Chlovalicin, a New Cytocidal Antibiotic Produced by *Sporothrix* sp. FO-4649

II. Physicochemical Properties and Structural Elucidation

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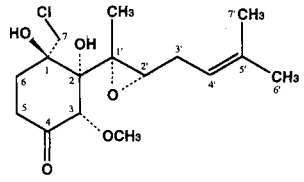
(Received for publication November 15, 1995)

A new growth inhibitor of IL-6 responsive MH60 cells, chlovalicin (MW; 332, $C_{16}H_{25}O_5Cl$), was found in cultures of *Sporothrix* sp. FO-4649, together with a known sesquiterpene, ovalicin. The structure of chlovalicin was elucidated by spectroscopic methods. Chlovalicin possesses a chlorinated methylene moiety at the C-1 position, and it corresponds to halogenated products derived from the epoxide ring attached to the C-1 position of ovalicin. The absolute configuration of chlovalicin was clarified as 1*S*, 2*R*, 3*S*, 1′*S*, 2′*R* by chemical transformation from ovalicin.

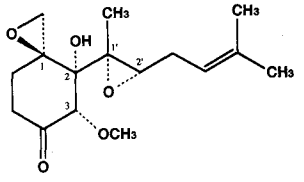
Interleukin 6 (IL-6) is a multifunctional cytokine produced by various cells irrespective of lymphoid and nonlymphoid lineage, which acts on immune response, hematopoiesis, acute phase reactions and the nervous system[1]. Thus, IL-6 plays one of the central regulatory roles in host defense mechanisms. Recently, it has been demonstrated that dysregulation of IL-6 gene expression is actually involved in the pathogenesis of autoimmune diseases and cancer cachexia accompanying lymphoid malignancies and multiple myelomas[2~5]. Therefore, it may be possible that the inhibition of IL-6 activity relieves cancer cachexia.

In the course of a search for suppressors of IL-6 activity of microbial origin, we found a new IL-6 inhibitor, chlovalicin (**1**), together with ovalicin (**2**) in cultures of *Sporothrix* sp. FO-4649, isolated from a soil sample (Fig. 1). Taxonomic studies of the producing strain, the isolation procedure and the biological characteristics of chlovalicin (**1**) were reported in a previous paper[6]. This paper describes physicochemical properties and structural determination of chlovalicin (**1**).

Fig. 1. Structures of chlovalicin (**1**) and ovalicin (**2**).



1 (1*S*, 2*R*, 3*S*, 1′*S*, 2′*R*)



2 (1*S*, 2*R*, 3*S*, 1′*S*, 2′*R*)

Results and Discussion

Chlovalicin (**1**) was isolated as a colorless oil. Physicochemical properties of **1** are summarized in Table 1. The EI-MS spectrum of **1** showed $[M^+]$ at *m/z* 332 (100%) and $[M+2]^+$ at *m/z* 334 (35%). These peaks are characteristic in the spectra of compounds containing a chlorine atom in the molecule. The molecular formula of **1** was determined to be $C_{16}H_{25}O_5Cl$ by HR-EI mass spectrometry. In the ^{13}C NMR spectrum of **1** (Table 2), the chemical shifts of carbon signals showed a similar pattern to that of ovalicin (**2**). Therefore, **1** was suggested to be a derivative of **2**. In the ^1H-^1H COSY spectrum of **1**, the side chain moiety of **1** was determined as shown in Fig. 2 (a, b). Other partial structures of **1** were determined by the DEPT experiments of **1** (Fig. 2, c~g). The signal of C-1 observed at δ 76.9 ppm was shifted downfield (14.7 ppm) compared to that of **2** (δ 62.2 ppm). In the ^1H NMR spectrum of **1** (Table 3), proton signals at H-7a (δ 3.68, br d J=11.2 Hz) and at

Table 1. Physicochemical properties of **1**.

Appearance:	Colorless oil
$[\alpha]_D^{20}$:	−16.0° (*c* 0.1, MeOH)
UV λ_{max}^{MeOH} nm:	End abs.
EI-MS (*m/z*):	334 (M^++2, 35%), 332 (M^+, 100%)
HR EI-MS (*m/z*):	Obsd. 332.1386 ($C_{16}H_{25}O_5Cl$)
	Calcd. 332.1390
Color reaction	
Positive:	50% $H_2SO_4+\Delta$, Iodine
Negative:	Dragendorff's reagent, ninhydrin reagent

H-7b (δ 3.55, br d J=11.2 Hz) were observed in the low-field, in the 0.71~0.92 ppm range, compared with those of **2**. Therefore, the methylene carbon at the C-7 position was assumed to be adjacent to chlorine in consideration of the ^{1}H NMR spectrum and the molecular formula. Furthermore, chemical shifts and coupling constants of chloromethyl carbon on ^{1}H and ^{13}C NMR spectra of **1** were in good agreement with those of an antibiotic AA-57[7] as shown in Fig. 3. Final elucidation of the structure of **1** was performed using HMBC (8 Hz) experiments as shown in Fig. 4. The presence of a methoxy group at C-3 and the epoxide ring on the side chain attached to the cyclohexanone ring at C-1 was supported.

The final confirmation of structure **1**, including the absolute configuration, was performed by chemical conversion from **2**, the stereochemistry of which was clarified by X-ray crystallographic techniques using the bromo derivative[8]. Reaction of **2** with hydrogen chloride in benzene gave **1**. This reaction was suggested that the dominant mode introduced chloride at the less substituted carbon[9]. Therefore, 1-chloromethyl-1-hydroxy derivative (**1**) was formed in preference to 1-chlorohydroxymethyl. Chemical shifts of **1** derived from **2** were same as those of natural **1** on ^{1}H NMR spectrum. Furthermore, the structure of synthetic **1** was confirmed by co-TLC and co-HPLC analyses with an authentic sample. In the CD spectrum, **1** showed a positive Cotton effect at 291 nm ($\Delta\varepsilon$+1.4). The synthetic **1** derived from **2** showed the same Cotton effect as that of the natural compound. Therefore, the absolute configuration of **1** was confirmed as 1*S*, 2*R*, 3*S*, 1′*S*, 2′*R*, identical to that of ovalicin (**2**).

As the result of screening for suppressors of IL-6-

Table 2. ^{13}C NMR chemical shifts of **1** and **2** in CD_3OD.

C	M	1	2	Δ (1~2)
1	s	76.9	62.2	+14.7
2	s	83.1	80.0	+3.1
3	d	86.1	87.7	−1.6
4	s	210.9	209.4	+1.5
5	t	36.3	37.6	−1.3
6	t	33.0	31.4	+1.6
7	t	52.5	52.0	+0.5
1′	s	62.3	61.9	+0.4
2′	d	58.1	58.2	−0.1
3′	t	28.0	28.4	−0.4
4′	d	119.6	120.0	−0.4
5′	s	136.3	136.4	−0.1
6′	q	25.9	26.2	−0.3
7′	q	18.1	18.4	−0.3
OMe				
3	q	59.5	59.6	−0.1
Me				
1′	q	16.2	15.3	+0.9

M: Multiplicity.

Fig. 2. Partial structures of **1**.

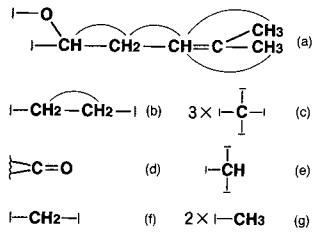
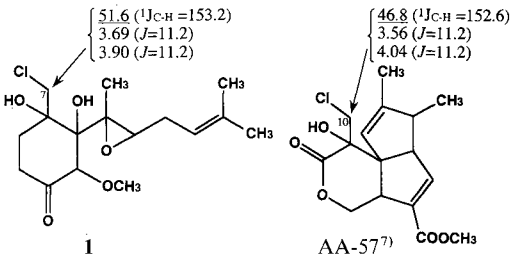


Table 3. ^{1}H NMR chemical shifts of **1** and **2** in CD_3OD.

H	1	2	Δ (1~2)
3	4.60 (1H, br s)	4.27 (1H, d, 0.7)	+0.33
5a	2.60 (1H, dt, 14.2, 7.6)	2.62 (1H, ddd, 12.5, 7.3, 0.7)	−0.02
5b	2.12 (1H, br d, 7.9)	2.27 (1H, m)	−0.15
6a	2.12 (1H, br d, 7.9)	2.41 (1H, dd, 13.2, 5.3)	−0.29
6b	1.95 (1H, dt, 14.2, 7.6)	1.42 (1H, ddd, 13.2, 7.3, 2.3)	+0.53
7a	3.68 (1H, br d, 11.2)	2.97 (1H, d, 4.3)	+0.71
7b	3.55 (1H, br d, 11.2)	2.63 (1H, d, 4.3)	+0.92
2′	2.85 (1H, t, 7.0)	2.80 (1H, t, 6.5)	+0.05
3′a	2.30 (1H, br t, 7.0)	2.27 (1H, m)	+0.03
3′b	2.12 (1H, br d, 7.9)	2.10 (1H, ddd, 14.5, 7.3, 2.0)	+0.02
4′	5.15 (1H, t, 7.0)	5.13 (1H, tq, 7.3, 1.3)	+0.02
6′	1.66 (3H, s)	1.66 (3H, s)	±0
7′	1.59 (3H, s)	1.58 (3H, s)	+0.01
OMe			
3	3.38 (3H, s)	3.39 (3H, s)	−0.01
Me			
1′	1.44 (3H, s)	1.26 (3H, s)	+0.18

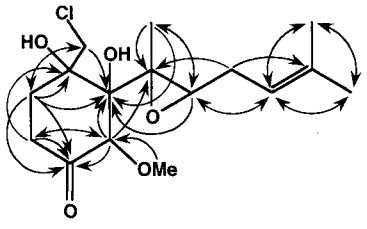
(Multiplicity, J value, Hz).

Fig. 3. Chemical shift comparison of chloromethyl carbon of **1** with those of AA-57 on ^{1}H and ^{13}C NMR in $CDCl_3$.



Values show δ_H, those underlined represent δ_C, and those in parentheses are coupling constants in Hz.

Fig. 4. ^{1}H-^{13}C long range couplings detected by HMBC experiments of **1**.



$^{1}H \rightarrow {}^{13}C$ (J=8 Hz).

activity, we isolated a new sesquiterpene, named chlovalicin (**1**), together with ovalicin (**2**). The absolute structure of **1** was confirmed by chemical transformation from **2**. Ovalicin (**2**), a metabolite of the fungus *Pseudorotium ovalis* Stolk[8,10], shows antitumor and immunosuppressive activity[11~13], and biosynthesis[14,15] and total synthesis[16] of ovalicin have been investigated. Compound **1** possesses a chlorinated methylene moiety at the C-1 position, which corresponds to halogenated products derived from the epoxide ring attached to the C-1 position of ovalicin (**2**). Therefore, it is of interest to consider the biosynthesis of its unique chlorinated sesquiterpene.

Experimental

Spectroscopic Studies

UV spectra were recorded on a Shimadzu model UV-160A spectrophotometer. MS were obtained with a JEOL model JMS DX-300 mass spectrometer. ^{1}H (270 MHz) and ^{13}C (67.8 MHz) NMR spectra were recorded on a JEOL JNM-EX 270. CD spectra were measured on a JASCO J-720 spectropolarimeter in MeOH. Analytical HPLC was carried out with CH_3CN-H_2O (40:60) using a Senshu Pak Pegasil ODS (5 μm, i.d. 4.6 × 250 mm) column employing a UV monitoring system (210 nm) at a flow rate of 0.8 ml/minute. Preparative HPLC was performed using a Senshu Pak Pegasil ODS (5 μm, i.d. 20 × 250 mm) column with a solvent system of CH_3CN-H_2O (40:60) at 7 ml/minute.

Reaction of 2 with Hydrogen Chloride[9]

Ovalicin (**2**) (4.0 mg) (CD: 291 nm; $\Delta\varepsilon$ +2.6) was dissolved in 1 ml of benzene. The benzene solution (450 μl) saturated with hydrogen chloride gas was added gradually to the former solution in an ice bath. After the reaction mixture was concentrated under reduced pressure, the products were purified by preparative HPLC. Synthetic **1** (CD: 291 nm; $\Delta\varepsilon$ +1.4) was obtained with a yield of 24.5% (1.1 mg).

Acknowledgments

This work was supported in part by Grants-in Aid from the Ministry of Education, Science and Culture, Japan and funds from the Japan Keirin Association.

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