SUPPLEMENTARY MATERIAL

Angucycline antibiotics and its derivatives from marine-derived actinomycete *Streptomyces* sp. A6H

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Abstract: Vineomycin A₁ (**1**) and B₂ (**2**) were isolated from the culture broth of marine actinomycete *Streptomyces* sp. A6H. Five hydrolysis products were obtained by rational hydrolysis and methanolysis of the fermentation extract. Their structures were characterized as aquayamycin (**3**), vineomycinone B₂ (**4**), 9-C-D-oliviosyltetrangulol (**5**), 7-O-methylgaltamycinone (**6**) and vineomycinone B₂ methyl ester (**7**). In addition to these compounds, two ester derivatives vineolactone A (**8**) and vineomycinone B₂ benzyl ester (**9**) of compound **4** were generated semisynthetically. Compound **6** is a new analogue of galtamycinone while compounds **8** and **9** are new members of vineomycins. Cytotoxic activities and antimicrobial activities were determined for all compounds. The results indicate that only compound **1** showed significant activities with IC₅₀ value of 0.34 μM against H1975 and MIC value of 4 μg/mL against *Staphylococcus aureus*.

Keywords: *Streptomyces* sp. A6H; angucycline; hydrolysis; semisynthesis;

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Experimental

General experimental procedures. HPLC analysis was performed on Shimadzu High Performance Liquid Chromatography (DGU-20A5 Degasser, LC-20AT Liquid Chromatography, SIL-20AC Auto Sampler, SPD-M20A Diode Array Detector, CTO-20AC Column Oven) using Inerstil ODS-SP (5 μ m, 4.6 $\times$ 250 mm) column. UV spectra were obtained from HPLC analysis. NMR spectra were recorded on a 600 MHz Bruker Advance NMR spectrometer at 298K in DMSO- d_6 or $CDCl_3$. High resolution mass spectra were acquired using an Agilent 1260 HPLC-6230 TOF tandem mass spectrometer. Preparative HPLC was performed on Beijing Chuangxintongheng LC3000 Semi-preparation Gradient HPLC System using Sepax Amethyst C-18 (5 μ m, 21.2 $\times$ 250 mm) column. TLC was carried out on Qingdao Puke Sil G/UV254 plates of 0.25 thickness, and spots were visualized by spraying with 10% H_2SO_4 /EtOH followed by heating.

Microorganism. The actinomycetes were isolated from a sediment sample collected in Taiwan Strait, China. The sample was collected in a 50 mL centrifuge tube containing 10 mL 40% glycerol water solution. After shaken at 3000 rpm for 30 min, the sample was diluted with saline (10^{-1} , 10^{-2} and 10^{-3} , respectively). Then 100 μ L aliquots were spread onto Gause's agar containing nalidixic acid (40 μ g/mL). The plates were incubated 10 days at 28°C, and the resulting colonies were transferred to Gause's agar. The taxonomic identity of the strain A6H was determined by 16S rDNA sequence analysis. The top sequence of A6H was 99% sequence similarity to two *Streptomyces cellulosa* strains NRRL B-2889 and NBRC 13027 (accession number: NR_043815.1 and NR_112346.1) and 99% sequence similarity to other nine *Streptomyces* sp. strains Table S1) in the GenBank database. Therefore, the taxonomy of actinomycete A6H was proposed to be *Streptomyces* sp. A6H.

Large-scale fermentation and extraction. Spores of strain A6H were inoculated to eighteen 500-mL Erlenmeyer flask containing 250 mL medium described above and shake-cultured for 3 days at 28°C. Afterwards, 5 mL portions of the culture were inoculated to 500-mL Erlenmeyer flasks containing 250 mL the same medium. A total of 180L fermentation was carried out at 28°C on a rotary shaker at 180rpm for 7 days.

Then the fermentation broth was combined and filtered. And the filtrate was extracted with EtOAc three times and dried *in vacuo* to provide the crude extract (15g).

Isolation. The crude extract (15g) was subjected to a silica gel column, eluting with a gradient of dichloromethane – methanol (100:1, 80:1, 60:1, 40:1, 20:1, 10:1 0:1) to give eleven fractions (Fr. A-Fr. K). Fr. D (3.3g) was subjected to another silica gel chromatography to give five fractions (Fr. D1-D5). Fr. D2 was separated by preparative HPLC using acetonitrile-H₂O as the mobile phase (60% CH₃CN maintained for 60 min, flow rate 10mL/min) to yield compound **1** (12 mg, *t_R*=58 min). Fr. E (1.5g) was subjected to preparative HPLC using acetonitrile – H₂O as the mobile phase (0-30min: 65%-100% CH₃CN, 30-40min: 100% CH₃CN, flow rate 10mL/min) to yield compound **2** (2mg, *t_R*=30min).

Hydrolysis. The fractions were recombined and delivered to hydrolysis. A mixture (2g) in 0.1N HCl-THF (50mL) was treated at 40°C for 24h. After that, the reaction mixture was concentrated and the residue was resuspended with 20 mL water, extracted with CH₂Cl₂ (20 mL × 3), washed with water and concentrated to dryness. The residue was subjected to a silica gel chromatography to give four fractions (Fr. 1-4). Fr. 2 was purified by preparative HPLC (CH₃OH/H₂O, 90% CH₃OH maintained for 35min, flow rate 10mL/min) to obtain compound **5** (2.4mg, *t_R*=26min) and compound **6** (2.3mg, *t_R*=29min). Fr. 3 was separated by preparative HPLC (CH₃OH/H₂O, 45% CH₃OH maintained for 30min, flow rate 10mL/min) to give compound **3** (5mg, *t_R*=21min). Fr. 4 was separated by preparative HPLC (CH₃CN/H₂O, 30% CH₃CN maintained for 60min, flow rate 10mL/min) to give compound **4** (70mg, *t_R* =45min).

Methanolysis. The combined mixture (100mg) was dissolved in 0.1N HCl-MeOH (5mL) and stirred at 40°C for 10h. The reaction mixture was neutralized with saturated NaHCO₃ and evaporated to dryness. The residue was resuspended with 10 mL water, extracted with EtOAc (10 mL × 3), washed with water and concentrated to dryness. The products were subjected to a silica gel chromatography to yield compound **7** (9mg) as a orange powder.

Synthesis of 8 from 4. A solution of compound **4** (24.3mg, 0.05mmol) and

trimethylamine (24 μ L, 0.175mmol) in 2mL THF and 8mL CH₂Cl₂ was added over 6h to a solution of 2,4,6-trichlorobenoyl chloride (11.8 μ L, 0.075mmol) and DMAP (6.5mg, 0.055mmol) in CH₂Cl₂ (1mL). The solution was then allowed to stir for 1h after complete addition after which time it was quenched with H₂O (10mL). The phases were separated and the aqueous phase was extracted with CH₂Cl₂ (2 $\times$ 10mL). The organic phases were combined and the solvent was removed via rotary evaporation to yield a brown solid which was purified by preparative HPLC to yield compound **8** (10mg, 42%) as a yellow powder.

Synthesis of 9 from 4. A solution of compound **4** (4.86mg, 0.01mmol) and KOH (1.8mg, 0.032mmol) in DMF (2mL) was treated with benzyl bromide (3 μ L, 0.025mmol) and stirred at 40°C for 4h. The reaction mixture was diluted with water and then extracted with CH₂Cl₂ three times. The organic phases were combined, washed with water ten times and dried *in vacuo* to yield a brown oil which was purified by preparative HPLC to yield compound **9** (3.2mg, 56%) as a yellow powder.

Antimicrobial assay

Antimicrobial activities were measured against *Staphylococcus aureus* ATCC25923, methicillin-resistant *Staphylococcus aureus* ATCC43300, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC10231 and *Fusarium graminearum* by broth microdilution method. The microbial cultures were prepared by diluting precultured broths to 5 $\times$ 10⁵ cfu/mL. The assay was performed in 96-well plates. Each compound were tested in dilution series ranging from 64 to 0.0625 μ g/mL. The plates were incubated at 35°C for 24 h. Then the MICs were recorded as the lowest concentrations that completely inhibited bacterial growth, assessed with visual inspection.

Cytotoxicity assay

Human cancer cell lines H1975, SGC-7901 and EC109 were cultured in 1640 medium (Gibco Company) with 10% FBS (Hyclone Company) at 37 °C in a humidified incubator (Thermo Company) with 5% CO₂. The cells (5 $\times$ 10³/well) were inoculated in 96-well plates and treated with compounds for 24h. Then 10 μ L of CCK8 (Dojindo Company) was added and reacted for 1-3h. The OD values were measured at 450nm using a multiskan spectrum (Plus 384, MD Company).

16S rDNA sequence of strain A6H

ACGCTGTGCGGCGTGCTTAACACATGCAAGTCCAACGATGAACCACTTCG
GTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGC
ACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGATCGCC
TTGGGCATCCTTGGTGATCGAAAGCTCCGGCGGTGCAGGATGAGCCCCGCG
GCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTA
GCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTG
ATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTT
TCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGG
AATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAA
AGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTT
CGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATC
AGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTG
AGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGC
CGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGCGGAGCATGTGGCT
TAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAA
AACCTTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGG
CTGTCGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCG
CAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCAC
GGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCA
TCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAA
TGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGT
TCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAAT
CGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACC

Table S₁. Sequences producing significant alignments

Description	Max score	Total score	Query cover	Accession
<i>Streptomyces cellulosae</i> NRRL B-2889	2590	2590	99%	NR_043815.1
<i>Streptomyces cellulosae</i> NBRC 13027	2590	2590	99%	NR_112346.1
<i>Streptomyces</i> sp. ASC764	2584	2584	99%	JQ358565.1
<i>Streptomyces</i> sp. OAct 68	2579	2579	99%	JX047040.1
<i>Streptomyces gancidicus</i>	2571	2571	99%	JX042473.1
<i>Streptomyces caelestis</i> AW9-9C	2571	2571	99%	JX204833.1
<i>Streptomyces pseudogriseolus</i> NRRL B-3288	2571	2571	99%	NR_043835.1
<i>Streptomyces</i> sp. 5361	2571	2571	99%	EF063470.1
<i>Streptomyces</i> sp. 11E-1290	2571	2571	99%	EF063449.1
<i>Streptomyces</i> sp. 3177	2571	2571	99%	DQ663177.1
<i>Streptomyces malachiticus</i> subsp. <i>griseospinosus</i>	2571	2571	99%	AB184540.1

Figure S₁. ¹H-NMR spectrum of compound **6** (in DMSO-*d*₆)

Figure S₂. ¹³C-NMR spectrum of compound **6** (in DMSO-*d*₆)

Figure S3. ^1H - ^1H COSY spectrum of compound **6** (in $\text{DMSO}-d_6$)

Figure S4. HSQC spectrum of compound **6** (in $\text{DMSO}-d_6$)

Figure S5. HMBC spectrum of compound **6** (in DMSO- d_6)

Figure S6. NOESY spectrum of compound **6** (in DMSO- d_6)

Figure S₉. HMBC spectrum of compound **8** (in DMSO-*d*₆)

Figure S₁₀. ¹H-NMR spectrum of compound **9** (in CDCl₃)

Figure S₁₁. ¹³C-NMR spectrum of compound **9** (in CDCl₃)

Figure S₁₂. HMBC spectrum of compound **9** (in CDCl₃)

Table S₂. ¹H and ¹³C NMR data of compounds **6**, **8** and **9**.

Position	6^a		8^a		9^b	
	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	119.8	7.68(d,7.8)	167.0		161.3	13.2(s,1-OH)
2	133.1	7.81(d,7.8)	45.6	2.52(d,13) 2.48(d,13)	134.6	
3	137.1		74.6	5.41(s,3-OH)	139.6	7.66(d,7.7)
4	156.9	12.4(s,4-OH)	43.5	2.96(d,14) 2.93(d,14)	118.8	7.79(d,7.7)
4a	113.9		138.4		131.7	
5	187.3		136.2	7.83(d,7.8)	158.9	13.1(s,5-OH)
5a	133.5					
6	99.8	7.57(s)	124.1	8.13(d,7.8)	138.2	
6a	120.4		133.3			
7	160.3		187.9		133.3	7.92(d,7.8)
7a			114.8			
8	113.0	7.60(s)	157.4	12.7(s,8-OH)	119.4	7.85(d,7.8)
8a					131.8	
9	140.7		136.7		188.1	
9a					115.6	
10	118.6	7.00(s)	133.5	7.86(d,7.8)	188.1	
10a	129.7				115.5	

11	155.2		118.8	7.67(d,7.8)		
11a	125.8		133.1			
12	185.7		180.3			
12a	135.8		123.2			
12b			150.1			
13	21.1	2.43(s,CH ₃)				
14	56.5	4.15(s,CH ₃)				
1'	70.6	4.79(d,7)	70.6	4.80(d,11)	71.2	4.95(d,11)
2'	40.0	2.28(dd;13,4)	40.0	2.25(dd;13,4)	39.3	2.54(dd;13,4)
		1.32(m)		1.32(m)		1.48(m)
3'	71.7	3.55(m)	71.7	3.54(m)	73.1	3.86(m)
		5.04(d,3'-OH)		5.03(s, 3'-OH)		
4'	77.0	2.90(td;8,4)	77.0	2.89(t,8.7)	75.9	3.22(t,8.8)
		4.95(d,4'-OH)		4.94(s, 4'-OH)		
5'	76.1	3.38(m)	76.2	3.37(m)	78.0	3.53(m)
6'	18.4	1.28(d,6,CH ₃)	18.4	1.26(d,6, CH ₃)	18.1	1.41(d,6,CH ₃)
1''					40.5	3.10(d,13)
						3.02(d,13)
2''					71.9	
3''					44.5	3.62(d,16)
						2.58(d,16)
4''					172.6	

5''	27.2	1.30(s,CH ₃)
Bn-CH ₂	66.6	5.15(s)
Bn-Ar	135.3	7.36(5H,m)
	128.6	
	128.6	
	128.4	
	128.4	
	128.5	

^a measured in DMSO-d₆ at 600 MHz; ^b measured in CDCl₃ at 600 MHz.

Table S₃. Antimicrobial activity of compounds **1-9** (except **2**).

Test organism	MIC (µg/mL)								
	1	3	4	5	6	7	8	9	+*
<i>Staphylococcus aureus</i>	4	16	>64	>64	>64	>64	>64	>64	2
<i>Staphylococcus aureus</i> (methicillin-resistant)	4	32	>64	>64	>64	>64	>64	>64	1
<i>Pseudomonas aeruginosa</i>	>64	>64	>64	>64	>64	>64	>64	>64	1
<i>Canidia albicans</i>	>64	>64	>64	>64	>64	>64	>64	>64	2
<i>Fusarium graminearum</i>	>64	>64	>64	>64	>64	>64	>64	>64	4

* + for *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* was vancomycin hydrochloride; for *Pseudomonas aeruginosa* was polymyxin B sulfate; for *Canidia albicans* was amphotericin B; for *Fusarium graminearum* was carbendazim.