments of Lucensimycins F (**6**) and G (**7**)[a]

	6 (CD_3OD)		**7** (CD_3OD)		7 (CD_3OD)
position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	HMBC
1	33.2	1.15, m	32.5	1.15, m	
		1.25, m		1.95, m	
2	76.5	4.42, dt, 4, 10	77.5	3.99, dt, 4, 10	C-1, 3, 23, 1′
3	46.1	1.55, m	45.8	1.52, m	C-1, 2, 4, 23
4	77.3	2.82, t, 10	77.6	2.86, t, 10	C-5, 6, 10
5	45.3	1.77, brt, 10	45.6	1.76, brt, 10	
6	127.4	6.00, brd, 10	127.2	6.00, brd, 10	C-4, 8, 10
7	130.1	5.77, ddd, 10, 4.8, 2.4	130.4	5.80, m	C-5, 9
8	44.8	2.40, m	44.7	2.45, m	C-6, 9, 10, 13, 14
9	52.8		53.9		
10	32.2	2.30, dt, 2.4, 10	33.6	1.95, m	C-1, 2, 5, 9, 24
11	202.7		201.5		
12	71.9		73.8		
13	48.0	3.21, m	45.1	3.20, m	C-8, 12, 14, 16, 22
14	30.9	2.25, m	31.4	2.40, m	C-7, 8, 9, 12, 13, 15
		1.92, m		1.94, m	C-7, 8, 9, 12, 13, 15
15	133.8	5.65, dd, 15, 9	135.9	5.79, dd, 15, 9	C-12, 13, 14, 16, 17
16	135.5	6.33, dd, 15, 11	134.1	6.29, dd, 15, 11	C-12, 13, 17, 18
17	140.3	6.55, dd, 15, 11	141.0	6.53, dd, 15, 11	C-15, 16, 18, 19
18	132.8	6.41, dd, 15, 11	132.1	6.37, dd, 15, 11	C-16, 17, 19, 20
19	145.8	7.25, dd, 15, 11	146.2	7.28, dd, 15, 11	C-17, 18, 20, 21
20	123.2	5.90, d, 15	122.6	5.87, d, 15	C-17, 18, 21
21	170.3		170.4		
22	204.1		203.0		
23	87.0	4.88, t, 4	84.4	4.94, t, 4	C-12, 22, 24, 25
24	35.7	3.17, m	32.3	3.25, dd, 15, 4	
		2.86, dd, 15.6, 6.6		3.05, dd, 15, 4.5	C-22, 23, 28
25	173.0		171.6		
26	14.7	1.05, d, 6.5	14.7	1.05, d, 6.5	C-2, 3, 4
27	17.1	1.10, s	16.5	1.07, s	C-8, 9, 10, 11
28	33.8	3.17, m	36.5	2.98, dd, 14, 6	C-30, 29, 24
		2.95, dd, 13.8, 9.6		2.89, dd, 14, 8	C-30, 29, 24
29	55.2	4.48, dd, 9, 4.8	54.9	4.49, dd, 8, 6	C-30, 28,1″
30	172.5		172.6		
32	100.1	5.13, d, 4.2	100.1	5.11, d, 4	C-33, 34, 36, 38
33	55.6	3.92, ddd, 10.8, 9, 4	55.6	3.92, ddd, 11, 9, 4	C-30, 32, 34, 35
34	72.9	3.74, dd, 11, 9	72.9	3.75, dd, 11, 9	C-33, 35, 36
35	72.4	3.31, dd, 9, 3.6	72.4	3.31, dd, 9, 2.5	C-33, 36, 37
36	74.3	3.82, ddd, 6, 3.6, 2	74.3	3.82, ddd, 6.5, 3	
37	62.8	3.85, dd, 11, 2	62.8	3.85, dd, 12, 2	
		3.64, dd, 11, 6		3.64, dd, 12, 6.5	
38	80.3	3.49, dd, 10, 2.5	80.4	3.47, dd, 10, 2.5	C-32, 43
39	73.4	4.13, t, 3	73.4	4.13, t, 3	C-41, 43
40	73.2	3.36, dd, 10, 3	73.2	3.35, dd, 10, 3	
41	74.0	3.60, t, 10	74.0	3.60, t, 10	C-40, 42
42	76.6	3.18, t, 10	76.4	3.18, t, 10	C-38, 41, 43
43	74.1	3.81, t, 10	74.1	3.80, t, 10	C-38, 42
1′	172.8		172.8		
2′	21.2	2.02, s	21.1	1.99, s	C-1′
1″	173.7		173.6		
2″	22.8	2.05, s	22.8	2.10, s	C-1″
31	NH	7.99, d, 9	NH	7.99, d, 9	

[a] The NMR assignments for **6** and **7** were secured by full suites of 2D (DQF-COSY, TOCSY, HSQC, HMBC) spectroscopic analysis. HMBC data of only **7** are listed here. Compound **6** showed similar HMBC correlations.

extract malt extract glucose medium (ISP2) and preserved as frozen agar plugs in 10% glycerol.

Morphological Characterization of the Strain. Sporulating characteristics were observed upon growth of the strain on different selective media after 21 days incubation at 28 °C. The strain grows well on all the tested media. The strain produces characteristic thin spore spirals borne at the end of the aerial hyphae on media ISP2, ISP3, ISP4, ISP5, and ISP7. Macroscopically, it produces a dense growth of gray, grayish-brown to brownish aerial mycelium, of cottony appearance on ISP7. On the contrary it exhibits flat growth and poor aerial development on ISP5. This macro- and micromorphology are in accordance with those of some members of the genus *Streptomyces*.

16S rDNA Sequencing and Phylogenetic Analysis. The complete 16S rDNA sequence (Genbank accession number EU909401) was aligned with *Streptomyces* nucleotide sequences from Genbank and confirmed the preliminary identification of the strain as *Streptomyces* sp. The taxonomic position of the strain was determined by phylogenetic analysis of the aligned 16S rDNA sequences of 29 validated *Streptomyces* species. The phylogenetic tree based on these 16S rDNA sequences was built using the maximum parsimony method. Bootstrap replicates from each grouping was used as a measure of statistical confidence. From the phylogenetic analysis the strain was found to be closely related to the species *Streptomyces lucensis* NBRC13056 (original producer of the antifungal polyene etruscomycin). This close relationship is highly supported by the bootstrapping value (85%) (see Supporting Information, Figure S1).

Production Conditions for Lucensimycins. For production of the secondary metabolites, a seed culture of the strain was prepared by inoculation from a frozen vial containing agar plugs in a 50 mL tube containing 10 mL of seed medium (in g/L: soluble starch 20.0; dextrose 10.0; NZ Amine type E 5.0; Difco beef extract 3,0; Difco Bacto peptone 5,0; Difco yeast extract 5.0; and $CaCO_3$ 1.0). After three days of incubation at 28 °C, 5% of the inoculum was transferred to 50 mL EPA tubes containing 10 mL of various production media, and the cultures were incubated at 28 °C with 220 rpm agitation, 70% humidity, for seven days. The strain was cultivated in three different production

media, and the activity was obtained only in the medium CLA (in g/L: AMBEREX PH 5.0; yellow corn meal 40.0; lactose 40.0).The production of the activity was confirmed in a 50 mL fermentation in the same CLA production medium. A 5% sample of the inoculum was transferred to a 250 mL flask containing 50 mL of the production medium, and the culture was incubated for seven days in a rotary shaker at 220 rpm, 70% humidity.

Extraction and Isolation of Lucensimycins. Fermentation broth grown for seven days in 250 mL × 60 shake flasks containing 50 mL of CLA production medium was pooled to give 3 L of broth (pH 5.0), which was diluted with 3 L of acetone and shaken for 60 min on a shaker. The extract was filtered through Celite, and cells were washed with 1 L of acetone. The combined filtrate was concentrated under reduced pressure to a volume of 3 L and was diluted with 1 L of water to make a total volume of 4 L. This solution was loaded at a flow rate of 5 mL/min onto a 100 mL medium grade Amberchrome column. After loading, the column was washed with 2 L of water and eluted with a 100 min linear gradient of 5−100% aqueous MeOH at a flow rate of 10 mL/min. The column was washed with 7:3 CH_3CN−*i*-PrOH. Each fraction was 50 mL, and a total of 25 fractions were collected. Fractions 14−21 contained various lucensimycins. These fractions were repooled into four fractions on the basis of analytical HPLC. Concentration under reduced pressured followed by lyophilization gave fractions 14, 15 (A, 0.26 g), fractions 16, 17 (B, 0.5 g), fraction 18 (C, 0.17 g), and fractions 19, 20 (D, 0.21 g) all as yellowish powders. Fraction D was chromatographed by reversed-phase HPLC on a Zorbax RX C_8 (21.2 × 250 mm) column eluting with a 37 min linear gradient of aqueous CH_3CN + 0.1% TFA at a flow rate of 12 mL/min. After two identical runs, the fractions containing the major peak eluting at 27 min were combined and lyophilized to afford lucensimycin A (**1**, 19.2 mg, 6.4 mg/L). Fractionation of fraction B by similar reversed-phase HPLC followed by lyophilization of fractions eluting at 13, 24−25, 29−30, and 33−34 min afforded lucensimycins F (**6**, 13.9 mg), D (**4a**, 68.5 mg), E (**5**, 63.6 mg), and C (**3**, 15.4 mg). Similar reversed-phase chromatography of fraction C followed by lyophilization produced lucensimycin E (**5**, 90 mg). Preparative reversed-phase HPLC of fraction A on the same Zorbax RX C_8 column and elution at 12 mL/min with a shallower 49 min linear gradient of 30−50% aqueous CH_3CN + 0.1% TFA and lyophilization of fractions eluting at 10, 13, 24−25, 29−30, and 33−34 min gave lucensimycins G (**7**, 15 mg, 5 mg/L), F (**6**, 10 mg, total 23.9 mg, 8 mg/L), D (**4a**, 13.7, total 82.2 mg, 27.4 mg/L), E (**5**, 7.7 mg, total 161.3, 53.7 mg/L), and C (**3**, 2.4 mg, total 17.8 mg, 5.9 mg/L).

Lucensimycin D (4a): colorless, amorphous powder; $[\alpha]^{23}_D$ −11.9 (*c* 0.67, MeOH); UV (MeOH) λ_{max} 285 (log ϵ 4.39) nm; IR (ZnSe) ν_{max} 3333, 2932, 1800 (w), 1747, 1688, 1616, 1547, 1378, 1221, 1136, 1065, 1010, 984, 931, 904, 860, 797, 740, 663 cm^{-1}; for ^1H and ^{13}C NMR, see Table 1; HRESIFTMS *m/z* 688.2435 (calcd for $C_{34}H_{41}NO_{12}S$+H, 688.2427), 670.2329 (calcd for $C_{34}H_{39}NO_{11}S$+H, 670.2322).

Lucensimycin E (5): colorless, amorphous powder; $[\alpha]^{23}_D$ +58.8 (*c* 2.5, MeOH); UV (MeOH) λ_{max} 285 (log ϵ 4.38) nm; IR (ZnSe) ν_{max} 3359, 2935, 1801 (w), 1749, 1690, 1617, 1379, 1239, 1137, 1066, 1011, 987, 894, 860, 739 cm^{-1}; for ^1H and ^{13}C NMR, see Table 1; HRESIFTMS *m/z* 688.2419 (calcd for $C_{34}H_{41}NO_{12}S$+H, 688.2427), 670.2311 (calcd for $C_{34}H_{39}NO_{11}S$+H, 670.2322).

Lucensimycin F (6): colorless, amorphous powder; $[\alpha]^{23}_D$ −6.25 (*c* 0.8, MeOH); UV (MeOH) λ_{max} 289 (log ϵ 4.63) nm; IR (ZnSe) ν_{max} 3329, 2931, 1802 (w), 1750, 1673, 1620, 1537, 1379, 1239, 1138, 1022, 721 cm^{-1}; for ^1H and ^{13}C NMR, see Table 3; HRESIFTMS *m/z* 1011.3639 (calcd for $C_{46}H_{62}N_2O_{21}S$+H, 1011.3643), 831.3018 (calcd for $C_{40}H_{50}N_2O_{15}S$+H, 831.3010).

Lucensimycin G (7): colorless, amorphous powder; $[\alpha]^{23}_D$ +84.2 (*c* 0.95, MeOH); UV (MeOH) λ_{max} 290 (log ϵ 4.65) nm; IR (ZnSe) ν_{max} 3329, 2938, 1804 (w, olefin), 1745, 1689, 1615, 1541, 1380, 1241, 1139, 1023, 669 cm^{-1}; for ^1H and ^{13}C NMR, see Table 3; HRESIFTMS *m/z* 1011.3657 (calcd for $C_{46}H_{62}N_2O_{21}S$+H, 1011.3643), 831.3021 (calcd for $C_{40}H_{50}N_2O_{15}S$+H, 831.3010).

Reaction of Lucensimycin A (1) with *N*-Acetyl-L-cysteine. To a solution of lucensimycin A (**1**, 2.7 mg) in 200 μL of CH_3CN was added a solution of *N*-acetyl-L-cysteine (10 mg) in 300 μL of CH_3CN. The solution was stirred at room temperature, and the product formation was quantitatively monitored by injecting an aliquot of the reaction mixture to an optimized HPLC (Zorbax RX C_8, 4.6 × 250 mm, 15 min linear gradient of 20−95% aqueous CH_3CN + 0.1% TFA, 1 mL/min). Retention times: **1** (11.9 min), **5** (9.9 min), and **4a** (8.9 min). The reaction was complete after 170 h, affording a 2:1 mixture of **5** and **4a**, which were confirmed by HPLC co-injections and LCMS analysis (Figure 1).

Reaction of Lucensimycin D (4a) with Diazomethane. To a solution of lucensimycin D **4a** (4 mg) in a 0.3 mL mixture of $CHCl_3$ + MeOH (4:1) was added 0.3 mL of a 2 M diethyl ether solution of TMS−diazomethane. The solution was stirred at room temperature for 4 h and concentrated to dryness, and the major products were purified by reversed-phase HPLC (Zorbax RX C_8, 21.2 × 250 mm, 37 min linear gradient of 40−65% aqueous CH_3CN + 0.1% TFA, 12 mL/min). Fractions eluting at 23 and 24 min were lyophilized to afford dimethyl ester **4b** (1 mg) and pyrazole **8** (1.2 mg). **4b**: ^1H NMR 500 MHz (CD_3OD, 25 °C) δ 1.10, 1.21 (1H each, m, H-1), 4.39 (1H, dt, *J* = 4.5, 10 Hz, H-2), 1.52 (1H, m, H-3), 2.91 (1H, t, *J* = 10 Hz, H-4), 1.77 (1H, brt, *J* = 10 Hz, H-5), 6.03 (1H, brd, *J* = 10 Hz, H-6), 5.78 (1H, ddd, *J* = 2.5, 4.5, 10 Hz, H-7), 2.40 (1H, m, H-8), 2.25 (1H, m*, H-10), 3.21 (1H, m, H-13), 2.26 (1H, m*, H-14), 1.91 (1H, dt, *J* = 15, 2.5 Hz, H-14), 5.66 (1H, dd, *J* = 15, 9 Hz, H-15), 6.32 (1H, dd, *J* = 15, 11 Hz, H-16), 6.56 (1H, dd, *J* = 15, 11 Hz, H-17), 6.41 (1H, dd, *J* = 15, 11 Hz, H-18), 7.28 (1H, dd, *J* = 15, 11 Hz, H-19), 5.95 (1H, d, *J* = 15 Hz, H-20), 4.85 (1H, HDO overlap, H-23), 3.10 (1H, dd, *J* = 15, 3.0 Hz, H-24), 2.79 (1H, dd, *J* = 15, 7 Hz, H-24), 1.04 (3H, d, *J* = 6.5 Hz, H-26), 1.09 (3H, s, H-27), 3.13 (1H, dd, *J* = 14, 4.5 Hz, H-28), 2.99 (1H, dd, *J* = 14, 8 Hz, H-28), 4.65 (1H, m, H-29), 8.31 (1H, d, *J* = 8 Hz, NH), 3.73, 3.71 (3H each, s, 2 × OCH_3); HRESIFTMS *m/z* 716.2734 (calcd for $C_{36}H_{45}NO_{12}S$+H, 716.2740).

Compound 8: ^1H NMR 500 MHz (CD_3OD, 45 °C) δ 1.15, 1.75 (1H each, m, H-1), 4.74 (1H, dt, *J* = 5, 11 Hz, H-2), 1.55 (1H, m, H-3), 3.03 (1H, t, *J* = 10 Hz, H-4), 1.80 (1H, brt, *J* = 10 Hz, H-5), 6.0 (1H, brd, *J* = 10 Hz, H-6), 5.79 (1H, m, H-7), 2.25 (1H, m, H-8), 2.13 (1H, ddd, *J* = 12.5, 10, 3 Hz, H-10), 4.93 (1H, brd, *J* = 12 Hz, H-12), 3.02 (1H, m, H-13), 1.9, 1.75 (1H each, m, H-14), 5.80 (1H, m, H-15), 6.24 (1H, ddd, *J* = 15, 11 Hz, H-16), 6.45 (1H, dd, *J* = 15, 11 Hz, H-17), 6.20 (1H, dd, *J* = 15, 11 Hz, H-18), 7.22 (1H, dd, *J* = 15, 11 Hz, H-19), 5.84 (1H, d, *J* = 15 Hz, H-20), 6.76 (1H, d, *J* = 2.5 Hz, H-24), 7.64 (1H, d, *J* = 2.5 Hz, H-25), 1.08 (3H, d, *J* = 6.5 Hz, H-26), 0.83 (3H, s, H-27), 2.05 (3H, s, $COCH_3$), 3.69 (3H, s, OCH_3); ^{13}C NMR 125 MHz (CD_3OD, 45 °C) δ 32.8 (C-1), 77.1 (C-2), 46.5 (C-3), 77.0 (C-4), 45.9 (C-5), 127.8 (C-6), 131.0 (C-7), 46.5 (C-8), 52.1 (C-9), 35.3 (C-10), 212.8 (C-11), 61.0 (C-12), 43.8 (C-13), 36.6 (C-14), 141.0 (C-15), 130.4 (C-16), 142.0 (C-17), 131.9 (C-18), 146.2 (C-19), 121.0 (C-20), 169.3 (C-21), 194.5 (C-22), 139.2 (C-23), 106.6 (C-24), 132.5 (C-25), 15.7 (C-26), 14.8 (C-27), 51.9 (OCH_3), 172.6 ($O\underline{C}OCH_3$), 21.0 ($OCO\underline{CH_3}$); HRESIFTMS *m/z* 537.2595 (calcd for $C_{30}H_{36}N_2O_7$+H, 537.2600).

Two-Plate Differential Sensitivity RPSD Assay. *S. aureus* cells (RN450) carrying plasmid S1-782B bearing antisense to RPSD (*rpsD* AS-RNA strain) or vector (control strain, EP167) were inoculated from a frozen vial source into a 13 mm culture tube containing 3 mL of Miller's LB broth (Invitrogen) plus 34 μg/mL chloramphenicol. Tubes were incubated at 37 °C at 220 rpm for 18−20 h and kept at room temperature until use. Miller's LB broth was supplemented with 1.2% Select agar (Invitrogen, autoclaved and cooled to 48 °C), 0.2% glucose, 15 μg/mL chloramphenicol, and 12 mM xylose (only for the antisense strain). The culture that grew to OD_{600} = 3.0 was diluted 1:1000 by addition of Miller's LB agar medium (measured OD 0.003). A 100 mL aliquot of each mixed culture (control and antisense strain) in the LB medium was poured into each 20 × 20 cm NUNC plate and well-caster templates placed into the agar, and the agar was allowed to solidify for 30 min. Then, 20 μL quantities of test samples were added to the wells and the plates incubated at 37 °C for 18 h, and zones of inhibition were measured. MDC (minimum detection concentration) values were determined by 2-fold serial dilution.

Antibiotic Assay (MIC). The MIC (minimum inhibitory concentration) against each of the strains was determined by National Laboratory Standard Institute (NLSI) protocol as previously described.[33] Cells were inoculated at 10^5 colony-forming units/mL followed by incubation at 37 °C with a 2-fold serial dilution of compounds in the growth medium for 20 h. MIC is defined as the lowest concentration of an antibiotic inhibiting visible growth.

Antibiotic Assay (Agar Plate Assay). The agar plate impregnated with *S. aureus* was prepared in identical conditions as described for the two-plate differential sensitivity assay except for the use of only *S. aureus* EP167 strain without xylose.

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Supporting Information Available: ^{1}H and ^{13}C NMR data of **4a**, **4b**, **5**, **6**, **7**, and **8**, ^{1}H and ^{13}C NMR of time-dependent conversion of **4a** to **5**, phylogenetic tree (Figure S1), and antibacterial test on agar plate (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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