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(*3R*,*6R*)-4-Methyl-6-(1-methylethyl)-3-phenylmethylperhydro-1,4-oxazine-2,5-dione: An Apoptosis-Inducer from the Fruiting Bodies of *Isaria japonica*

Abstract

(3*R*,6*R*)-4-Methyl-6-(1-methylethyl)-3-phenylmethylperhydro-1,4-oxazine-2,5-dione (**1**) was isolated from the fruiting bodies of *Isaria japonica* as an apoptosis-inducing agent. The complete structural assignment of the compound was accomplished on the basis of spectroscopic methods and chemical transformations. Compound **1** induced apoptotic cell death of the human leukemia cells (HL-60) in a dose-dependent manner, ranging from 5.0 μg/ml to 100.0 μg/ml.

Key words
Isaria japonica · fungal metabolite · dixomorpholine · human HL-60 leukemia cell · apoptosis

Introduction

The genus *Isaria*, which is an anamorph stage of *Cordyceps*, infects live insects in the larval and pupal stages, and grows out of the dead host [1]. The fruiting bodies of these fungi have been traditionally used in oriental medicine to treat numerous illnesses [2]. A number of bioactive metabolites were reported from *Cordyceps* species, including cordycepin [3] and other antibacterial and antitumor adenosine derivatives [4]. However, the constituents of *Isaria* species are relatively unknown. The representative metabolite from the genus *Isaria* is a potent immunosuppressive agent myriocin from *I. sinclairii* [5].

Apoptosis (programmed cell death) is a distinctive type of cell death, which is regulated by the genetic process [6], [7]. This physiological process includes cell shrinkage, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, and chromatin condensation [7]. Although the involvement of apoptosis in various diseases is not always straightforward, there is accumulated evidence suggesting that the malfunction of this death mechanism could lead to proliferative or degenerative diseases. For example, cancer could be viewed as a result of ineffective apoptosis or unrestricted proliferation [8]. In cancer therapy, it is now recognized that many effective chemotherapeutic agents might trigger the tumor to kill itself by activation of an apoptotic pathway [6]. Therefore, apoptosis-inducing natural compounds would have a great therapeutic potential in cancer therapy.

Our previous studies of *Isaria japonica* Yasuda as a source of anticancer and/or other bioactive secondary metabolites have resulted in the isolation of a potent apoptosis-inducing compound 4-acetyl-12,13-epoxy-9-trichothecene-3,15-diol [9]. Continuing investigations on the fungus have led to the isolation of an additional apoptosis-inducing agent, (3*R*,6*R*)-4-methyl-6-(1-methylethyl)-3-phenylmethylperhydro-1,4-oxazine-2,5-dione (**1**). Two fungal metabolites, lateritin and bassiatin (**3**), have been reported to have the same planar structure as that of **1** [10], [11]. Particularly, the NMR data of compound **1** matched very closely with those of lateritin. However, the stereochemistry of lateritin has not been elucidated. In addition, the physico-chemical properties of either bassiatin or three other synthetic stereo-isomers reported to be different from those for lateritin, questioning the proposed structure for lateritin.

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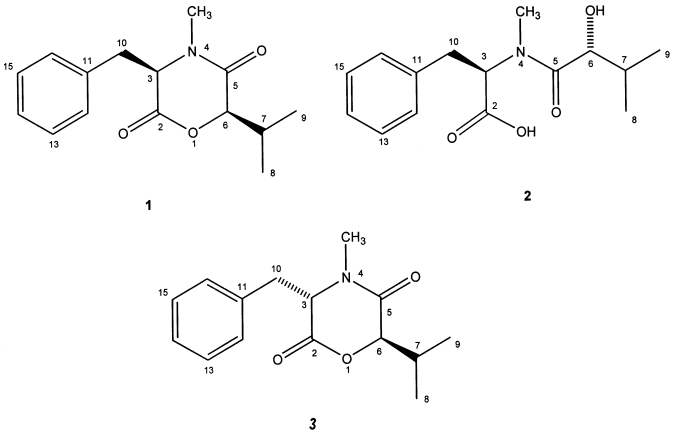

This paper describes the isolation, the complete assignment of compound **1** as (3*R*,6*R*)-4-methyl-6-(1-methylethyl)-3-phenylmethylperhydro-1,4-oxazine-2,5-dione by spectroscopic analysis and chemical transformations, and its biological activity.

Materials and Methods

Fungal material

Details of the fungal species used in this study and fermentation conditions were described in our previous paper [9].

Extraction and isolation

The air-dried fruiting bodies (1 kg) from the fermentation of *I. japonica* were extracted with MeOH (5 l) for 48 h. The MeOH extract was concentrated, suspended in H_2O, and sequentially partitioned with *n*-hexane, EtOAc, and butanol. The EtOAc-soluble fraction (2.9 g) was subjected to C_{18} flash column chromatography (3 × 20 cm) with a stepwise gradient of 0%, 20%, 40%, 50%, 60%, 70%, 90%, and 100% (v/v) MeOH in H_2O (200 ml each, 8 fractions). The fraction eluted at 90% MeOH (305 mg) was applied to silica gel (Merck Kieselgel 60; 0.063 – 0.2 mm particle size; 3 × 20 cm) column chromatography with CH_2Cl_2-MeOH gradient system [0% to 50% MeOH in CH_2Cl_2 (v/v), 50 ml each, 15 fractions]. Based on the TLC analysis (silica gel, CH_2Cl_2/MeOH, 9 : 1), fractions eluted at 4% and 5% MeOH in CH_2Cl_2 were combined (between 300 – 400 ml, 162.4 mg) and subjected to semi-preparative reversed-phase HPLC using a gradient from 60 to 100% CH_3CN in H_2O over 60 min [Alltech HS Hyperprep 100 BDS C_{18} (1.0 × 25 cm; 8-μm particle size; 2 ml/min; UV detection at 210 nm)] to yield **1** (42.5 mg, t_R = 36.8 min).

(3*R*,6*R*)-4-Methyl-6-(1-methylethyl)-3-phenylmethylperhydro-1,4-oxazine-2,5-dione (1)

$[\alpha]_D^{23}$: –48.8° (*c* 0.43, $CHCl_3$); UV (MeOH): λ_{max} (ε) = 209 (5056) nm; IR (film): ν_{max} = 2964, 1745, 1660, 1483, 1413, 1371, 1263, 1180, 1109, 1018 cm^{-1}; ^{1}H- and ^{13}C-NMR: see Table **1**; LRESIMS: *m/z* = 262 $[M + H]^+$.

(3*R*,6*R*)-*N*-Methyl-*N*-(1-hydroxy-2-methylpropyl)-phenylalanine (2)

$[\alpha]_D^{23}$: +25° (*c* 0.12, $CHCl_3$); UV (MeOH): λ_{max} (ε) = 206 (5296) nm; IR (film): ν_{max} = 3474, 2966, 1747, 1664, 1496, 1377, 1253, 1182, 1136, 1043 cm^{-1}; ^{1}H- and ^{13}C-NMR: see Table **1**; LRESIMS: *m/z* = 278 $[M - H]^-$.

Nuclear staining with DAPI/PI and flow cytometric analysis

Nuclear staining and flow cytometric procedures are described in our previous paper [9].

Results and Discussion

Compound **1** was obtained as a white powder. The molecular formula of the compound **1** was determined to be $C_{15}H_{19}NO_3$ based on the analysis of ESIMS and ^{13}C-NMR data. The ^{1}H-, ^{13}C-NMR data of **1** (Table **1**) were almost identical with those of lateritin.

Table **1** NMR data of **1** and **2** in $CDCl_3$

	1				**2**	
C/H no.	***13C[a] δ***	***1H[b] δ (multiplicity, J in Hz)***	***HMBC***	***NOESY***	***13C[a] δ***	***1H[b] δ (multiplicity, J in Hz)***
2	170.2	–	–	–	165.9	
3	57.6	5.47 (br d)	2	10	61.9	4.40 (dd, 5.1, 5.1)
4-CH_3	32.6	3.00 (s)	3, 5	–	32.9	2.99 (s)
5	169.5		–	–	164.5	–
6	75.7	4.92 (d, 8.7)	2, 7, 8	7	83.8	4.36 (d, 7.4)
7	29.9	2.02 (m)	5, 6, 8	6	32.4	1.18 (m)
8	17.8	0.42 (d, 6.9)	6, 7, 9	9, 12, 13	17.5	0.59 (d, 6.5)
9	18.5	0.80 (d, 6.9)	6, 7, 8	8	18.9	0.85 (d, 6.9)
10	35.0	3.36 (dd, 14.5, 5.1) 2.98 (dd, 14.5, 11.9)	2, 3	3	38.1	3.34 (dd, 14.2, 5.1) 3.30 (dd, 14.2, 5.1)
11	136.9	–	–	–	134.8	–
12, 16	129.1	7.23 – 7.26 (m)	10	8	129.8	7.26 – 7.34 (m)[c]
13, 15	128.8	7.23 – 7.26 (m)	10	8	129.1	7.15 – 7.16 (m)[c] 7.26 – 7.34 (m)[c]
14	127.0	7.17 (m)	–	–	127.8	7.15 – 7.16 (m)[c]

[a] Recorded at 500 MHz.
[b] Recorded at 125 MHz.
[c] Assignments may be interchanged.

Although the proposed structure for lateritin was questioned [11], analysis of 1D and 2D NMR data of compound **1** (Table **1**) indeed supported the proposed structure. The proposed structure of **1** was further supported by the formation of compound **2** (72% yield) upon hydrolysis of **1** under mild alkaline conditions (1 N NaOH in MeOH, rt, 80 min). The structure of **2** was identified by the analysis of ^{1}H- and ^{13}C-NMR data, along with the ESIMS result ($[M-H]^-$ at *m/z* 278). The numbering system shown in **2** was chosen by analogy to that used in **1**. The relative stereochemistry of **1** was proposed based on the NOESY data (Table **1**). NOESY correlations of the signals for the aromatic protons (δ = 7.23 – δ 7.26) with the signal for the methyl group (δ = 0.42) suggested their spatial proximity. Therefore, the benzyl group and the methyl groups were located on the same side of the 1,4-oxazine ring, indicating that the relative configurations at C-3 and C-6 to be different from those in bassiatin. Comparison of ^{1}H-NMR data of **1** and those of bassiatin [11] revealed significant chemical shift differences for signals corresponding to H-6 (δ = 2.98 vs. δ 4.92) and H_3 – 9 (δ = 0.74 vs. δ 0.42). Comparison of the X-ray structure of bassiatin [9] and the Drieding model of **1** was undertaken to examine the correlation between the chemical shift differences and the stereochemistries of the compounds. The X-ray structure of bassiatin showed that the H-6 is positioned at shielding zone of the benzyl group, thus, explaining the relatively up-field che-

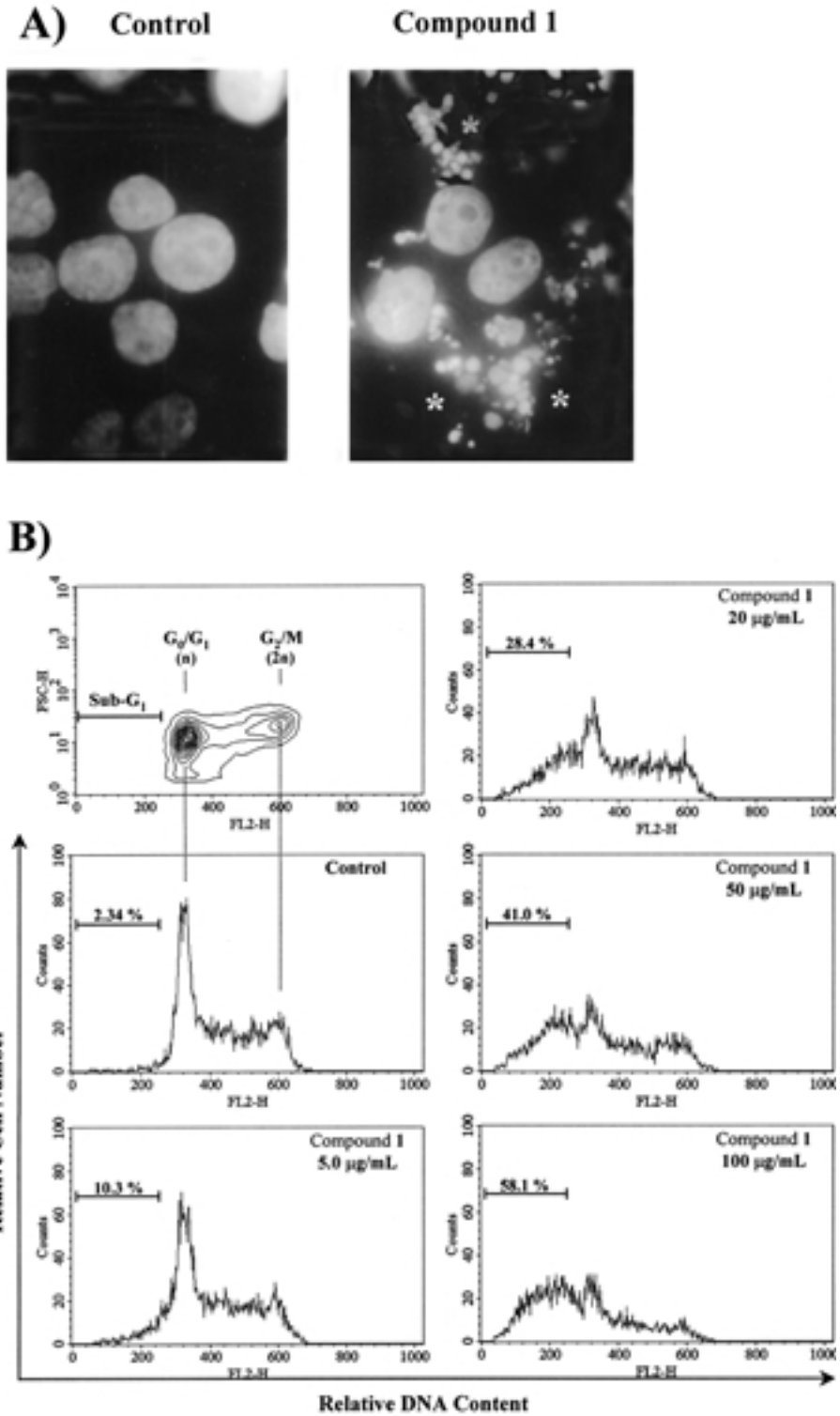

Fig. **1** Apoptotic effects of compound **1** on the human leukemia cells (HL-60). **A**) Morphological features of the cell nuclei after the incubation with 50 μg/ml of **1** for 4 h. Asterisks indicate apoptotic bodies. **B**) Flow cytometric analysis of cell cycles after 6 h treatment of the cells with indicated dose of **1**; sub-G_1 represents fragmented DNA contents (%). Values are representative of three independent experiments.

mical shift of H-6 in bassaitin as compared to the chemical shift of H-6 in **1**. On the other hand, the methyl group (H_3–9), rather than H-6, was positioned in the shielding zone of the benzyl group in compound **1**, thereby resonated at relatively up-field region as compared to H_3–9 in bassiatin. Therefore, the disparity in chemical shifts could be explained by the different orientations of the benzyl group relative to the H-3 and the methyl groups of **1** and bassiatin.

In order to determine the absolute configurations of C-3 and C-6 in **1**, the optical rotations of the hydrolysates were examined. Acidic hydrolysis of compound **1** (6 N HCl, 103 °C, 12 h) resulted in the formation of *N*-methyl phenylalanine and 2-hydroxy-3-methylbutyric acid, which were identified by the analysis of ^{1}H-NMR and ESIMS data. The optical rotation of the obtained *N*-methylphenylalanine ($[\alpha]_D^{23}$ –20 °, *c* 0.05, 1 M HCl) suggested the absolute configuration of C-3 in **1** to be *R*, since the value was consistent with the literature value of *R*-*N*-methylphenylalanine ($[\alpha]_D^{25}$ –24.7 °, *c* 1.6, 1M HCl). Likewise, the absolute configuration of 2-hydroxy-3-methylbutyric acid was suggested to be *R* based on the comparison of the observed optical rotation ($[\alpha]_D^{23}$ –50 °, *c* 0.02, $CHCl_3$) with the literature value of *S*-2-hydroxy-3-methylbutyric acid ($[\alpha]_D^{25}$ + 19 °, *c* 1, $CHCl_3$). In contrast to this assignment, the optical rotation of **1** ($[\alpha]_D^{23}$ –48.8º, *c* 0.43, $CHCl_3$) matched with the reported value for the (3*S*, 6*S*) isomer of bassiatin ($[\alpha]_D^{23}$ –24.3 °, *c* 1.0, $CHCl_3$). At this point, as the planar structure and relative stereochemistry of compound **1** were clearly determined by NMR data analysis and chemical transformations, the NMR data among reported bassiatin isomers [11], **1**, and **2** were closely compared. The NMR data of compound **1,** especially the chemical shifts of H-3 and H-6, were quite different from those of reported both (3*R*,6*R*) and (3*S*,6*S*) isomers of bassiatin. Instead, the NMR data of compound **2** (Table **1**) were almost identical with those reported for the (3*S*,6*S*) and (3*R*,6*R*) isomers of bassiatin. This observation suggested that the previously reported NMR data for the (3*S*,6*S*) and (3*R*,6*R*) isomers of bassiatin could be the NMR data for the morpholine-2,5-dione ring-opened products (i.e., *N*-methyl-*N*-(1-hydroxy-2-methylpropyl)-phenylalanates) of the respective bassiatin isomers. Moreover, the reported optical rotation of (3*R*,6*R*)- 4-methyl-6-(1-methylethyl)-3-phenylmethylperhydro-1,4-oxazine-2,5-dione was consistent with the observed optical rotation of compound **2** ($[\alpha]_D^{23}$ +25°, *c* 0. 12, $CHCl_3$). Therefore, the complete structure of **1** was assigned as (3*R*,6*R*)-4-methyl-6-(1-methylethyl)-3-phenylmethylperhydro-1,4-oxazine-2,5-dione, a morpholine derivative.

To determine the apoptotic cell death of human leukemia HL-60 cells induced by compound **1**, HL-60 cells were incubated for 4 h in the presence of 50 μg/ml of **1**. After the cells were stained with DAPI/PI, morphological alteration was examined under a fluorescence microscopy. As shown in Fig. **1A**, the morphological changes characteristic of apoptosis such as condensation of nuclear chromatin, nuclear fragmentation, and apoptotic bodies were clearly observed. Since the biological hallmark of apoptosis is degradation of DNA, the degree of apoptosis induced by **1** was quantified by measuring the fragmented DNA contents. Quantification of the sub-G1 peak (apoptotic peak) determined by using flow cytometry indicated that **1** increased the apoptotic peak in a concentration-dependant manner (Fig. **1B**). These results strongly suggest that compound **1** induced apoptotic cell death in a dose-dependent manner, ranging from 5.0 μg/ml to 100.0 μg/ml.

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