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Deoxyverrucosidin, a Novel GRP78/BiP Down-regulator, Produced by *Penicillium* sp.

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Abstract Glucose-regulated protein 78 (GRP78) resides in endoplasmic reticulum (ER) and plays a role in protecting tumor cells against the toxic effects of anticancer agents. During the search for down-regulators of GRP78 using a reporter gene (luciferase) assay system, we isolated a novel compound designated as deoxyverrucosidin (**1**), a congener of verrucosidin (**2**), from *Penicillium* sp. and identified it as a down-regulator of the *grp*78 gene. The structure of **1** was determined by mainly ESI-mass and two-dimensional NMR spectra. **1** dose-dependently inhibited the expression of GRP78 promoter with an IC_{50} of 30 nM.

Keywords glucose-regulated protein 78 (GRP78), anticancer agents, deoxyverrucosidin, *Penicillium* sp.

GRP78 resides in the endoplasmic reticulum (ER) and functions as a molecular chaperone by aiding the folding and transport of proteins it associates with transiently as they traverse the ER [1–3]. Strikingly, pathological conditions such as tumor growth correlate with GRP78 overexpression [4]. This could be partly caused by the activation of *grp* gene expression through glucose starvation, acidosis and hypoxia, which are hallmarks of the microenvironment of poorly vascularized solid tumors [5]. These correlations are supported by the observed increases in the levels of GRP78 expression in fibrosarcomas, in which GRP78 expression strongly correlates with tumor growth [6, 7]. The observation that GRP78 plays a role in protecting tumor cells against intracellular-mediated cytotoxicity and from the toxic effects of anticancer agents *in vitro* suggests that the induction of GRP78 may protect tumor cells *in vivo* [8]. Thus, substances that directly down-regulate GRP78 induction might be of potential use in cancer therapy.

During the course of our screening program for chaperone modulators using a reporter gene (luciferase) assay system, we have isolated a novel compound designated as deoxyverrucosidin (**1**) from *Penicillium* sp. and identified it as a down-regulator of the *grp*78 gene (Fig. 1). In this paper, we report upon the isolation, structure elucidation and biological activities of **1**. The producing strain *Penicillium* sp. was cultivated in a seed medium consisting of glucose 2%, yeast extract 0.2%, peptone 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05% and KH_2PO_4 0.1% (pH 5.6) for 3 days at 28°C on a rotary shaker. The seed culture was transferred into a production medium composed of wheat bran - H_2O (1 : 1) and fermentation was carried out in 500 ml flasks for 7 days at 28°C [9, 10].

The mycelium obtained from 10 liters culture was extracted with acetone and the extract was concentrated *in vacuo* to eliminate acetone. The aqueous resultant was extracted with ethyl acetate. The organic layer was concentrated and applied to a column of silica gel eluted with hexane - ethyl acetate (10 : 1~1 : 1). The active eluate was then chromatographed on a Sephadex LH-20 column

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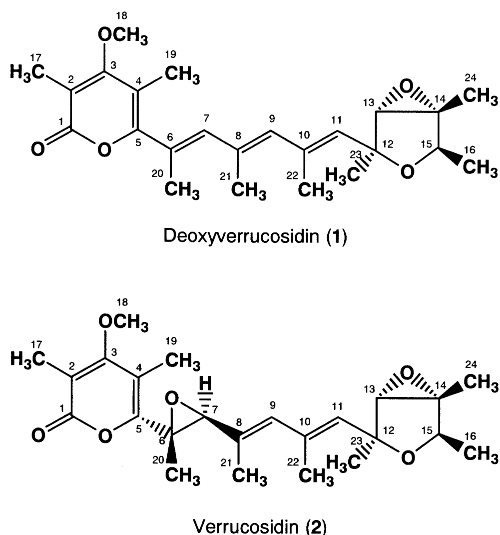

Fig. 1 Structures of deoxyverrucosidin (**1**) and verrucosidin (**2**).

eluted with MeOH. Finally, pure deoxyverrucosidin (**1**), a congener of verrucosidin (**2**), was obtained by HPLC using a YMC-pack ODS-A column (4.6 mm i.d. ×150 mm) eluted with 60% CH_3CN [11].

The UV spectra of **1** (λ_{max} nm in MeOH: 278, 326) and **2** (λ_{max} nm in MeOH: 238, 295) indicated that two compounds have different chromophores. The molecular formula of **1** was established as $C_{24}H_{32}O_5$ by ESI-MS spectrum in combination with ^1H and ^{13}C NMR data. The ^1H and ^{13}C NMR spectra of **1** exhibited signals from 14 protons and 24 carbons. A heteronuclear multiple-quantum coherency (HMQC) experiment established all one-bond ^1H-^{13}C connectivities as shown in Table 1. A COSY experiment revealed two spin networks to generate partial structures **A** and **B** (Fig. 2). The heteronuclear multiple-bond correlation (HMBC) spectrum displayed ^1H-^{13}C long-range couplings from methyl protons 20-H (δ_H 2.07) to C-5 (δ_C 159.0), C-6 (δ_C 127.4) and C-7 (δ_C 139.9), from methyl protons 21-H (δ_H 1.97) to C-7 (δ_C 139.9), C-8 (δ_C

Table 1 ^1H and ^{13}C NMR data for deoxyverrucosidin and verrucosidin in chloroform-d_1

Position	Deoxyverrucosidin		Verrucosidin	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
1	165.5		165	
2	110		111	
3	168.5		167.8	
4	109		111	
5	159		155.9	
6	127.4		60.7	
7	139.9	6.08 (s)	64.7	3.49 (s)
8	134		127.7	
9	136.3	5.87 (s)	131.6	5.87 (s)
10	132		127.7	
11	133.4	5.51 (s)	133	5.47 (s)
12	80		80	
13	67.5	3.45 (s)	67.4	3.43 (s)
14	67.4		67.4	
15	76.7	4.14 (q, J=6.8 Hz)	76.7	4.13 (q, J=6.8 Hz)
16	18.8	1.21 (d, J=6.8 Hz)	18.8	1.20 (d, J=6.8 Hz)
17	10.3	2.06 (s)	10.4	2.05 (s)
18	60.2	3.83 (s)	60	3.84 (s)
19	11.8	2.01 (s)	9.2	2.05 (s)
20	16.7	2.07 (d, J=1.2 Hz)	15.6	1.43 (d, J=1.8 Hz)
21	18.6	1.97 (br)	15.3	1.91 (s)
22	18.5	1.97 (br)	18.5	1.96 (s)
23	21.9	1.44 (s)	21.9	1.43 (s)
24	13.8	1.48 (s)	13.8	1.48 (s)

Chemical shifts in ppm from TMS as internal standard.
^1H and ^{13}C NMR were measured at 400 MHZ and 100 MHz, respectively.

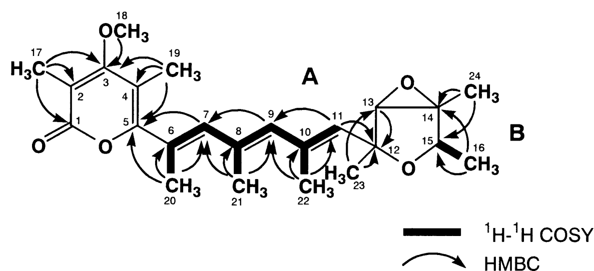

Fig. 2 ^{1}H-^{1}H COSY and HMBC analysis of **1**.

134.0) and C-9 (δ_C 136.3) and from methyl protons 22-H (δ_H 1.97) to C-9 (δ_C 136.3), C-10 (δ_C 132.0) and C-11 (δ_C 133.4), thus establishing the existence of a triene moiety (Fig. 2). The ^{13}C chemical shifts of five downfield signals corresponding to C-1 (δ_C 165.5), C-2 (δ_C 110.0), C-3 (δ_C 168.5), C-4 (δ_C 109.0) and C-5 (δ_C 159.0) were well matched to those of an α-pyrone unit [12–14]. Two singlet methyl protons of 17-H (δ_H 2.06) and 19-H (δ_H 2.01) exhibited HMBC correlations to their adjacent carbons in the α-pyrone moiety, as shown in Fig. 2. Additionally, methoxy protons 18-H (δ_H 3.83) revealed ^{1}H-^{13}C long-range coupling to a quaternary carbon C-3 (δ_C 168.5), indicating the connection between methoxyl group and C-3 position in the α-pyrone substructure. The long-rang couplings from methyl protons 19-H (δ_H 2.01) to C-5 (δ_C 159.0), from methyl protons 20-H (δ_H 2.07) to C-5 and from olefin proton 7-H (δ_H 6.08) to C-5 indicated a connection between partial structure **A** and an α-pyrone moiety. The long-range couplings from methyl protons 16-H (δ_H 1.21) to C-14 (δ_C 67.4) and C-15 (δ_C 76.7) and from singlet methyl protons 24-H (δ_H 1.48) to C-14 (δ_C 67.4) and C-15 (δ_C 76.7) linked the quaternary carbon C-14 to partial structure **B** (Fig. 2). ^{1}H-^{13}C long-range couplings from methyl protons 23-H (δ_H 1.44) to C-11 (δ_C 133.4) C-12 (δ_C 80.0) and C-13 (δ_C 67.5), from an oxygenated methine proton 13-H (δ_H 3.45) to C-12 (δ_C 80.0) and from an olefin proton 11-H (δ_H 5.51) to C-12 (δ_C 80.0) connected the quaternary carbon C-12 to partial structure **A** (Fig. 2). By the process of elimination, two oxygenated carbons C-13 (δ_C 67.5) and C-14 (δ_C 67.4) should form an epoxide ring. Stereochemistry of tetrahydrofuran in **1** was proposed to be same as that of verrucosidin by identical chemical shift values and coupling constant of both compounds.

Biological activity of **1** and **2** were evaluated by reporter gene assay system utilizing luciferase gene. In brief, HT 1080 cells were transformed with luciferase gene under the control of *grp*78 promoter and the cells (20,000 in each well of 96-well plates) were incubated for 8 hours and treated for 18 hours with various concentrations of **1** or **2** in the presence or absence of the 2-DG. Firefly luciferase activity was determined using the luciferase assay kit. As expected, treatment with 2-DG resulted in a 4-fold increase of *grp*78 promoter activity in HT1080 cells. **1** and **2** dose-dependently inhibited the expression of GRP78 promoter with IC_{50} of 30 nM and 25 nM, respectively. These results indicate that **1** and **2** are specific inhibitors of the GRP78 promoter under low glucose conditions. Thus, it is hoped that these compounds are chemotherapeutically active against solid tumors, and/or that it can be used as a specific tool in studies addressing the molecular mechanisms of mammalian ER stress response. Detailed studies on biological activities are under the investigation.

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