

Article

A New Anthracene Derivative from Marine *Streptomyces* sp. W007 Exhibiting Highly and Selectively Cytotoxic Activities

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Abstract: A new anthracene derivative, 3-hydroxy-1-keto-3-methyl-8-methoxy-1,2,3,4-tetrahydro-benz[α]anthracene, was isolated from the marine strain *Streptomyces* sp. W007, and its structure was established by spectroscopic analysis including mass spectra, 1D- and 2D-NMR (¹H–¹H COSY, HMBC, HSQC and NOESY) experiments. 3-hydroxy-1-keto-3-methyl-8-methoxy-1,2,3,4-tetrahydro-benz[α]anthracene showed cytotoxicity against human lung adenocarcinoma cell line A549.

Keywords: *Streptomyces*; anthracene; structure establishment; cytotoxicity

1. Introduction

Natural products remain either the source or inspiration for a significant proportion of the new small-molecule chemical entities introduced as drugs [1]. Microbial natural products are an important

source of both existing and new drugs [2]. Among the producers of commercially important metabolites, actinomycetes have proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds. Secondary metabolites produced by actinomycetes possess a wide range of biological activities [3–7], and the vast majority of these compounds are derived from the single genus *Streptomyces* [8].

Streptomyces species are distributed widely in marine habitats and are of commercial interest due to their unique capacity to produce novel metabolites. Our group has done research on novel natural products of marine *Streptomyces* species with potential antitumor activity for 12 years [9–12]. As part of this work, in our screening of marine *Streptomyces* isolates for more active secondary metabolites, a new anthracene derivative, 3-hydroxy-1-keto-3-methyl-8-methoxy-1,2,3,4-tetrahydro-benz[α]anthracene (compound **1**) was obtained from the fermentation broth of marine *Streptomyces* sp. W007 for the first time. The structure of compound **1** was determined by LC-MS, ^1H , ^{13}C and 2D NMR spectroscopy and by comparison of the NMR data with other anthraquinone compounds, the cytotoxicities of compound **1** against human leukemic cells line HL-60, human lung adenocarcinoma cell line A549 and human hepatoma cell line BEL-7402 were studied, the details of structure and cytotoxicities of compound **1** are presented here.

2. Results and Discussion

2.1. Structure Analysis of Compound **1**

Compound **1** (Figure 1, Table 1) was obtained as yellow powder and showed a quasi-molecular ion $[\text{M} + \text{H}]^+$ at m/z 307 in the LC-MS, which was consistent with the molecular formula $\text{C}_{20}\text{H}_{18}\text{O}_3$ (calcd for 306.3551), and required 12 degrees of unsaturation. Its ^1H NMR spectrum exhibited an aliphatic methyl singlet at δ_{H} 1.46 (3H, s), one methoxy group at δ_{H} 4.09 (3H, s), two methylene signals at δ_{H} 3.35 (2H, dd, 15.0, 20.0 Hz) and δ 2.97 (2H, dd, 10, 15 Hz) and 7 aromatic proton signals at δ_{H} 10.10 (1H, s), 8.78 (1H, s), 8.26 (1H, d, 8.6 Hz), 7.65 (1H, d, 8.25 Hz), 7.45 (1H, t, 8.25, 7.45 Hz), 7.37 (1H, d, 8.6 Hz), 6.89 (1H, d, 7.55 Hz).

Figure 1. (A) Structure of compound **1**; and (B) Selected ^1H – ^1H COSY, HMBC and NOESY correlations for compound **1**.

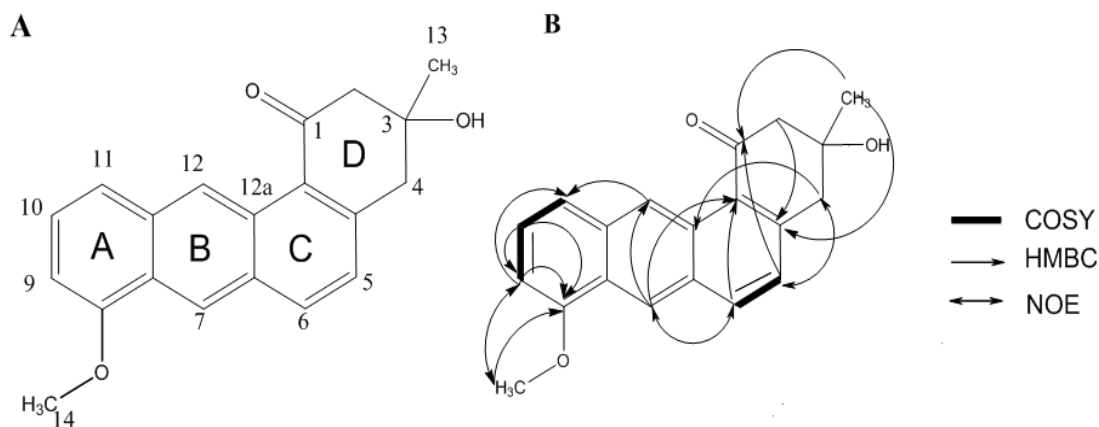


Table 1. ^1H and ^{13}C NMR data of compound **1** in CDCl_3 (500 MHz).

Position	d (J in Hz)	δ_{C}
1		198.76
2	2.97 (2H, dd, 10, 15)	54.28
3		70.55
4	3.35 (2H, dd, 15.0, 20.0)	45.41
4a		145.61
5	7.37 (1H, d, 8.6)	127.37
6	8.26 (1H, d, 8.6)	135.19
6a		130.69
7	8.78 (1H, s)	121.52
7a		124.29
8		155.38
9	6.89 (1H, d, 7.55)	102.5
10	7.45 (1H, t, 8.25, 7.45)	126.35
11	7.65 (1H, d, 8.25)	121.13
11a		134.54
12	10.10 (1H, s)	125.17
12a		128.74
12b		125.46
13	1.46 (3H, s)	28.47
14	4.09 (3H, s)	55.10

The ^{13}C NMR, DEPT135 and HMBC spectra of compound **1** displayed two methyl resonances at δ_{C} 28.47 (C-13, 1-Me), 55.10 (C-14), two methylenes at δ_{C} 45.41 (C-4), 54.28 (C-2), methines at δ_{C} 135.19 (C-6), 127.37 (C-5), 102.5 (C-9), 125.17 (C-12), 121.52 (C-7), 121.13 (C-11), 126.35 (C-10), quaternary carbons at δ_{C} 155.38 (C-8), 145.61 (C-4a), 134.54 (C-11a), 130.69 (C-6a), 128.74 (C-12a), 125.46 (C-12b), 124.29 (C-7a), 70.55 (C-3), in addition to one carbonyl at δ_{C} 198.76 (C-1). By detailed comparison of the ^1H and ^{13}C NMR data of compound **1** with 1,4,8,10-tetramethoxyanthracene-2-carbaldehyde [13], the similar NMR data of the aromatic moieties confirmed that compound **1** has the anthracene skeleton. Because compound **1** required 12 degrees of unsaturation, there is a six-carbon ring next to the anthracene skeleton. In the NOESY spectrum, the proton at δ_{H} 3.35 (H-4) showed a correlation with the proton at δ_{H} 7.37 (H-5) and the proton at δ_{H} 8.26 (H-6) showed a correlation with the proton at δ_{H} 8.78 (H-7). It confirmed the combination of the B, C and D rings, also implied carbonyl group was at position 1 not position 4. The methoxyl group at δ_{H} 4.09 (H-14) showed a NOE correlation with the proton at δ_{H} 6.89 (H-9). It indicated the combination of the A and B rings.

In the COSY spectra, δ_{H} 10.10 (1H, s) and δ_{H} 8.78 (1H, s) there is no correlation with other protons, they connected with C-12 (δ_{C} 125.17), C-7 (δ_{C} 121.52), respectively. The full assignment of compound **1** was supported by the HMBC correlations. The observed long range correlations from δ_{H} 1.46 (13-Me) to δ_{C} 145.61 (C-4a) and 198.76 (C-1) in the HMBC spectrum showed that a methyl group was attached to C-3, the HMBC correlation from δ_{H} 4.06 (OH, s) to δ_{C} 28.47 (C-13, 1-Me), 45.41 (C-4), 54.28 (C-2) and 70.55 (C-3) indicated that the hydroxyl group was attached to C-3. HMBC correlation from δ_{H} 4.09 (–O–CH₃) to δ_{C} 155.38 (C-8) indicated that the methoxy group was attached to C-8. The

HMBC correlation from δ_H 3.35 (H-4), 7.37 (H-5) and 2.97 (H-2) to δ_C 198.76 (C-1) indicated that a carbonyl unit was in C-1. As shown in Figure 1B, the linkage of the Six-carbon ring with the anthracene skeleton was established by the correlations from δ_H 3.35 (H-4) and 2.97 (H-2) to δ_C 145.61 (C-4a), δ_H 2.97 (H-2) to δ_C 125.46 (C-12b).

Consequently, the structure of compound **1** was established to be 3-hydroxy-1-keto-3-methyl-8-methoxy-1,2,3,4-tetrahydro-benz[α]anthracene (Table 1).

2.2. Cytotoxicity and Antifungal Activities

In the cytotoxicity test, compound **1** showed no cytotoxicity against human leukemic cells line HL-60 (Table 2), a weaker cytotoxicity against human hepatoma cell line BEL-7402 compared with adriamycin (Table 2). However, compound **1** exhibited a potent inhibitory activity against human lung adenocarcinoma cell line A549. Although compound **1** showed a weaker cytotoxicity at higher concentrations than the positive control, adriamycin, while at lower concentrations, the effect of compound **1** was found to be stronger than adriamycin (Table 2).

Table 2. Cytotoxicity of compound **1** against human leukemic cells line HL-60, human hepatoma cell line BEL-7402, human lung adenocarcinoma cell line A549.

Cancer cell line	Rate of inhibition of sample (%)	Concentration (M)				
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
human leukemic cells line HL-60	Rate of inhibition of compound 1	0	0	0	0	0
	Rate of inhibition of adriamycin	100	88.5	89.5	88.2	0
human hepatoma cell line BEL-7402	Rate of inhibition of compound 1	37.5	37.0	25.7	19.5	0
	Rate of inhibition of adriamycin	80.9	85.7	63.4	32.8	15.5
human lung adenocarcinoma cell line A549	Rate of inhibition of compound 1	65.5	62.8	61.8	47.8	48.8
	Rate of inhibition of adriamycin	100	61.8	50.8	21.4	4.3

The results demonstrated that the compound **1** almost showed no antifungal activities against the *Monilinia fructicola* (Mf) and *Colletotrichum lagenarium* (Cl) (Table 3).

Table 3. Antifungal activity of compound **1**.

Microbial activities	The radius of the zone of inhibition (mm)
Blank	6
Antifungal (Mf)	8
Antifungal (Cl)	9

3. Experimental Section

3.1. General Experimental Procedures

NMR spectra were recorded on Bruker AVANCE IIITM 500 spectrometers and TMS was used as internal standard. Column chromatography was carried out on silica gel (200–300 mesh) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden). LC-MS were obtained by ESI on a Thermo Fisher Scientific LCQ Fleet mass spectrometer. All reagents were of analytic grade.

Precoated silica gel plates (F-254, 0.2 mm) were used for analytical TLC. HPLC was performed on a JASCO PU-2087 HPLC apparatus with a Zorbax Eclipse XDB-C18 column (250 × 9.4 mm, 5 µm; 2 mL/min; 254 nm; Agilent, Palo Alto, CA, USA).

3.2. Strain and Medium for Isolation and Fermentation

Marine *Streptomyces* sp. W007 isolated on Gause's synthetic agar containing 50% natural seawater was from the marine sediments of Kiaochow Bay, Qingdao. Marine *Streptomyces* sp. W007 was characterized according to the 16S rRNA gene sequence (Accession No. JN180126 in GenBank). *Monilinia fructicola* (Mf) and *Colletotrichum lagenarium* (Cl) were from Qingdao Agricultural University (Shandong, China).

Gause's synthetic agar (for separation of streptomycete): soluble starch 20 g, KNO₃ 1 g, K₂HPO₄ 0.5 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 0.01 g, K₂Cr₂O₇ 0.3 g, seawater 500 mL, deionized water 500 mL, pH 7.4. M2+ medium (for fermentation): malt extract 10 g, yeast extract 4 g, anhydrous glucose 4 g, deionized water 500 mL, seawater 500 mL, pH 7.8.

3.3. Fermentation

Well-grown agar cultures of *Streptomyces* sp. W007 were served to inoculate 1 L-Erlenmeyer flasks each containing 300 mL M2+ medium. The liquid medium of *Streptomyces* sp. W007 were incubated for 2 days with 180 rpm at 28 °C, then they were used to inoculate 100 Erlenmeyer flasks each containing 300 mL M2+ medium, inoculum size was 20% (v/v). At last, 100 Erlenmeyer flasks were held at 28 °C for 4 days (pH 7.8, 220 rpm).

3.4. Extraction and Separation

The culture broth (30 L) was filtered to give the mycelium and culture filtrate, and the filtrate was extracted by ethyl acetate. The mycelium was dried at 45 °C, and ultrasonically extracted three times by ethyl acetate and acetone respectively (15 min/time), then they were dried at 45 °C under reduced pressure. Three parts of organic layers were combined and defatted with cyclohexane to give a crude extract (8.4 g). The defatted extract was separated with silica gel column, five fractions were obtained. Fraction 1 was further purified by Sephadex LH-20 to afford A1–A5, A5 was purified by reverse column with a stepwise gradient of methanol/water (2:8–8:2) and detected by TLC to give four fractions (B1–B4). B4 was subjected to PTLC, during this step, three fractions (C1–C3) was obtained, C3 was separated by semi-preparative HPLC (JASCO PU-2087, Zorbax Eclipse XDB-C18, 250 × 9.4 mm; 2 mL/min; 254 nm; Agilent, Palo Alto, CA, USA) eluting with CH₃OH/H₂O(80:20, v/v) to afford compound **1** (retention time 9.37 min, 12 mg).

3.5. Cytotoxicity Tests and Antifungal Activities

Cytotoxic activity was evaluated using the human leukemic cell line HL-60 by the MTT (Methyl-Thiazol-Tetrazolium) method [14] and the human lung adenocarcinoma cell line A549 and human hepatoma cell line BEL-7402 by the SRB (sulforhodamine B) method [15]. The antifungal

activities of compound **1** were evaluated using the agar diffusion assay [16]. Inhibition ratio was calculated according to the formula:

$$\text{Inhibition ratio} = (1 - \frac{A_{\text{sample}}}{A_{\text{blank}}}) \times 100\%$$

A_{sample} : the absorbance of the sample at 540/515;

A_{blank} : the absorbance of the blank at 540/515.

In the MTT assay, the cell line was grown in RPMI-1640 supplemented with 10% FBS (Fetal bovine serum) under a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cell suspensions (200 µL) at a density of 5×10^4 cells/mL were plated in 96-well microtiter plates and incubated for 24 h. The compound **1** solutions (2 µL in MeOH) at different concentrations (0, 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} M) were added to each well and further incubated for 72 h under the same conditions. MTT solution (20 µL of a 5 mg/mL solution IPMI-1640 medium) was added to each well and incubated for 4 h. RPMI-1640 medium (150 µL) containing MTT was then gently replaced by DMSO (dimethyl sulfoxide) and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 540 nm.

In the SRB assay, cell suspensions (200 µL) were plated in 96-cell plates at a density of 2×10^5 cells/mL. Then compound **1** solutions (2 µL in MeOH) at different concentrations (0, 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} M) were added to each well and further incubated for 24 h. Following drug exposure, the cells were fixed with 12% trichloroacetic acid and the cell layer was stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm.

Agar diffusion assay: compound **1** was dissolved in MeOH/CH₂Cl₂ (1:1) at concentrations of 5 µg/µL. Fifty microliters of each solution was pipetted onto a sterile filter disk, which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the respective test organism. The disks (6 mm) were air-dried in bechtop, placed on an inoculated agar plate, and incubated at 28 °C overnight, the radius of the zone of inhibition was measured in mm.

4. Conclusions

The results of cytotoxic tests suggested that cytotoxicities of compound **1** are selective to the tested cell lines. Adriamycin plays an antitumor function: the interaction of adriamycin and type II DNA topoisomerase causes the DNA to break. Compound **1** showed a stronger cytotoxicity at low concentration, and weaker cytotoxicity at high concentration, than positive control adriamycin; such a phenomenon implied that antitumor mechanisms of adriamycin and compound **1** were different. Possible mechanisms for cytotoxic activity against human lung adenocarcinoma cell line A549 of compound **1** are as follows: compound **1** selectively induces apoptosis in human lung adenocarcinoma cell line A549 while sparing other cells; Compound **1** has negative effect on cancer cell proliferation and the cell cycle, maybe as an enzyme inhibitor; Compound **1** can inhibit metabolism of tumor cell lines and kill them.

Therefore, further studies on the bioactivity of compound **1** are being carried out and the results described in this study suggest that compound **1** could potentially lead to antitumor agents.

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Samples Availability: Available from the authors.

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