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Discovery of Lucensimycins A and B from *Streptomyces lucensis* MA7349 Using an Antisense Strategy

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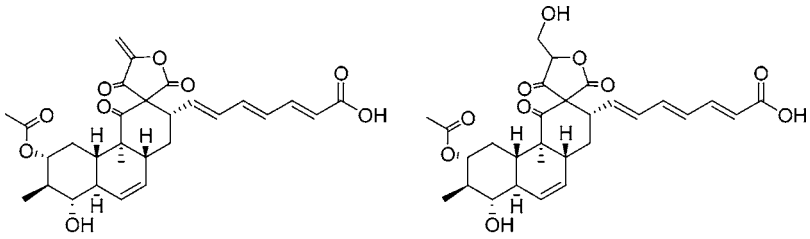
ABSTRACT



Lucensimycin A **Lucensimycin B**

Inhibition of protein synthesis is one of the validated and highly successful targets for inhibition of bacterial growth; this mechanism is a target of a large number of clinical drugs. Ribosomal protein S4, a primary protein, is a potential target for the discovery of antibacterial agents. We describe, using an antisense-sensitized *rpsD Streptomyces aureus* strain, the discovery and activity of lucensimycins A and B.

The incidence of infections by drug-resistant bacteria continues to grow and remains a serious threat to human lives. Protein synthesis is one of the most effective antibacterial drug targets that has led to a number of clinically useful agents.[1] These include macrolides, aminoglycosides, tetracyclines, streptogramins, lincosamides, and oxazolidinones. Protein synthesis is catalyzed by ribosomes which are composed of two asymmetric macromolecular components, the large (50S) and small (30S) subunits. The large subunit consists of two ribosomal RNAs (rRNAs), 23S and 5S, and 34 unique ribosomal proteins (r-proteins), L1−L34.[1a,2] The small subunit is composed of 16S rRNA and 21 r-proteins, S1−S21.[2] All but protein S1 of the small subunit appear to be essential for normal ribosomal functioning and protein synthesis.[2] The catalytic power of protein synthesis resides in the ribosome itself. Ribosomal proteins help the ribosome maintain its tertiary structure. Most of the drugs that are in clinical use today bind not only to rRNA but also to one or more r-proteins. Alteration of the binding to either rRNA or r-proteins inactivates the action of drugs.[1a] Therefore, it is expected that selectively altering the conformation of a particular r-protein or inhibiting the synthesis of an r-protein would potentially result in the loss of function and may lead to the inhibition of protein synthesis. Small ribosomal protein S4 is encoded by the *rpsD* gene in both Gram-positive and Gram-negative bacteria.[3]

S4 is a primary protein that is required for functioning of the ribosome. Its structure appears to be conserved across

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Table 1. ^{1}H and ^{13}C NMR Data of Lucensimycin A (**1a**) and B (**2a**) in CD_3OD

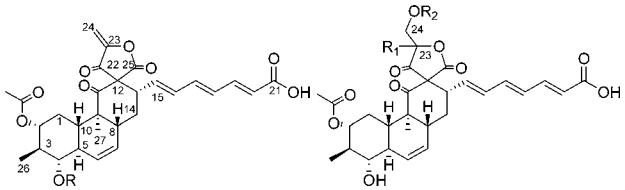
	1a			**2a**[a]		
no.	δ_C	δ_H (J in Hz)	all HMBC	δ_C	δ_H (J in Hz)	key HMBC
1	32.2	1.12, m		32.5	1.16, m	
		1.00, m			1.06, m	
2	76.7	4.32, dt, 4.5, 11	C-1, 3, 4, 10, 26, 1′	77.6	4.09, dt, 4.5, 11	
3	45.8	1.55, m	C-1, 2, 4, 5, 26	45.8	1.56, m	
4	77.2	2.94, t, 10	C-2, 3, 5, 6, 10	77.5	2.91, t, 10	
5	45.3	1.80, m	C-4, 6, 7, 10	45.5	1.79, brt, 10	
6	127.4	6.06, brd, 10	C-4, 5, 8, 10	127.2	6.04, brd, 10	
7	130.2	5.83, ddd, 10, 4.5, 2.5	C-5, 9	130.4	5.83, ddd, 10, 4.5, 2.5	
8	44.7	2.47, md, 13	C-6, 7, 10, 13, 14	44.9	2.43, md, 13	
9	53.3	--		53.7	--	
10	32.9	2.19, dt, 2.5, 12	C-1, 2, 5, 6, 8, 9, 11	33.3	2.06, dt, 2.50, 12	
11	202.1	--		201.7	--	
12	72.8	--		73.6	--	
13	46.0	3.29, ddd, 12, 8, 3.5	C-8, 11, 12, 14, 16, 22, 25	45.6	3.21, ddd, 13, 9, 4	C-8, 12, 15, 16, 23, 25
14	32.7	2.44, q, 13	C-7, 8, 9, 12, 13, 15	31.6	2.44, q, 13	
		1.99, m	C-7, 8, 9, 12, 13, 15		1.96, m	
15	134.0	5.69, dd, 15, 9	not used due to overlap	133.9	5.75, dd, 15, 9	
16	135.2	6.33, dd, 15, 11	C-13, 15, 17, 18	135.7	6.28, dd, 15, 11	
17	139.9	6.51, dd, 15, 11	C-15, 16, 18, 19	140.7	6.47, dd, 15, 11	
18	132.8	6.41, dd, 15, 11	C-16, 17, 19, 20	131.8	6.36, dd, 15, 11	
19	145.3	7.25, dd, 15, 11	C-17, 18, 20, 21	145.4	7.22, dd, 15, 11	
20	123.8	5.93, d, 15	C-17, 18, 21	123.5	5.90, d, 15	
21	170.7	--		171.1	--	
22	190.1	--		203.1	--	
23	151.1	--	C-2, 3, 4	86.0	4.72, t, 2.5	C-12, 22, 25
24	99.2	5.29, d, 3	C-12(w), 22, 23, 25(w)	60.2	3.92, dd, 13, 2.5	C-12 (w), 22, 23
		5.62, d, 3	not used due to overlap		4.04, dd, 13, 2.5	C-22, 23
25	169.4	--		172.3	--	
26	14.7	1.07, d, 6.5	C-2, 3, 4	14.7	1.09, d, 6	
27	16.9	1.11, s	C-8, 9, 10, 11	16.6	1.10, s	
1′	172.0	--		172.6		
2′	20.9	2.00, s	C-1′	21.0		C-1′

[a] **2a** exhibited HMBC correlations identical to those for **1a**. Only key correlations are listed here.

bacterial species and therefore represents a broad spectrum target. In a genomewide strategy for the identification of essential genes in *Streptomyces aureus* using antisense, Forsyth et al.[4] showed that the *rpsD* gene function is essential for bacterial growth.

Recently, we reported the design and application of a two-plate whole-cell differential sensitivity screening assay using an antisense-sensitized *S. aureus* strain in which the FabF target was dialed down.[5] This led to the discovery of platensimycin, a novel and potent FabF inhibitor with in vivo antibiotic properties.[6] A similar two-plate assay with a dialed down *rpsD* gene by antisense was used to screen natural product extracts. This strategy led to the identification of an extract derived from a strain of *Streptomyces lucensis* MA7349. Bioassay-guided fractionation of this extract led to the isolation of lucensimycin A (**1a**) and B (**2a**). The isolation, structure elucidation, and activities of these compounds are herein described.

Streptomyces lucensis MA7349 was isolated from a soil sample collected in Martinique Island.[7] One liter of fermentation broth (pH 5.0) was extracted with ethyl acetate, concentrated and dissolved in methanol−water (4:1), washed with hexanes, and chromatographed on Sephadex LH20 in methanol and by reversed-phase C_{18} HPLC to afford 3.6 mg (3.6 mg/L) of **2a** and 21.8 mg (21.8 mg/L) of **1a** as amorphous colorless powders.[8]



HRESIFTMS analysis of **1a** produced a molecular formula of $C_{29}H_{32}O_9$ (obsd m/z 547.1952; calcd for M + Na, 547.1944) suggesting 14 degrees of unsaturation. ^{1}H and ^{13}C NMR spectral data were in full agreement with the assigned molecular formula. The UV spectrum showed an absorption maximum at λ_{max} 280 nm suggesting a high degree of conjugation. The IR spectrum showed key absorption bands for hydroxy (3417 cm^{-1}), ene-γ-lactone (1816 cm^{-1}), and

ester (1740 cm^{-1}) groups. The ^{13}C NMR spectrum in CD_3OD (Table 1) displayed signals for 29 carbons which when coupled with a DEPT spectrum suggested the presence of three methyls, three methylenes (one is olefinic), 15 methines (seven sp^3 including two oxygenated and eight sp^2), three quaternary carbons (one olefinic), three ester/lactone carbonyls , and two ketones (an enone and a saturated ketone). The $^1H-^1H$ spin-coupling network was determined by a DQCOSY experiment, and one-bond proton−carbon connectivity was determined by an HMQC experiment. The ^1H NMR spectrum of **1a** displayed a doublet for a methyl at δ_H 1.07 (J = 6.5 Hz) suggesting the presence of a secondary methyl group. The remaining methyl groups appeared as singlets, one resonating at δ_H 1.11 and the other at δ_H 2.00, indicating an angular and an acetyl methyl, respectively. These methyls correlated to δ_C 14.7, 16.9, and 20.9 ppm, respectively, in the HMQC experiment. Six of the eight olefinic methines provided extended conjugation terminating with a doublet for H-20 (δ_H 5.93, J = 15 Hz) at one end and a doublet of doublets for H-15 (δ_H 5.69, J = 15, 8.5 Hz) at the other. The H-15 exhibited COSY correlation to the H-13 (δ_H 3.29, ddd, J = 12, 8, 3.5 Hz) which showed correlations to H_2-14 that in turn displayed COSY cross peaks to the H-8 (δ_H 2.47, md, J = 13 Hz). Careful analysis of the COSY correlations of H-7 led to its connectivity to H-8−H_2-14−H-13 on one side and to H-6−H-5 on the other leading to the formation of ring A (H-5[H-10]−H-4−H-3−H-2−H-1−[H-10]) and establishing the connectivity of the major part of the molecule (Figure 1).

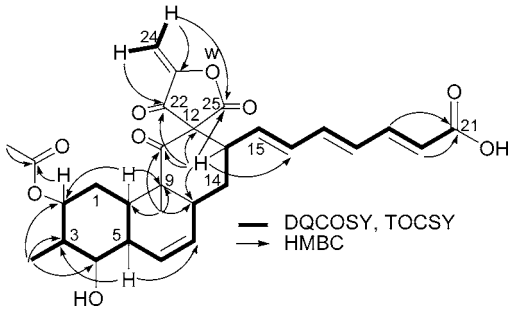
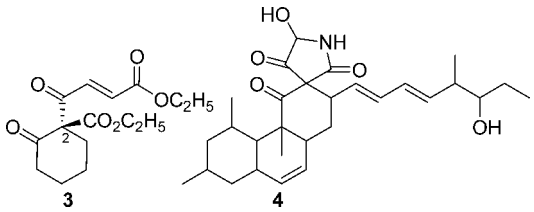


Figure 1. COSY and HMBC correlations of **1a**.

The COSY/HMQC correlations also established the oxygenations at C-2 (δ_H 4.32, dt, J = 4.5, 11 Hz, δ_C 76.7) and C-4 (δ_H 2.94, t, J = 10 Hz, δ_C 77.2). The remaining part of the molecule was connected to the COSY-derived fragment by HMBC correlations. The downfield ^1H NMR shift of H-2, its HMBC correlation to C-1′ (δ_C 172.0), and its HMBC correlation of CH_3-2′ to C-1′ confirmed the location of the acetate group at C-2. The angular methyl protons H_3-27 (δ_H 1.11) displayed two- and three-bond HMBC correlations to C-10 (δ_C 32.9), C-9 (δ_C 53.3), C-8 (δ_C 44.7), and C-11 (δ_C 202.1) thus linking three additional carbons and producing ring B. H-13 showed HMBC correlations to C-8, C-11, C-12 (δ_C 72.8), C-22 (δ_C 190.1), and C-25 (δ_C 169.4) along with correlations from both protons of the olefinic methylene to C-22 and C-23 (δ_C 151.1), and a four-bond correlation, albeit weak, to C-25 helped in assembling the C-ring and the γ-lactone enone leading to the assignment of the planar structure of **1a**. The downfield ^{13}C NMR shift of C-12 (δ_C 72.8) was initially surprising; however, a similar shift is not unprecedented (for example, synthetic compound **3**, δ_C 73.7, C-2[9] and the acid-catalyzed product of delaminomycin A (**4**)).[10]



The relative configuration of **1a** was determined by NOESY, NOE difference, molecular modeling (ChemDraw 3D), and magnitude of the scalar couplings. H-2 exhibited NOESY correlations to H-4 and H-10 suggesting their 1,3,5-triaxial relationships in a chair conformation of ring A which was further supported by the NOESY correlation of H-3 to H-5 (Figure 2). H-10 displayed a NOESY correlation to H-8 which in turn showed NOESY correlation to H-13 thus

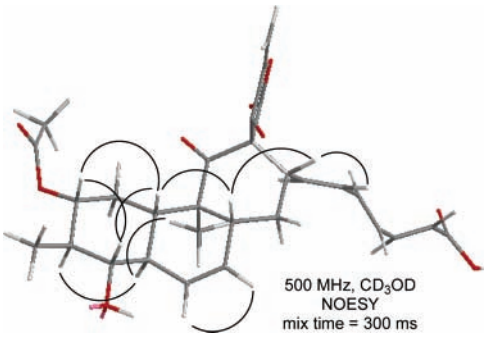


Figure 2. NOESY and NOE difference data of **1**.

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establishing their 1,3-diaxial relationships and a trans−trans A/B/C ring fusion. The scalar couplings of these protons showed at least one large coupling (J = >10 Hz) confirming their axial assignments in chair-twisted-chair-twisted-chair conformations. The *Z*-olefin geometry was assigned for $\Delta^{6,7}$, and an all-*E*-geometry was assigned to the trienoic acid chain at C-13 by respective J values of 10 and 15 Hz. The relative configuration of the *spiro*-γ-lactone could not be determined. Not surprisingly, the enone of γ-lactone showed reactivity to nucleophiles. For example, Michael addition of CD_3OD was observed when a solution (60 mg/mL) of **1a** in CD_3OD was stored for 5 days leading to the formation of 30% of a deuterated adduct (**2b**, ESIMS m/z 578 (M + NH_4) and 559 (M − H)). Compound **1a** produced a monoacetate **1b** (ESIMS m/z 567 (M + H); ^{1}H NMR (CD_3OD) δ_{H-4} 4.53, t, J = 10.5 Hz, δ_{H-4Ac} 2.13, s) upon acetylation with Ac_2O/C_5D_5N. On the basis of these data, structure **1a** was assigned for lucensimycin A.

Lucensimycin B (**2a**) was isolated as a minor congener of **1a**. Mass spectral analysis of **2a** showed a molecular formula of $C_{29}H_{34}O_{10}$ (obsd m/z 565.2024; calcd for M + Na, 565.2049) and differed from **1a** by an extra molecule of water. Comparisons of ^{1}H and ^{13}C NMR spectra indicated the absence of signals for the olefinic methylene group of the enone and exhibited the presence of an oxymethine (δ_H 4.72, t, J = 2.5 Hz; δ_C 86.0) and an oxymethylene (δ_H 3.92, 4.04, each dd, J = 13, 2.5 Hz; δ_C 60.2) group. The hydration of the olefin resulted in elimination of the conjugation leading to the expected downfield shift of C-22 (δ_C 203.1). The HMBC correlations of H-23 and H-24 to respective carbons (Table 1) confirmed the hydration of C-23−C-24 and confirmed the structure **2a** for lucensimycin B.

Lucensimycin A at 10 μg exhibited a 14 mm hazy zone of clearance on agar plates seeded with a sensitized antisense *rpsD* strain compared to a 12.1 mm hazy zone of clearance on a control *S. aureus* strain. The zone of clearance was dose dependent with decreasing diameter and quality of zone of clearance, tested at 2-fold dilutions. At 0.63 μg, the zones of clearance were 8 and 7.3 mm, respectively, with very hazy qualities. Lucensimycin B was significantly less active and only showed zones of inhibition of 9.3 and 6.8 mm, respectively, at 10 μg. These compounds did not show a significant zone differential between antisense and control plates indicating a lack of target specificity. Niether of these two compounds showed activity in a liquid MIC assay at the highest concentration of 250 μg/mL against wild-type *S. aureus*.

Delaminomycins A−C were reported from *Streptomyces albulus* MJ202-72F3 with antibacterial activity (MIC 25 μg/mL against *S. aureus*), inhibition of cell adhesion to an extracellular matrix receptor, and immunomodulator and cytotoxicity activities.[11] Acid treatment of delaminomycin A produced compound **4**. Lucensimycins are natural products and are not derived from acid treatment during isolation as evidenced by direct HPLC analysis of the EtOAc extract.[12]

In summary, we have described here the discovery and structure of two new natural products by using a new antisense-based antibacterial discovery method. Unlike platensimycin that was identified in the FabF program using this method,[6] these compounds display weak antibiotic activity and may potentially weakly bind/inhibit the S4 protein. Weak activity prevented additional work on these compounds.

Supporting Information Available: ^{1}H and ^{13}C NMR data of compounds **1a** and **2a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) It was identified as *Streptomyces lucensis* MA7349 by 16S rDNA analysis. Phylogenetic analysis of the strain suggested a close relationship with *Streptomyces lucensis* NBRC13056 with a bootstrapping value of 85%. The strain was grown on a 10 mL seed medium {composition in grams/liter: 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} for 3 days at 28 °C. A portion of 5% of seed inoculum was transferred to a 50 mL production medium {composition in grams/liter: yellow corn meal 40.0, lactose 40.0, Amberex 5.0} in a 250 mL Erlenmeyer flask and was incubated at 28 °C for 7 days at 220 rpm while maintaining 70% relative humidity.

(8) Physical properties (**1a**): $[\alpha]^{23}_D$ +9 (c 4.75, MeOH), UV (MeOH) λ_{max} 280 (ϵ = 18 570) nm; IR ν_{max} (ZnSe) 3417, 2937, 1816, 1740, 1690, 1648, 1618, 1451, 1380, 1238, 1219, 1137, 1098, 1072, 1008, 986, 934, 898, 860, 820, 740, 665 cm^{-1}. (**2**): $[\alpha]^{23}_D$ + 41.1 (c 1.8, MeOH); λ_{max} 285 (ϵ = 19 150) nm; IR ν_{max} (ZeSe) 3384, 2930, 1801, 1750, 1691, 1617, 1452, 1381, 1241, 1137, 1097, 1063, 1009, 984, 860, 809, 739 cm^{-1}.

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(12) Whether compound **2a** is a natural product or an artifact of hydration of **1a** is not clear.