SUPPLEMENTARY MATERIAL

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

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Abstract: Vineomycin A\(_1\) (1) and B\(_2\) (2) were isolated from the culture broth of marine actinomycete *Streptomyces* sp. A6H. Five hydrolysis products were obtained by rational hydrolysis and methanolsysis of the fermentation extract. Their structures were characterized as aquayamycin (3), vineomycinone B\(_2\) (4), 9-C-D-olivosyltetragulol (5), 7-O-methylgaltamycinone (6) and vineomycinone B\(_2\) methyl ester (7). In addition to these compounds, two ester derivatives vineolactone A (8) and vineomycinone B\(_2\) 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 IC\(_{50}\) 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 μm, 4.6 × 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 μm, 21.2 × 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 μL aliquots were spread onto Gause’s agar containing nalidixic acid (40μg/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 cellulosae 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, tR=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, tR=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, tR=26min) and compound 6 (2.3mg, tR=29min). Fr. 3 was separated by preparative HPLC (CH3OH/H2O, 45% CH3OH maintained for 30min, flow rate 10mL/min) to give compound 3 (5mg, tR=21min).Fr. 4 was separated by preparative HPLC (CH3CN/H2O, 30% CH3CN maintained for 60min, flow rate 10mL/min) to give compound 4 (70mg, tR=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 (24µL, 0.175mmol) in 2mL THF and 8mL CH₂Cl₂ was added over 6h to a solution of 2,4,6-trichlorobenzylo chloride (11.8µL, 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 (2×10mL). 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 (3µL, 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, Canidia albicans ATCC10231 and Fusarium graminearum by broth microdilution method. The microbial cultures were prepared by diluting precultured broths to 5×10⁵ cfu/mL. The assay was performed in 96-well plates. Each compound were tested in dilution series ranging from 64 to 0.0625 µg/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 (5×10³/well) were inoculated in 96-well plates and treated with compounds for 24h. Then 10µL 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
ACGCTGTGCGGCGGTCTTAACACATGCAAGTCCAACGAGTGAACCTTCG
GTGGGGATTTAGTGGCGAAGCTCCCGGCGGTGCAAGATGAGCCC
ACTCTGGGACAAGCCTGGAAACGGGTCTCTAATACCGGATGCTG
TTGGGCATCTCTGTTGGTGAATTCACTACAAGGCGAAACGGCGCCAG
GCCTACTACGGGAGGCGAGCTGAAATTTGGCAATGGGGCGAAAGCCTG
GCCTAAGCTTGTTGGTGAGGTAAATGGCTCACCAATGGGGCGAAAGCCTG
AGCCCGGCTTAACCCCGGCTGCTCTGCAGGCAGCTACTTGCAAGGCGAAACGGCGCCAG
AACACTACGGCGACGCAGCGCCTTAATACGCTAGGGCGGCGAGCTTTGTCGC
AGCAGCGAATTCCCGGCAGTACCGCTAGGGCGGCTCTTGAAATCGCCAGATAC
AGGAGGAACACCGTGCGCAGGCGGCAAGCCCGATCTCCTGGGGCGGACACATG
GCCGTAAAACCGTGCGCAGCTCGCTCGCTTGCTGCTGCTCGCTTGCGTG
CGCAGCTAACCAGATTGCCCCCGCTGGGAGTACCGGCAAGGCGACAGCTG
AAATCCAAAGGAAATTTGACGGGGGCGCCCCACAGGCGAGGAGCTTGCT
TAATTGCAGCGAACCGGAAAACCTTACCAAGGCTGTACATACAGGGCGGCAAGC
AACCCCTTGAGACAGGTCCCCCTTGTGCTGCTGTAAGGGTGTGGTACAGG
CTGTCGTACCTCGTGCTGATTATGTTGTTGATATTGGTAAGTCCCAGGCAAGC
AAAATTCCTTTCTCCCGGCTTGGCAGCAGGCCTTTGTGCTGGTGGGTACACT
GGGAGACCGCGGGGTCACGTGGAGGAAGGTGGGCTCAGGACGTCAAGTCA
TGCTGTCGTACCTCGTGCTGATTATGTTGTTGATATTGGTAAGTCCAGTC
CGGAGTGTGGTCGGGCTCTGAGGAAATCCATGGAATCGGAGTCGCTAGTAAT
TGAGCTGCGATACCCCGGGAAGTCGAATCTCAGACCCGACGTGAAGT
TCGGATTGCGGTTGCTCGAATTCAACCTGCATCGGGGCTTCTGTATAC
CGCAGATCGATCCTGTGCGGGAATCGTTCGCCCGGCTTGTACACACC
| Description                        | Max score | Total score | Query cover | Accession       |
|-----------------------------------|-----------|-------------|-------------|----------------|
| *Streptomyces cellulosae* NRRL B-2889 | 2590      | 2590        | 99%         | NR_043815.1    |
| *Streptomyces cellulosae* NBRC 13027 | 2590      | 2590        | 99%         | NR_112346.1    |
| *Streptomyces* sp. ASC764         | 2584      | 2584        | 99%         | JQ358565.1     |
| *Streptomyces* sp. OAct 68        | 2579      | 2579        | 99%         | JX047040.1     |
| *Streptomyces gancidicus*         | 2571      | 2571        | 99%         | JX042473.1     |
| *Streptomyces caelestis* AW9-9C   | 2571      | 2571        | 99%         | JX204833.1     |
| *Streptomyces pseudogriseolus* NRRL B-3288 | 2571 | 2571        | 99%         | NR_043835.1    |
| *Streptomyces* sp. 5361           | 2571      | 2571        | 99%         | EF063470.1     |
| *Streptomyces* sp. 11E-1290       | 2571      | 2571        | 99%         | EF063449.1     |
| *Streptomyces* sp. 3177           | 2571      | 2571        | 99%         | DQ663177.1     |
| *Streptomyces malachiticus* subsp. griseospinosus | 2571 | 2571        | 99%         | AB184540.1     |
Figure S1. $^1$H-NMR spectrum of compound 6 (in DMSO-$d_6$)

Figure S2. $^{13}$C-NMR spectrum of compound 6 (in DMSO-$d_6$)
Figure S3. $^1$H-$^1$H COSY spectrum of compound 6 (in DMSO-$d_6$)

Figure S4. HSQC spectrum of compound 6 (in DMSO-$d_6$)
Figure S5. HMBC spectrum of compound 6 (in DMSO-\textit{d}_6)

Figure S6. NOESY spectrum of compound 6 (in DMSO-\textit{d}_6)
Figure S7. $^1$H-NMR spectrum of compound 8 (in DMSO-$d_6$)

Figure S8. $^{13}$C-NMR spectrum of compound 8 (in DMSO-$d_6$)
Figure S9. HMBC spectrum of compound 8 (in DMSO-$d_6$)

Figure S10. $^1$H-NMR spectrum of compound 9 (in CDCl$_3$)
Figure S11. $^{13}$C-NMR spectrum of compound 9 (in CDCl$_3$)

Figure S12. HMBC spectrum of compound 9 (in CDCl$_3$)
Table S2. $^1$H and $^{13}$C NMR data of compounds 6, 8 and 9.

| Position | 6<sup>a</sup> | 8<sup>b</sup> | 9<sup>b</sup> |
|----------|--------------|--------------|--------------|
|          | $\delta_C$   | $\delta_H$ (J in Hz) | $\delta_C$   | $\delta_H$ (J in Hz) | $\delta_C$   | $\delta_H$ (J in Hz) |
| 1        | 119.8        | 7.68(d, 7.8) | 167.0        | 161.3                 | 13.2(s, 1-OH) | 13.2(d, 13) |
| 2        | 133.1        | 7.81(d, 7.8) | 45.6         | 2.52(d, 13)           | 134.6        | 2.48(d, 13)  |
| 3        | 137.1        | 74.6         | 5.41(s, 3-OH) | 139.6                 | 7.66(d, 7.7) | 7.66(d, 7.7) |
| 4        | 156.9        | 12.4(s, 4-OH)| 43.5         | 2.96(d, 14)           | 118.8        | 7.79(d, 7.7) |
|          |              |              |              | 2.93(d, 14)           |              |              |
| 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<sub>3</sub>) |   |
| 14 | 56.5 | 4.15(s, CH<sub>3</sub>) |   |
| 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<sub>3</sub>) | 18.4 | 1.26(d, 6, CH<sub>3</sub>) | 18.1 | 1.41(d, 6, CH<sub>3</sub>) |
| 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 |
$$^5$$

| Compound   | δ (ppm)      | J (Hz)       |
|------------|--------------|--------------|
| Bn-CH₂     | 66.6         | 5.15(s)      |
| Bn-Ar      | 135.3        | 7.36(5H,m)   |

128.6

128.6

128.4

128.4

128.5

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[^a]: measured in DMSO-$d_6$ 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 | +* |
|--------------------------------|-------------|---|---|---|---|---|---|---|---|----|
| Staphylococcus aureus          |             | 4 | 16| &lt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| 2  |
| Staphylococcus aureus (methicillin-resistant) |             | 4 | 32| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| 1  |
| Pseudomonas aeruginosa         |             | &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| 1  |
| Canidia albicans               |             | &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| 2  |
| Fusarium graminearum           |             | &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;64| &gt;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.