Arch Pharm Res Vol 29, No 8, 624-626, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Four Butenolides are Novel Cytotoxic Compounds Isolated from the Marine-Derived Bacterium, *Streptoverticillium luteoverticillatum* 11014

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(Received March 9, 2006)

Four known butenolides were isolated from the ethyl acetate extracts of the culture broth of the marine-derived bacterium, *Streptoverticillium luteoverticillatum*, by bioassay-guided fractionation. The structures were identified on the basis of spectral data. The absolute configuration of compound (**1**) was determined by CD spectrum for the first time. Compounds **1-4** showed *in vitro* cytotoxicity against the murine lymphoma P388 and human leukemia K562 cell lines. This is the first report on the isolation of butenolides from the marine bacterium, *Streptoverticillium luteoverticillatum*, and their cytotoxic activities.

Key words: Marine-derived bacteria, *Streptoverticillium luteoverticillatum*, Butenolides, Anti-tumor activities

INTRODUCTION

Natural products with novel structures and distinct biological activities have attracted much attention in the development of new medicines, and numerous such products have been discovered as secondary metabolites of marine-derived microbes (Blunt *et al.*, 2005; Tayyab *et al.*, 2004). In the continuing search for active anti-tumor compounds from marine-derived microorganisms, we found that crude extracts from bacteria strain 11014, identified as *Streptoverticillium luteoverticillatum*, exhibited cytotoxicity against the mouse tsFT210 cancer cell line. Further studies on the active constituents of this bacterium led to the isolation of four butenolides (Fig. 1) by bioassay-guided fractionation. Their structures were determined to be (4*S*)-4, 10-dihydroxy-10-methyl-undec-2-en-1, 4-olide (**1**), (4*S*)-4, 10-dihydroxy-10-methyl-dodec-2-en-1, 4-olide (**2**), and two diastereomeric (4*S*)-4, 11-dihydroxy-10-methyl-dodec-2-en-1, 4-olides (**3/4**). Compounds **1** and **2** had been previously isolated from a marine-derived streptomycete, and their plane structures were identified (Cho *et al.*, 2001). In addition, the absolute configurations of **2-4** at C-4 have been reported (Mukku *et al.*, 2000). In this paper, we describe the isolation and evaluation of the *in vitro* cytotoxicity of these four butenolides (**1-4**) against the K562 and P388 cell lines and the absolute stereochemical identification of compound **1** for the first time.

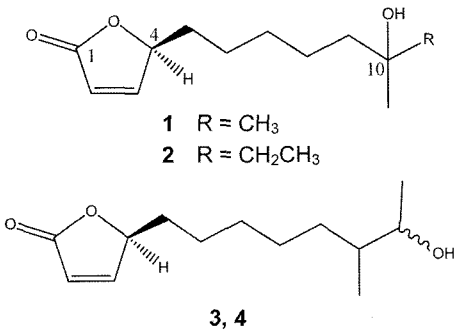

Fig. 1. Structures of compounds **1-4**

MATERIALS AND METHODS

General experimental procedures

1D (^{1}H, ^{13}C, DEPT) and 2D NMR spectra were recorded on a JEOL Eclips-600MHz NMR spectrometer using TMS

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as an internal standard; chemical shifts were recorded as δ values. ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semi-preparative HPLC was performed using an ODS column [Shin-pak ODS (H), 20×250 mm, 5 μm, 4 mL/min].

Collection and taxonomic identification

The bacterial strain 11014 was isolated from underwater sediment (depth 20 m), rich with organic materials, collected off the coast of TaiPingJiao, QingDao, China, in October 2001. The strain was maintained on Gause's Starch agar with natural aged seawater. It was identified as *Streptoverticillium luteoverticillatum* by the China Center for Type Culture Collection.

Fermentation and extraction

A slant culture of strain 11014 was inoculated in a 500 mL Erlenmeyer flask containing 100 mL of media (2% glucose, 0.05% NaCl, 0.05% $MgSO_4$, 0.05% K_2HPO_4, 0.3% beef extract, 0.3% corn steep liquor, 1% yeast extract, 1% starch, and 0.2% $CaCO_3$) in aged seawater, adjusted to pH 7.0. The culture was incubated at 28°C for 3 days on a rotary shaker (110 rpm). Ten milliliters of the seed culture was transferred to a 500 mL Erlenmeyer flask containing 100 mL of the same media and incubated for 6 days under similar conditions for fermentation. Two hundred liters of whole broth was filtered to separate the broth supernatant and mycelia. The former was extracted with ethyl acetate, while the latter was extracted with acetone. The acetone extraction was evaporated under reduced pressure to produce an aqueous solution, which was then extracted with ethyl acetate. The two ethyl acetate extractions were combined and concentrated *in vacuo* to yield 5.0 g of crude extract.

Isolation and purification

The crude extract (5.0 g) showed cytotoxic activity against tsFT210 cells and was subsequently chromatographed on a silica gel column using a gradient elution with petroleum/EtOAc/ MeOH to obtain 20 fractions. Fraction 13 (197 mg), the active fraction, was then chromatographed by semi-preparative HPLC with 50% aqueous MeOH to yield compounds **1** (8 mg, t_R=12.5 min), **2** (10 mg, t_R=32.1 min) and **3/4** (11 mg, t_R=33.4 min).

Compound (1)

Yellow syrup; IR (KBr) max: 3469, 2968, 2935, 1750, 1600, 1465, 1165 cm^{-1}; positive ESI-MS *m/z*: 235.2 $[M + Na]^+$; CD $[\theta]_{204.1}$ (MeOH) +25 488; ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.44 (1H, dd, *J* = 5.9, 1.4 Hz, H-3), 6.12 (1H, dd ,*J* = 5.9, 1.1Hz, H-2), 5.05 (1H, m, H-4), 1.78 (1H, m , H-5), 1.67 (1H, m, H-5), 1.48 (2H, m, H-6),1.46 (2H, m, H-9), 1.36 (4H, m, H-7, H-8), 1.21 (6H, s, H-11, H-12); ^{13}C-NMR: see Table I.

Compound (2)

Yellow syrup; IR (KBr) max: 3467, 2965, 2935, 1751, 1601, 1462, 1376, 1166 cm^{-1}; positive ESI-MS *m/z*: 249.2 $[M + Na]^+$; CD $[\theta]_{203.5}$ (MeOH) +26 526; ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.44 (1H, dd, *J* = 5.9, 1.4 Hz, H-3), 6.11 (1H, dd , *J* = 5.9, 1.4 Hz, H-2), 5.04 (1H, m, H-4), 1.78 (1H, m , H-5), 1.68 (1H, m, H-5), 1.63 (2H, m, H-6), 1.48 (2H, m, H-11), 1.42 (2H, m, H-9), 1.35 (4H, m,H-7, H-8), 1.14 (3H, s, H-13), 0.89 (3H, t, *J* = 7.3 Hz, H-12); ^{13}C-NMR: see Table I.

Compounds (3/4)

Yellow syrup, inseparable mixture of two diastereomers; IR (KBr) max: 3466, 2930, 2858, 1750, 1461, 1376, 1165, 1101 cm^{-1}; positive ESI-MS *m/z*: 227.2 $[M + H]^+$; CD $[\theta]_{220.6}$ (MeOH) +22 152; ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.46 (1H, dd, *J* = 5.8, 1.6 Hz, H-3), 6.11 (1H, dd, *J* =5.8, 1.6 Hz, H-2), 5.05 (1H, m, H-4), 3.72, 3.65 (each 1H, m, H-11of **3** and **4**), 1.82-1.25 (11H, m), 1.13, 1.15 (each 3H, 2 d, *J* = 6.2 Hz, CH_3-12 of **3** and **4**), 0.88, 0.86 (each 3H, 2d, *J* = 6.8 Hz, CH_3-13 of **3** and **4**); ^{13}C-NMR: see Table I.

Biological assays

The ability of compounds **1-4** to inhibit K562 (human myeloid leukemia cell) and P388 (mouse lymphoma cell) proliferation was measured using the SRB assay (Skehan *et al.*, 1990). Briefly, 200 μL of cell suspensions were plated in 96-well plates at a density of 2×10^5 cells mL^{-1}. Two μL of each test compound at various concentrations in MeOH was then added to each well and further incubated for 24 hours. Following drug exposure, the cells were fixed with 12% trichloroacetic acid, and the cell layer was then stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm. Dose-response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of log-dose-response curves.

RESULTS AND DISCUSSION

Compounds **1** and **2**, each yellow syrups, had pseudo-molecular ion peaks at *m/z* 235.2 $[M + Na]^+$ and 249.2 $[M + Na]^+$ in the positive ESI-MS, corresponding to their molecular compositions of $C_{12}H_{20}O_3$ and $C_{13}H_{22}O_3$, respectively. Through combined interpretation of the ^{1}H- and ^{13}C-NMR spectra data (Table I), their structures were identified as 4, 10-dihydroxy-10-methyl-undec-2-en-1, 4-olide (**1**) and (4*S*)-4, 10-dihydroxy-10-methyl-dodec-2-en-1, 4-olide (**2**), according to reported data in the literature (Cho *et al.*, 2001).

Butenolide **1** also showed a positive π-π^* cotton effect at 204.1 nm, therefore C-4 must be in the *S* configuration, as it is in compound **2** (Mukku *et al.*, 2000). This is the first time that the absolute configuration of compound **1** has been established.

An inseparable mixture of the two diastereomers, **3** and **4**, had a pseudomolecular ion peak at *m/z* 227.2 $[M + H]^+$ in the positive ESI-MS. Corresponding to their molecular composition of $C_{13}H_{22}O_3$, these compounds were subsequently identified as two diastereomers, (4*S*)-4, 11-dihydroxy-10-methyl-dodec-2-en-1, 4-olides, following the analysis of their CD and NMR data (Table I), as well as comparison of their spectral data with those in the literature (Mukku *et al.*, 2000).

The cytotoxicity of compounds **1-4** was evaluated in the human leukemia K562 and murine lymphoma P388 cell lines by the SRB method. Compounds **1-4** inhibited the proliferation of K562 cells with IC_{50} values (Table II) of 8.73, 6.29, and 1.05 μmol/mL and of P388 cells with IC_{50} values of 0.34, 0.19, and 0.18 μmol/mL, respectively. In addition, the cells were observed under a light microscope for morphological changes following incubation with these compounds. In agreement with the SRB assay results, compounds **1-4** exhibited cytotoxicity at high concentrations. All of the compounds were more active on the P388 cell line than on the K562 cell line. The mixture of compounds **3** and **4** was the most active, but it remains unclear if both of the diastereomers are active. None of the compounds display any cell-cycle inhibitory or apoptosis-inducing activity on K562 and P388 cells, as assessed by flow cytometry analysis. Detailed studies on their anti-tumor activities and the related mechanisms of action are currently underway.

Table I. ^{13}C-NMR data (150 MHz) of compounds **1-4** in $CDCl_3$

position	1	2	3	4
1	173.2 s	173.1 s	173.2 s	173.2 s
2	121.6 d	121.5 d	121.5 d	121.5 d
3	156.3 d	156.3 d	156.3 d	156.3 d
4	83.4 d	83.4 d	83.4 d	83.4 d
5	33.1 t	33.1 t	33.1 t	33.1 t
6	25.0 t	24.9 t	24.9 t	24.9 t
7	30.0 t	29.8 t	29.6 t	29.6 t
8	24.1 t	23.5 t	27.0 t	27.1 t
9	43.7 t	41.0 t	32.4 t	32.4 t
10	71.0 s	72.8 s	39.6 d	39.9 d
11	29.3 q	34.2 t	71.3 d	71.7 d
12	30.3 q	8.2 q	19.4 q	20.2 q
13		26.3 q	14.2 q	14.5 q

Table II. Cytotoxicity of **1-4** in two cancer cell lines

Compound	IC_{50} ($\bar{X}$ ± SD μmol/mL, n=3)*	
	K562	P388
1	8.73 ± 1.44	0.34 ± 0.27
2	6.29 ± 3.25	0.19 ± 0.09
3/4	1.05 ± 0.54	0.18 ± 0.11
CDDP	0.078 ± 0.044	0.039 ± 0.008

IC_{50} is defined as the concentration that results in a 50% decrease of viable cell numbers. Data represent mean values of three independent experiments and were determined by the SRB method.

Butenolides are often generated in fungi, bacteria, and gorgonians (Mukku *et al.*, 2000). Their saturated analogues act as signaling molecules in bacteria to enhance spore formation in streptomycetes or induce metabolite production. Some butenolides have also shown anti-tumor activity (Tayyab *et al.*, 2004), antibiotic activity against *Pseudomonas aeruhinosa*, and inhibition of the chitinase from *Serratia marcescens* (Braun *et al.*, 1995). To the best of our knowledge, this is the first report of the isolation of butenolides from the marine-derived bacterium, *Streptoverticillium luteoverticillatum*, and of the anti-tumor activities of these four compounds (**1-4**).

ACKNOWLEDGEMENTS

This work was founded by the Chinese Ocean Mineral Resource R & D Association (DY105-2-04).

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