Brief Report

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16,17-dihydroxycyclooctatin, a new diterpene from *Streptomyces* sp. LZ35

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ABSTRACT: Genome sequence analysis of *Streptomyces* sp. LZ35 has revealed a large number of secondary metabolite pathways, including a complete gene cluster for the biosynthesis of cyclooctatin. This cluster contains four genes, cotB1–4, located in a 5-kb region. Optimization of fermentation medium for LZ35Δ*heng* (SR107) led to the identification of cyclooctatin (1) and 16,17-dihydroxycyclooctatin (2), a new diterpene. The structures of these substances were elucidated on the basis of 1D-, 2D-NMR, and HRESIMS data. Cytotoxicity against MDA-MB-231 and A549 cell lines was also evaluated. Results demonstrated that gene cluster and pathway analysis are key to guided isolation of new natural products.

Keywords: *Streptomyces* sp. LZ35, gene cluster, cyclooctatin, 16,17-dihydroxyl-cyclooctatin

1. Introduction

Cyclooctatin, a diterpene characterized by a novel 5-8-5-fused ring system, is a potent inhibitor of lysophospholipase, which was previously isolated from *Streptomyces melanosporofaciens* MI614-43F2 (*1,2*). The complete cyclooctatin biosynthesis gene cluster was cloned from *S. melanosporofaciens* MI614-43F2 and heterologously expressed with variations of cytochrome P450 genes in *Streptomyces albus* to produce cyclooctatin together with two new intermediates, cyclooctat-9-en-7-ol and cyclooactat-9-en-5,7-diol (*3*). Additionally, 17-hydroxycyclooctatin was isolated from *Streptomyces* sp. MTE4a (*4*). To date, only four compounds with this novel 5-8-5-fused ring backbone have been isolated thus far.

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Genome sequence analysis of *Streptomyces* sp. LZ35 has revealed a complete cyclooctatin biosynthesis gene cluster containing four genes, cotB1–4, located in a 5-kb region (unpublished data). This cluster is highly homologous to that reported by Kim *et al*. (*3*). Therefore, an interesting question was whether *Streptomyces* sp. LZ35 produces cyclooctatin-type diterpenoids. To facilitate the isolation of minor components such as putative cyclooctatin derivatives, four gene clusters involved in the biosynthesis of hygrocin, elaiophylin, nigericin, and geldanamycin were deleted to afford a clean background mutant strain LZ35Δ*heng* (SR107) of *Streptomyces* sp. LZ35 (Supporting Information) (*5*). The fermentation of LZ35Δ*heng* on SSY agar medium led to the isolation of cyclooctatin (**1**) and 16,17-dihydroxycyclooctatin (**2**).

2. Materials and Methods

2.1. *General experimental procedures*

Mass spectra were measured using a Bruker BioTOF-Q spectrometer; NMR spectra were measured on Bruker DRX-600 MHz NMR spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) with tetramethylsilane (TMS) as an internal standard. The IR spectra (KBr) were obtained on a Nicolet iN10 Micro FT-IR Microscope (Thermo Scientific). Reversed-phase (RP) C_{18} silica gel for column chromatography (CC) was obtained from Merck (Darmstadt, Germany) and *Sephadex* LH-20 was obtained from GE Amersham Biosciences (Piscataway, NJ, USA). Silica gel (200–300 mesh) for CC and silica gel GF_{254} for TLC were purchased from Qingdao Marine Chemical, Ltd. (Qingdao, Shandong, China).

2.2. *Microorganisms*

The strain *Streptomyces* sp. LZ35 was isolated from the intertidal soil collected in Ji'mei, Xia'men, China (*6*). The mutant strain LZ35Δ*heng* (SR107) was constructed by deleting parts of *hgcA*, *elpA*, *nigA* and *gdmAI* that are the first module of PKS genes in the gene clusters for biosynthesis of hygrocin, elaiophylin, nigericin, and

geldanamycin, respectively (*5*).

2.3. *Fermentation and isolation*

The strain LZ35Δ*heng* was cultured for 14 d on SSY agar media (2.5% Soluble starch, 1.5% Soybean, 0.2% Yeast extract, pH 7.2) at 28°C.

The medium (12 L) was collected and extracted three times with an equal volume of EtOAc/MeOH/AcOH 80:15:5 (*v/v/v*) at room temperature. The organic solutions were collected by filtration and removed under vacuum at 40°C to obtain the crude extract. This was partitioned three times between 95% MeOH and petroleum ether (1:1) to remove lipids. The 95% MeOH solution was concentrated under vacuum at 40°C to afford MeOH extract (4.5 g).

The MeOH extract (4.5 g) was subjected to column chromatography (CC) over Sephadex LH-20 (120 g) eluted with MeOH-CH_2Cl_2 (2:1, *v/v*) to obtain 3 fractions, Fr. A, Fr. B, and Fr. C. Fr B was further subjected to CC over Sephadex LH-20 (120 g) eluted with MeOH to obtain 6 subfractions, Fr. B1-B6. Fr. B2 (302 mg) was subjected to MPLC over RP-18 silica gel (20 g) eluted with 15%, 40%, 60%, 80% and 100% MeOH, resp., (300 ml for each gradient) to afford 5 fractions: Fr. B2A – B2E. Fr. B2D (40 mg) obtained from 80% MeOH eluent was further purified by CC over silica gel (1.6 g) eluted with gradient CH_2Cl_2-MeOH (60:1, 50:1) to yield Fr. B2D1 (3.8 mg). Fr. B2D1 was finally purified by HPLC (Agilent 1200 equipped with a ZORBAX XDB-C18 5 μm, column ID: 9.4 × 250 mm, elution: 85% MeOH, flow rate: 4 mL/min, UV detection monitored at 220 and 273 nm) to afford **1** (RT 9.2 min, 3.0 mg). Fr. B2B (34 mg) obtained from 40% MeOH eluents was further purified by CC over silica gel (0.85 g) eluted with gradient CH_2Cl_2-MeOH (50:1, 30:1, 20:1) to yield **2** (2.3 mg).

2.4. *Cell lines*

The human breast cancer cells MDA-MB-231 and A549 were obtained directly from the American Type Culture Collection (ATCC) and maintained in DMEM containing 10% fetal bovine serum (FBS, Gibco). All cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

2.5. *Cell growth assay*

The cytotoxicities of compounds **1** and **2** were evaluated using the sulforhodamine B (SRB) assay as described previously (*7*). Briefly, 3000-4000 MDA-MB-231 human breast cancer cells and A549 human lung cancer cells were seeded in 96-well plates overnight and exposed to either investigational compounds or etoposide at various concentrations for 72 h in 5% CO_2 at 37°C. After the medium was discarded, a 100 μL solution of 10% trichloroacetic acid (TCA) was added to cell monolayers and cells were stained for 1 h at 4°C. The plates were washed five times with distilled water. Then, 100 μL 4 mg/mL of SRB (Sigma-Aldrich) was added to each well, and cells were stained at room temperature for 10 min. The excess stain was removed by washing cells five times with 1% acetic acid. The protein-bound stain was dissolved in 200 μL of 10 mM Tris base solution per well. The optical density was measured at 570 nm using a microplate reader (Bio-Rad, iMark680). All experiments were performed in triplicate. The rate of growth inhibition for each well was calculated as follows: Inhibition rate (%) = $[A_{570}(\text{control}) - A_{570}(\text{ sample})]/[A570(\text{control}) - A_{570}(\text{blank})] \times 100$.

3. Results and Discussion

Geldanamycins and related type I polyketides, such as nigericins, elaiophylins, and hygrocins, are normally major endogenous secondary metabolites produced by wild-type *Streptomyces* sp. LZ35 (*6*). Biosynthesis gene clusters encoding hygrocins (*hgc*), elaiophylins (*elp*), nigericins (*nig*), and geldanamycins (*gdm*) were disrupted using a REDIRECT technique in accordance with a previously described protocol with modifications (*8*) (Table S1, Figures S1-4, *http://www.ddtjournal.com/docindex.php?year=2013&kanno=5*). The portion of the *gdmAI* gene was first deleted to yield the mutant LZ35Δ*gdmAI* (SR101) (Figure S1), and double cross-over deletion mutants were obtained by screening for loss of yellow pigment production (data not shown). The *nigAI* gene was then deleted to yield the mutant LZ35Δ*nigAI*Δ*gdmAI* (SR105) (Figure S2), and double cross-over deletion mutants were confirmed by the detection of nigericins and geldanamycins with thin-layer chromatography (TLC) (data not shown). The *elpA* gene was deleted to yield the mutant LZ35Δ*elpA*Δ*nigA*Δ*gdmAI* (SR106) (Figure S3). Finally, the portion of the *hgcA* gene was deleted to yield the mutant LZ35Δ*hgcA*Δ*elpA*Δ*nigA*Δ*gdmAI* (LZ35Δ*heng*, SR107) (Figure S4). HPLC analysis of the EtOAc extract of whole broth confirmed the absence of hygrocins, elaiophylins, nigericins, and geldanamycins and revealed more absorbance peaks with similar densities (data not shown), suggesting that minor components could be readily isolated from the mutant strain LZ35Δ*heng*.

Compound **1** was obtained as a colorless amorphous powder. The molecular formula of **1** was determined to be $C_{20}H_{34}O_3$ on the basis of HR-Q-TOF MS (*m/z* 345.4718, calcd. 345.4714 for $C_{20}H_{34}O_3Na^+$) and NMR data (Table 1). Its UV spectrum revealed maximum absorption in MeOH. Its IR spectrum (KBr) revealed the presence of a hydroxyl group (3420 cm^{-1}). The ^{13}C NMR spectrum revealed 20 signals, corresponding to four CH_3, six CH_2, seven CH, and three quaternary C-atoms. Complete assigment of NMR data with the aid of HMQC and HMBC experiments revealed that compound **1** was identical to cyclooctatin (Figure 1),

Table 1. The NMR data for 1 and 2. Recorded at 600/150 MHz (δ in ppm, J in Hz)

	1[a]		**2**[a]		cyclooctatin[c]	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	1.53 (m), 1.40 (m)	45.5t	1.61 (dd, 5.9, 12.6), 1.35 (m)	46.6t	1.68 (br d, 12.8), 1.20 (t, 12.8)	45.6t
2	2.56 (m)	24.6d	2.58 (m)	35.8d	2.56 (m)	35.8d
3	2.66 (m)	44.1d	2.64 (m)	44.9d	2.61 (m)	44.9d
4	1.75 (m), 1.38 (m)	38.9t	1.71 (m), 1.32 (m)	39.7t	1.71 (br dd, 12.6, 5.0), 1.38 (dt, 12.6, 3.4)	39.7t
5	4.50 (d, 3.4)	75.0d	4.45 (t, 5.0)	75.6d	4.44 (br dd, 5.0, 3.4)	75.7d
6	1.98 (t, 5.2)	58.0d	1.99 (t, 4.7)	57.9d	1.97 (t, 5.0)	58.0d
7	/	77.3s	/	78.3s	/	78.4s
8	2.69 (m), 1.96 (m)	41.7t	2.79 (t, 11.6), 1.95 (m)	42.2t	2.72 (br t, 11.6), 1.91 (dd, 12.8, 7.4)	42.2t
9	5.25 (t, 7.8)	117.8d	5.45 (t, 8.5)	118.7d	5.28 (ddd, 10.3, 7.4, 2.2)	119.1d
10	/	153.1s	/	153.9s	/	154.5s
11	/	44.7s	/	45.8s	/	45.9s
12	1.53 (m), 1.49 (m)	45.5t	1.90 (m), 1.63 (dd, 6.0, 12.0)	45.6t	1.59 (m), 1.42 (m)	46.6t
13	1.49 (m), 1.36 (m)	23.2t	1.68 (q, 5.7), 1.35 (m)	25.3t	1.56 (m), 1.38 (m)	24.3t
14	2.26 (m)	53.7d	2.68 (m)	47.1d	2.30 (m)	55.1d
15	1.79 (m)	28.9d	1.23 (t, 6.7)	45.7d	1.83 (m)	30.2d
16	0.96 (d, 6.7, 3H)	22.2q	3.85 (dd, 5.1, 8.8), 3.66 (d, 10.1)	63.7t	0.96 (d, 6.6, 3H)	22.5q
17	0.77 (d, 6.7, 3H)	17.3q	3.60 (m) 3.39 (t, 10.1)	61.3t	0.79 (d, 6.6, 3H)	17.8q
18	3.77 (t, 10.2), 3.62 (dd, 7.6, 10.2)	63.3t	3.60 (m, 2H)	63.4t	3.66 (dd, 10.8, 7.4), 3.55 (dd, 10.8, 6.8)	63.4t
19	1.33 (s, 3H)	26.3q	1.32 (s, 3H)	26.7q	1.33 (br. s, 3H)	26.7q
20	1.23 (s, 3H)	24.8q	1.27 (s, 3H)	25.4q	1.25 (s, 3H)	25.2q

[a] Measured in $CDCl_3$; [b] Measured in CD_3OD; [c] The NMR data listed (CD_3OD) were reported previously by Aoyama *et al*. (*2*).

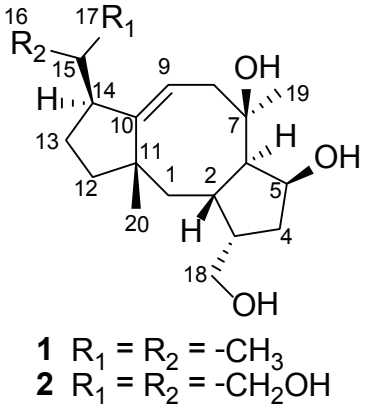

Figure 1. The chemical structures of compounds 1 and 2.

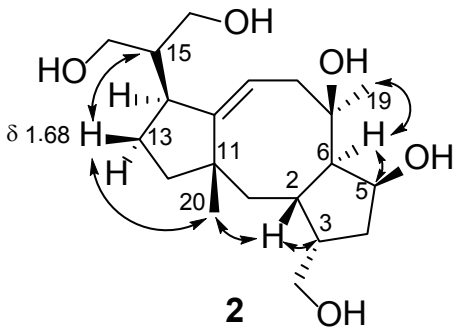

Figure 2. Selected NOE correlations for compound 2.

which is consistent with the literature (*2*).

Compound **2** was obtained as a colorless amorphous powder. The molecular formula of **2** was determined to be $C_{20}H_{34}O_5$ on the basis of HR-Q-TOF MS (*m/z* 377.4706, calcd. 377.4702 for $C_{20}H_{34}O_5Na^+$) and NMR data (Table 1). Its UV spectrum revealed maximum absorption in MeOH. Its IR spectrum (KBr) revealed the presence of a hydroxyl group (3430 cm^{-1}). The ^{13}C NMR spectrum revealed 20 signals, corresponding to two CH_3, eight CH_2 (three of which were oxygenated), seven CH, and three quaternary C-atoms.

The ^{1}H and ^{13}C NMR spectra of **2** closely resembled those of **1** except that the isopropylidene at C-14 in **1** was replaced with a 1,3-dihydroxyisopropyl group in **2**, as indicated by the two oxygen-bearing methylenes at δ_H 3.85 (dd, 5.1, 8.8) and 3.66 (d, 10.1), δ_C 63.7 (t) and at δ_H 3.60 (m) and 3.39 (t, 10.1), δ_C 61.3 (t). Extensive analysis of HMQC and HMBC spectra indicated that **2** had a structure of 16,17-dihydroxycyclooctatin (Figure 1). The relative configuration (*2S*,3S*,5S*,6R*,7R*,11S*,14S**) was determined from the NOESY correlations of H-6 with H-5 and H-19, H-2 with H-3 and H-20, and H-13β with H-15 and H-20 as those for 1 and 17-hydroxycyclooctatin (Figure 2) (*4*).

Cyclooctatins have a unique tricyclic diterpene skeleton (C_{20}) characterized by a 5-8-5-fused ring system. Prior to the current study, only four compounds of this type were identified, including cyclooctatin (*2*), cyclooctat-9-en-7-ol, cyclooctat-9-en-5,7-diol (*3*), and 17-hydroxycyclooctatin (*4*). Previous studies suggested that cyclooctatin was a potent inhibitor of lysophospholipase and exhibited no significant antimicrobial activity at 100 μg/mL (*1*). The current results suggested that compounds **1** and **2** showed no cytotoxicity against MDA-MB-231and A549 cell lines at concentrations up to 50 μm.

In conclusion, the current findings have revealed important new additions to the small family of cyclooctatin diterpenoids with a novel 5-8-5-fused ring backbone. The current work has also demonstrated that gene cluster and pathway analysis are key to guided isolation of new natural products.

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