Concanamycin H from the soil actinomycete Streptomyces sp. R1706-8

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Abstract
The actinomycete strain R1706-8 is isolated from a soil sample collected from the nest of the horned-face bee (Osmia cornifrons) and identified as Streptomyces sp. based upon the results of 16SrRNA sequence analysis. Two concanamycin derivatives obtained from the solid fermentation have been determined by analysis of the infrared, high-resolution electrospray ionization mass spectrometry, 1D and 2D NMR spectra as well as by comparison with literature data. Of the two derivatives, one is a new compound, named concanamycin H, and the other is the known compound, concanamycin G. These compounds are assayed for antibacterial activity, with concanamycins H and G displaying inhibitory activity against Bacillus subtilis (minimum inhibitory concentration = 0.625 µg mL⁻¹).

Keywords
antimicrobial, concanamycin, Osmia cornifrons, secondary metabolites, Streptomyces

Introduction
To date, seven concanamycin derivatives (A–G) have been isolated from the mycelia of Streptomyces.¹–³ These compounds possess antimalarial,³–⁵ antifungal,⁶–⁸ antiviral,⁹,¹⁰ anticancer,¹¹,¹² and other biological activities. This family of compounds features an 18-membered macrolide ring¹³ and a pyran ring,¹⁴,¹⁵ but the side-chain structures are rare. Structure–activity relationship studies showed that molecules of the concanamycin family are responsible for potent inhibitory activity. Many researchers have studied the synthesis,⁷ semisynthesis,¹⁶ and biosynthesis¹⁷ of these concanamycin derivatives.

In this paper, we report that a sample of the Streptomyces sp. R1706-8, which was isolated from the soil found in the front wall of the honeycomb chamber of the nest of the horned-face bee (Osmia cornifrons), located between Yantai and Weihai in Shandong Province, China, yielded one new compound, concanamycin H (1), and the known compound, concanamycin G (2) (Figure 1). These compounds were identified by analytical techniques, and their antibiotic activity has been investigated.

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Results and discussion

Compound 1 was isolated as a colorless oil. The high-resolution electrospray ionization mass spectrometry (HR-ESIMS) data showed a sodiated molecular ion at m/z (HR-ESIMS) 697.4279 [M + Na]⁺ (calcd for C₃₉H₆₂O₉Na: 697.4286) (see Figure S9 in the Supporting information), which in conjunction with ¹³C NMR data (Table 1) indicated a molecular formula of C₃₉H₆₂O₉Na, requiring nine degrees of unsaturation. The IR spectrum showed characteristic absorption bands attributed to hydroxy (3425 cm⁻¹), carbonyl (1712 cm⁻¹), and alkenyl (1456 cm⁻¹) functionalities (see Figure S10 in the Supporting information). The ¹H, ¹³C, and heteronuclear single quantum coherence (HSQC) NMR (Table 1) data demonstrated the presence of eleven methyl, two methylene, twenty-one methine, and five quaternary carbons, including six olefinic, two carboxyl, five oxymethine, and two methoxy groups. Correlated analysis indicated ¹H−¹H Correlated Spectroscopy (COSY) interactions between H₃-28/H-27/H-26/H-25/H-24/H-24a and H-24/H-23/H-22 and H₂-20a/H-20/H-19/H-18/H-18a and H-18/H-17/H-16/H-15/H-14/H-13, while the heteronuclear multiple bond correlation (HMBC) spectrum of H-22/C-21 and H-20/C-21 indicated the presence of a carbonyl group (δC = 203.0) at C-21 together with correlations of H-11/H-10/H-10a, H-10/H-9/H-8/H₁⁻/H₁⁻-8a/H₁⁻-8b, and H-8/H-7/H-6/H-6a (H-5) in the ¹H−¹H COSY spectrum, and HMBC correlations from H-17 to C-1, H-3 to C-1/C-2/C-4, H-4a to C-4/C-5, and H-12a to C-13/C-12/C-11 (Figure 2) completed the assignment of the concanamycin skeleton. The presence of an ethyl group at C-8 was suggested by the signals at δC = 22.7 and 11.6; δH = 1.29 (m) and 0.89 (t, 7.4). In the Nuclear Overhauser Effect Spectroscopy (NOESY) spectrum (Figure 2), the cross-peaks of H-6 with H-8 and H-7 and of H-17 with H-19, H-20, and H-24 indicated that H-6, H-8, H-7, H-17, H-19, H-20, and H-24 were α-oriented and that H-9, H-10, H-16, H-18, and H-25 were β-oriented. The coupling constants between 14-H and 15-H, 22-H and 23-H, and 26-H and 27-H indicated that these disubstituted double bonds had E configurations. Accordingly, the structure of 1 (Figure 1) was finally determined as the anhydroaglycone of concanamycin A¹ and named concanamycin H.

Compound 2 was isolated as a colorless oil. The HR-ESIMS data showed a sodiated molecular ion at m/z 683.4120 [M + Na]⁺ (calcd for C₃₈H₆₀O₉Na: 683.4129) (see Figure S19 in the Supporting information). The ¹H and ¹³C NMR data of compound 2 closely resembled those of 1 except for the C-8 ethyl signal disappeared and replaced by a methyl group (δC = 10.0; δH = 0.94 (d, 7.0)) in the ¹³C NMR of 2. Thus, compound 2 was assigned as the anhydroaglycone of concanamycin B¹ (Figure 1) and named concanamycin G.¹

Concanamycin H (1) and concanamycin G (2) showed weak inhibitory activity against Bacillus subtilis with clear inhibition zones in disk diffusion assays (Table 2). Accurate data on the inhibitory activity were obtained through determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (Table 3). Compounds 1 and 2 demonstrated minor inhibitory activity against Bacillus subtilis (MIC = 0.625 mg mL⁻¹ and MBC = >1 mg mL⁻¹).

Conclusion

Two concanamycin derivatives have been isolated from Streptomyces sp. R1706-8, including a new macrocycle named concanamycin H (1) along with the reported concanamycin G (2). The antibacterial activity compounds 1 and 2 have been evaluated by the disk diffusion method against four bacteria. Concanamycins H (1) and G (2)
exhibited inhibitory activity against Bacillus subtilis and displayed moderate activity (MIC = 0.625 μg mL−1 and MBC = >1 μg mL−1).

**Table 1.** 1H NMR (500 MHz) and 13C NMR (125 MHz) data of compounds 1 and 2 δ_C [mult., J (Hz)].

| Position | 1  | 2  |
|----------|----|----|
|          | δ_C | δ_H | δ_C | δ_H |
| 1        | 165.8 |     | 164.3 |     |
| 2        | 139.6 |     | 139.4 |     |
| 2a       | 59.6  | 3.66 (3H, s) | 60.0  | 3.64 (3H, s) |
| 3        | 130.7 | 6.43 (s) | 129.4 | 6.28 (s) |
| 4        | 132.8 |     | 132.9 |     |
| 4a       | 14.2  | 1.99 (br s) | 14.4  | 2.02 (s) |
| 5        | 139.5 | 5.71 (br d, 10.5) | 139.1 | 5.88 (br d, 10.9) |
| 6        | 34.5  | 2.74 (m) | 37.5  | 2.13 (m) |
| 6a       | 17.8  | 1.07 (d, 7.0) | 17.7  | 1.03 (d, 6.8) |
| 7        | 74.7  | 3.83 (dd, 8.0, 2.5) | 72.8  | 3.73 (m) |
| 8        | 43.6  | 1.49 (m) | 37.1  | 1.26 (m) |
| 8a       | 22.7  | 1.29 (m) | 10.0  | 0.94 (d, 7.0) |
| 8b       | 11.6  | 0.89 (t, 7.4) | 80.1  | 3.08 (br d, 10.0) |
| 9        | 79.6  | 3.22 (br d, 11.0) | 31.7  | 2.18 (m) |
| 10       | 31.8  | 2.19 (m) | 22.7  | 1.03 (d, 6.8) |
| 10a      | 21.0  | 1.06 (d, 6.8) | 45.3  | 2.05 (m) |
| 11       | 44.6  | 1.96 (m) |     |     |
| 12       | 142.5 |     |     |     |
| 12a      | 16.2  | 1.84 (br s) | 16.5  | 1.80 (br s) |
| 13       | 123.3 | 5.79 (br d, 10.5) | 125.9 | 5.84 (d, 10.9) |
| 14       | 132.8 | 6.51 (dd, 15.1, 10.5) | 133.0 | 6.51 (t, 15.8, 10.9) |
| 15       | 127.4 | 5.23 (dd, 15.1, 8.8) | 128.1 | 5.21 (dd, 15.1, 9.3) |
| 16       | 82.8  | 3.76 (t, 8.8) | 82.5  | 3.76 (t, 9.3) |
| 16a      | 55.7  | 3.23 (s) | 55.7  | 3.23 (s) |
| 17       | 75.3  | 5.15 (dd, 8.8, 1.5) | 74.8  | 5.22 (dd, 8.7, 1.5) |
| 18       | 37.9  | 2.11 (ddq, 9.0, 7.0, 1.5) | 37.5  | 2.13 (ddq, 9.0, 7.0, 1.5) |
| 18a      | 10.6  | 0.94 (d, 7.0) | 10.4  | 0.94 (d, 7.0) |
| 19       | 72.3  | 3.72 (dd, 9.0, 3.8) | 72.3  | 3.71 (dd, 9.0, 3.8) |
| 20       | 46.3  | 2.93 (q, 7.0, 3.8) | 46.4  | 2.92 (q, 7.1, 3.8) |
| 20a      | 10.1  | 1.20 (d, 7.0) | 9.92  | 1.20 (d, 7.0) |
| 21       | 203.1 |     |     |     |
| 22       | 129.1 | 6.26 (dd, 15.8, 0.9) | 129.3 | 6.28 (dd, 15.8) |
| 23       | 148.7 | 6.84 (dd, 15.8, 7.7) | 148.7 | 6.87 (dd, 15.8, 8.0) |
| 24       | 42.8  | 2.40 (m) | 42.8  | 2.40 (m) |
| 24a      | 15.4  | 0.99 (d, 7.0) | 15.6  | 1.03 (d, 6.8) |
| 25       | 76.2  | 3.91 (t, 7.0) | 78.2  | 3.91 (t, 7.0) |
| 26       | 131.7 | 5.44 (ddq, 15.5, 7.0, 1.5) | 131.9 | 5.47 (ddq, 15.3, 7.0, 1.6) |
| 27       | 128.9 | 5.68 (ddq, 15.5, 6.5) | 129.1 | 5.70 (ddq, 15.3, 6.5, 0.9) |
| 28       | 17.8  | 1.71 (dd, 6.5, 1.5) | 17.7  | 1.71 (dd, 6.5, 1.6) |

Experimental section

**General experimental procedures**

NMR spectra—including HSQC, HMBC, and COSY—were recorded on a Bruker AVANCE-500 instrument with tetramethylsilane (TMS) as an internal standard (Bruker BioSpin Group, Germany). NMR spectra recorded on a Bruker Avance III HD spectrometer (500 MHz; Swis Brook Bierspring Co. Ltd; Switzerland). UV spectra were obtained on a Shimadzu UV-2450 spectrometer (Shimadzu, Japan). HR-ESIMS was conducted on a Bruker APEX II spectrometer (Bruker, Germany). The IR spectra were obtained using a Nicolet IS5 FTIR spectrometer (Thermo Scientific, MA, USA). 1H, 13C, and 2D NMR spectra were recorded at 25 °C on a Bruker ARX 500 NMR spectrometer. GE Sephadex LH-20 (25−100μm; Pharmacia Biotek, USA) silica gel (200−300 and 300−400 mesh) was used for column chromatography (CC). Silica gel GF254 plates (Qingdao Haiyang Chemical Group Corp., Qingdao, China) were used for TLC.

**Media and culture conditions**

The culture medium used Gauze’s Synthetic Medium No. 1 that was prepared from soluble starch (20 g), KNO₃ (1 g), K₂HPO₄ (0.5 g), MgSO₄·7H₂O (0.5 g), NaCl (0.5 g), FeSO₄·7H₂O (0.01 g), agar (20 g), and pH = 7.0−8.0.
sterilization by steaming at 121 °C for 31 min and reducing the temperature to room temperature, actinomycete colonies were purified by streaking on the same agar medium. The solid medium was cultured at a constant temperature of 32 °C for around 10 days. Genomic DNA was extracted using a DNA isolation kit (Shanghai Sangon Biotech Co., China). Polymerase chain reaction (PCR) amplification of the 16S ribosomal RNA (rRNA) was performed using primers Fd2 (5'-GAGTTTGATCATGGCTCAG-3') and 16Sr (5'-TTGCGGGACTTAACCCAACAT-3') and sequenced by Shanghai Sangon Biotech Co., China. The nucleotide sequence was submitted to GenBank (accession numbers KX588720) and compared with reference 16SrRNA gene sequences in databases by BLAST searching. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 5.0 software (Figure 3). Streptomyces sp. R1706-8 was deposited at the Laboratory of Natural Products Chemistry, Shandong University, Weihai, P.R. of China.

### Extraction and isolation

The Streptomyces sp. R1706-8 was conserved as a glycercin spore suspension at −80 °C. Streptomyces sp. R1706-8 was grown in Petri dishes containing 50 mL of Gauze’s Synthetic Medium No. 1 (20 g L⁻¹ amylo-gen, 1 g L⁻¹ KNO₃, 0.5 g L⁻¹ NaCl, 0.5 g L⁻¹ KH₂PO₄·H₂O, 0.5 g L⁻¹ MgSO₄·H₂O, and 0.01 g L⁻¹ FeSO₄·H₂O adjusted to pH 7.0–8.0). The plates were cultured for 10 days at 32 °C. After fermentation, the culture medium was extracted four times with a mixture of ethyl acetate and methanol (10:1). The extracting solution was concentrated in vacuo to yield the crude extract (4.2 g), which was separated using silica gel CC (50 g, 200–300 mesh) eluting with n-hexane: acetone (5:1, 2:1, 1:1, and 0:1) and 100% methanol to give five fractions (Fr.1–Fr.5). Fr.3 (680.0 mg) was passed through a silica gel column eluting with (Fr.1–Fr.4) n-hexane acetone (5:1, 2:1, 1:1, and 0:1)

| Table 2. Antibiotic activities of compounds 1 and 2. |
|-----------------|---|---|---|
|                | 1  | 2  | Chloramphenicol |
| E. coli        | 25 | 25 | 30 |
| S. aureus      | 17 | 17 | 19 |
| C. subtilis    | 9  | 9  | 28 |
| P. aeruginosa  | –  | –  | 24 |

The diameter of the inhibition zones (mm), including the disk diameter (6 mm), is given as the mean of triplicate experiments. – denotes none.

Figure 2. ¹H−¹H COSY (blue bold), key HMBC (red arrows), and NOESY (black arrows) correlations in compounds 1 and 2.
to obtain four sub-fractions (Fr.3.1–3.4). Fr.3.1 (170.0 mg) was purified on a reverse C-18 column, using 60% MeOH/H₂O as the solvent to give four further fractions (Fr.3.1.1–Fr.3.1.4). Fraction 3.1.1 (35.0 mg) was fractionated by silica gel CC eluting with CH₂Cl₂/MeOH (8:1) to yield 1 (13.9 mg) and 2 (8.3 mg).

Antibacterial assay

The antibacterial activities of compounds 1 and 2 were evaluated by the disk diffusion method. Four bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, and four fungi *Aspergillus flavus*, *Mucor mucedo*, *Phytophthora parasitica var. nicotianae*, and *Candida albicans*, were used as indicator strains. The compounds were dissolved in acetone in which paper disks with a diameter of 6 mm had been soaked (bacteria: 50 μg disk⁻¹, fungi: 100 μg disk⁻¹). Chloramphenicol (10 μg disk⁻¹) was used as a positive control. The paper disks were dried under sterile conditions and placed on agar plates. After culture at 37°C for 48 h (bacteria), the diameters of the inhibition zones were measured (Table 3). The MIC values were determined using 96-well microplates. The MIC is defined as the lowest concentration of a compound that inhibits visible growth. For determination of the MBC, a sample of 100 μL from each well (without any color alteration) was sub-cultured on the agar plates and incubated at 36°C for 18–24 h (overnight). The MBC value is defined as the lowest concentration without any bacterial growth. Triplicate experiments were conducted for each test.

|           | 1 MICa | MBCb | 2 MICa | MBCb | Chloramphenicol MICc | MBCc |
|-----------|--------|------|--------|------|---------------------|------|
| *coli*    | >1     | >1   | >1     | >1   | 0.008               | 0.031|
| *aureus*  | >1     | >1   | >1     | >1   | 0.008               | 0.063|
| *subtilis*| 0.625  | >1   | 0.625  | >1   | 0.008               | 0.063|
| *aeruginosa*| >1   | >1   | >1     | >1   | 0.063               | 0.063|

*aMIC (μg mL⁻¹): minimum inhibitory concentration.
*bMBC (μg mL⁻¹): minimal bactericidal concentration.*

Table 3. The MIC and MBC values of compounds 1 and 2.

Figure 3. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strains R1706-8 and related members of the family Streptomyces. The percentage numbers (>50 are given) at the nodes are the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets. The tree is rooted with *Streptacidiphilus albus* NBRC 100918T (NR024930).

Bar: 0.005 nucleotide substitution per position.

**Compound characterization**

**Compound 1:** Colorless oil, [α]D 26.7 3.0 (c 1.0, CH₂Cl₂), Rf 0.53 (CH₂Cl₂/MeOH (4:1)), UV (CDCl₃): λmax (log ε) = 235, 284 nm. IR (KBr): νmax = 3425, 3057, 2968, 2926, 2370, 1712, 1624, 1456, 1412, 1377, 1263, 1200, 1105, 982, 771, 737, 704, 619 cm⁻¹; 1H and 13C NMR data (see Table 1); HR-ESIMS m/z [M + Na]+ calcd for C₃₉H₆₂O₉Na: 697.4286; found: 697.4279.
Compound 2: Colorless oil, $[\alpha]_D^{25} = 7.5 \ (c \ 0.1, \text{CH}_2\text{Cl}_2)$, $R_f = 0.41$ (CH$_2$Cl$_2$/MeOH (4:1)). UV (CDCl$_3$): $\lambda_{\text{max}}$ (log $\varepsilon$) = 237, 283 nm; IR (KBr)$\nu_{\text{max}} =$ 3455, 3418, 2968, 2934, 2877, 1692, 1622, 1453, 1376, 1358, 1247, 1201, 1102, 972, 920, 861, 765 cm$^{-1}$; $^1$H and $^{13}$C NMR data (see Table 1); HR-ESIMS $m/z$ [M + Na]$^+$ calcd for C$_{38}$H$_{60}$O$_9$Na: 683.4129; found: 683.4120.

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Supplemental material
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