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Butenolide derivatives from the fungus *Aspergillus terreus* and their radical scavenging activity and protective activity against glutamate-induced excitotoxicity

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Abstract

The organic extract of cultured *Aspergillus terreus* displayed scavenging activity against ABTS^{•+} and DPPH free radicals, and protective activity against glutamate-induced excitotoxicity in N18-RE-105 neuroblastoma-retina hybrid cells. Bioassay-guided fractionation of the active organic extract led to the isolation of total six butenolide derivatives, including one new metabolite, named butyroscavin (**1**), and five previously described metabolites, butyrolactones I (**2**), II (**3**), III (**4**), and VII (**5**), and aspernolide E (**6**). The planar structure of butyroscavin (**1**) was determined by the analysis of spectroscopic data including ESIMS (electrospray ionization mass spectrometry), and 1D and 2D NMR (nuclear magnetic resonance). The absolute configuration of butyroscavin (**1**) was assigned by comparison of the specific rotation with those of known compounds that share the same chiral carbon. All isolated compounds were active in the radical scavenging assay, whereas only butyrolactones I (**2**) and VII (**5**) exhibited protective activity against the glutamate-induced excitotoxicity with the EC₅₀ of 130.1 and 91.9 μM, respectively.

Keywords: Butenolides, Natural products, Antioxidants, Neuroprotection, *Aspergillus*

Introduction

High glutamate concentration in the synaptic cleft can cause excitotoxicity in neurons that has been known to be one of the major mechanisms of neurodegeneration in progressive neurological disorders such as Parkinson's disease and Alzheimer's disease [4]. Underlying molecular mechanism of excitotoxicity involves excessive generation of reactive oxygen species in hyperexcited neurons [1]. This suggests that the administration of antioxidants could be effective in slowing the progression of neurological disorders by protecting neurons from oxidative damages [9, 12]. As a part of our ongoing effort to search for neuroprotective metabolites from natural sources, we screened our library of fungal extracts for antioxidant activity as well as protective activity against the glutamate

induced excitotoxicity, and the extract of the cultured *Aspergillus terreus* exhibited significant activities in our screening. Thus, bioassay-guided fractionation was performed to identify active metabolites from the active extract. Here, we report the isolation of six butenolide derivatives from the culture of a soil fungus *A. terreus* that include one new metabolite, named butyroscavin (**1**), and five previously reported metabolites, butyrolactones I (**2**) [8], II (**3**) [10], III (**4**) [10] and VII (**5**) [5] and aspernolide E (**6**) [6].

Materials and methods

General experimental procedures

UV (Ultraviolet) spectra was acquired on a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrometer. Shimadzu 8400 s FT-IR spectrometer was used to obtain FT-IR (Fourier transform infrared) spectra. 1D and 2D NMR (nuclear magnetic resonance) spectra of all isolated compounds were obtained using a Varian INOVA-400 NMR spectrometer. MeOH-*d*₄ was used as a solvent

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for all NMR experiments, and signals were referenced against solvent signals. Finnigan Navigator 30086 and a JMS-700 MStation high-resolution mass spectrometer systems were used to acquire low resolution ESIMS (electrospray ionization) and high resolution EIMS (electron ionization) spectra, respectively. All preparative HPLC (high-performance liquid chromatography) experiments were performed using a Waters HPLC system equipped with a Waters 996 photodiode array detector using a reversed-phase column (J'sphere ODS-H80, 150 × 20 mm, 4 μm, YMC Co.).

Extraction and isolation

The fungus *A. terreus* was acquired from the KRIIBB microbial culture collection (Deposit No. KCTC 08095BP). For seed culture, the spores were inoculated into 150 ml YM liquid media (0.3% yeast extract, 0.3% malt extract, 0.5% tryptone, and 1% glucose) and cultured for three days at 28 °C with shaking (147 rpm). Three milliliter of the seed culture was used for the inoculation into each fresh 150 mL YM media. Total 1.5 L (10 × 150 mL) culture was incubated in the same condition as those used for the seed culture. For compound isolation, the cultures were harvested and separated between mycelium and broth by filtration using the Whatman filter paper. The mycelium was extracted with 80% aqueous acetone overnight and dried in vacuo. The mycelium extract was combined back with the broth, and then the mixture was extracted stepwise with hexane, CHCl₃ (chloroform), EtOAc (ethylacetate) and BuOH (butanol). Compound isolation was guided by radical scavenging activity against both ABTS^{•+} and DPPH radicals. The EtOAc layer showed the highest activity in both assays, and was thus fractionated by silica column chromatography with a solvent gradient (CHCl₃:MeOH) from 50:1 to 1:1. Active fractions were combined, and then further subjected to a Sephadex LH-20 size exclusion column chromatography eluting with MeOH. Lastly, reversed-phase HPLC (flow rate 7.0 ml/min) of the active fractions using the C₁₈ column (cosmosil, 5C₁₈-MS-II column, 150 × 20 mm) and 45% aqueous ACN (acetonitrile) led to the isolation of pure compounds **1** (25.2 mg), **2** (76.5 mg), **3** (103.2 mg), **4** (5.6 mg), **5** (20.9 mg), and **6** (19.5 mg).

Structural elucidation of compound 1

Butyroscavin (**1**). Yellow, amorphous powder; $[\alpha]_D^{20} + 49.41$ (c 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 203.7 (4.54) and 305.8 (4.18) nm; IR (KBr) 3366, 1739, 1610, 1515, and 1442 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; ESIMS (negative ion mode) m/z 369.5 [M-H]⁻; HREIMS m/z 370.1065 [M]⁺ (calcd. for C₂₀H₁₈O₇, 370.1053).

Table 1 NMR data for butyroscavin (**1**)

Position	Butyroscavin (1)		
	¹³ C ^a	¹ H (mult.) ^a	HMBC
1	169.6		
2	138.6		
3	128.0		
4	85.7		
5	38.3	3.42 (2H, s)	C-4, C-1'', C-2''
6	169.8		
7	62.5	4.25 (2H, q, J = 7.2 Hz)	C-8, C-6
8	13.0	1.21 (3H, t, J = 7.2 Hz)	C-7
1'	121.9		
2'	129.2	7.60 (2H, d, J = 8.8 Hz)	C-3, C-2', C-4'
3'	115.4	6.87 (2H, d, J = 8.8 Hz)	C-1', C-3', C-4'
4'	158.2		
1''	124.1		
2''	131.3	6.64 (2H, d, J = 8.8 Hz)	C-5, C-2'', C-4''
3''	114.4	6.51 (2H, d, J = 8.8 Hz)	C-1'', C-3'', C-4''
4''	156.3		

^a ¹H and ¹³C NMR were measured at 400 MHz and 100 MHz, respectively, in CD₃OD and solvent signals were used as reference

Radical scavenging activity assay

Radical scavenging activity of the crude extracts as well as pure compounds was evaluated using stable ABTS^{•+} [15] and DPPH [7] free radicals. α -Tocopherol, BHA (Butylated hydroxyanisole), and Trolox were used as reference compounds. Reference and test compounds were dissolved in DMSO at various concentrations. ABTS^{•+} radical scavenging activity was measured using the method previously published [15]. ABTS^{•+} free radicals were produced by combining 7.0 mM ABTS and 2.45 mM potassium persulfate solutions. The ABTS^{•+} free radical solution was diluted with water to an absorbance of 0.7 ± 0.025 at 734 nm, and then 190 μL of the solution was mixed with 10 μL of each test compound in DMSO. After 7 min, the absorbance was recorded at 734 nm. For the DPPH assay, 150 μL of a 0.15 mM DPPH solution in EtOH was mixed with 10 μL of each test compound in DMSO. The reaction mixtures were left at room temperature for 10 min, and then absorbance was measured at 517 nm on an ELISA plate reader.

The radical activity was expressed as a percentage activity using the following equation:

$$\text{Scavenging activity (\%)} = (1 - A_{\text{test}}/A_{\text{control}}) \times 100$$

where A_{test} is the absorbance of a sample at a given concentration, and A_{control} is the absorbance recorded for a blank (DMSO). EC₅₀ is defined as the concentration of a sample that causes 50% loss of the ABTS^{•+} and DPPH radicals.

Glutamate induced excitotoxicity assay

Protective activity against glutamate-induced excitotoxicity was evaluated using N18-RE-105 neuroblastoma-retina hybrid cells [2]. The cells were propagated at 37 °C in high glucose DMEM (Dulbecco's modified Eagle's) medium containing 9.7% FBS (fetal bovine serum), 4.8% Horse Serum, 1.9% HAT (hypoxanthine-aminopterin-thymidine) and 0.1% Sodium bicarbonate at 37 °C in 5% CO₂. Cells growing in log phase were collected by trypsinization. Then, a total of 50,000 cells were seeded onto each well of a 96-well plate and incubated overnight at 37 °C in 5% CO₂. Samples dissolved in DMSO were sequentially diluted with phosphate buffered saline, and added to appropriate wells together with the 400 mM glutamate solution (7 μL of each). The cells were incubated for 24 h, and the cell viability was measured using EZ-cytox Cell Viability Kit (DoGen). Briefly, each well was added with 10 μL of the Ez-cytox solution and incubated for 3 h. Then, 70 μL of the supernatant in each well was transferred to a new 96 well plate, and the absorbance was recorded at 450 nm. The protective activity was evaluated using the following equation:

$$\text{Protective activity (\%)} = \frac{(A_{\text{glutamate and sample}} - A_{\text{glutamate}})}{(A_{\text{no treatment}} - A_{\text{glutamate}})} \times 100$$

where $A_{\text{glutamate and sample}}$ is the absorbance of cells treated with both glutamate and sample at a given concentration, $A_{\text{glutamate}}$ is the absorbance for cells treated with only glutamate, $A_{\text{no treatment}}$ is the absorbance for cells with no treatment, and $A_{\text{glutamate}}$ is the absorbance for cells treated with only glutamate. EC_{50} is defined as the concentration of a sample that exhibited 50% cell viability compared to that of the positive control (cells with no treatment).

Results and discussion

Bioassay-guided fractionation

For compound isolation, the fungus *A. terreus* was cultured in 1 L scale, and the culture was harvested after three days. The mycelium, separated from the broth by filtration, was extracted with 80% aqueous acetone overnight, dried in vacuo and combined with the broth again. The combined mixture of mycelium and broth was extracted stepwise with hexane, ethyl acetate (EtOAc) and butanol (BuOH). The EtOAc extract displayed the highest radical scavenging activity, and thus fractionated using a silica column chromatography eluting with chloroform/methanol (CHCl₃/MeOH) gradient from 50:1 to 1:1. Active silica fractions were further separated by a Sephadex LH-20 size exclusion column chromatography eluting with MeOH. Final purification of active fractions was achieved using reversed-phase HPLC (high

performance liquid chromatography), yielding six pure compounds (compounds 1–6). In the ESIMS analysis, the molecular weights of compounds 2–6 were identical to those of previously reported butenolide derivatives. Detailed comparison of the ¹H and ¹³C NMR spectra with those of known butenolide derivatives confirmed that compounds 2, 3, 4, 5, and 6 were butyrolactones I, II, III and VII, and aspernolide E, respectively. Although the physical property of compound 1 was nearly identical to those of compounds 2–6, the molecular weight of 1 did not match with that of any known butenolide derivative, suggesting 1 to be a potentially new butenolide derivative (Fig. 1). Thus, the structure determination of 1 was carried out using combination of spectroscopic methods including the HREIMS, and 1D and 2D NMR (Fig. 2).

Structure determination of compound 1

Butyrosavin (1) was isolated as a yellow, amorphous powder. The HREIMS spectrum of 1 exhibited a molecular ion peak at *m/z* 370.1065, suggesting a molecular formula as C₂₀H₁₈O₇ (calcd. 370.1053). The ¹H NMR spectrum of 1 (MeOH-*d*₄) indicated the presence of four aromatic protons (δ_{H} 7.60, 6.87, 6.64 and 6.51), two methylene protons (δ_{H} 4.25 and 3.42), and one methyl protons (δ_{H} 1.21). The ¹³C NMR spectrum in combination with HMQC (Heteronuclear Multiple-Quantum Correlation) indicated the presence of two carbonyl (δ_{C} 169.8 and 169.6), ten olefinic (δ_{C} 158.2, 156.3, 138.6, 131.3, 129.2, 128.0, 124.1, 121.9, 115.4 and 114.4), one oxygenated quaternary (δ_{C} 85.7), two methylene (δ_{C} 62.5, and 38.3), and one methyl (δ_{C} 13.0) carbons. Four aromatic doublet proton signals that each integrate as two protons indicated the presence of two symmetric 1,4-disubstituted benzene rings. The carbon chemical shifts of C-4' (δ_{C} 158.2) and C-4'' (δ_{C} 156.3) together with HMBC correlations from H-2' (δ_{H} 7.60) to C-4' (δ_{C} 158.2), and from H-2'' (δ_{H} 6.64) to C-4'' (δ_{C} 156.3) determined one of the substitutions as hydroxyl groups in both of the benzene rings. HMBC correlation from H-2' (δ_{H} 7.60) to C-3 (δ_{C} 128.0) suggested that one of the benzene rings is connected to an olefinic carbon. A methylene substitution in another benzene ring was inferred by HMBC correlations from H-5 (δ_{H} 3.42) to C-1'' (δ_{C} 124.1) and C-2'' (δ_{C} 131.3), and an HMBC correlation from H-5 (δ_{H} 3.42) to C-4 (δ_{C} 85.7) connected the methylene to an oxygenated quaternary carbon. Although direct connection between the two benzene rings could not be achieved by HMBC correlations, three broad signals that account for one carbonyl, and two olefinic carbons were observed in the ¹³C NMR spectrum, suggesting the presence of a butenolide core structure in 1. This was further supported by the fact that many butenolide derivatives have been isolated from the fungus *A. terreus* [11, 13, 14]. Detailed comparison of

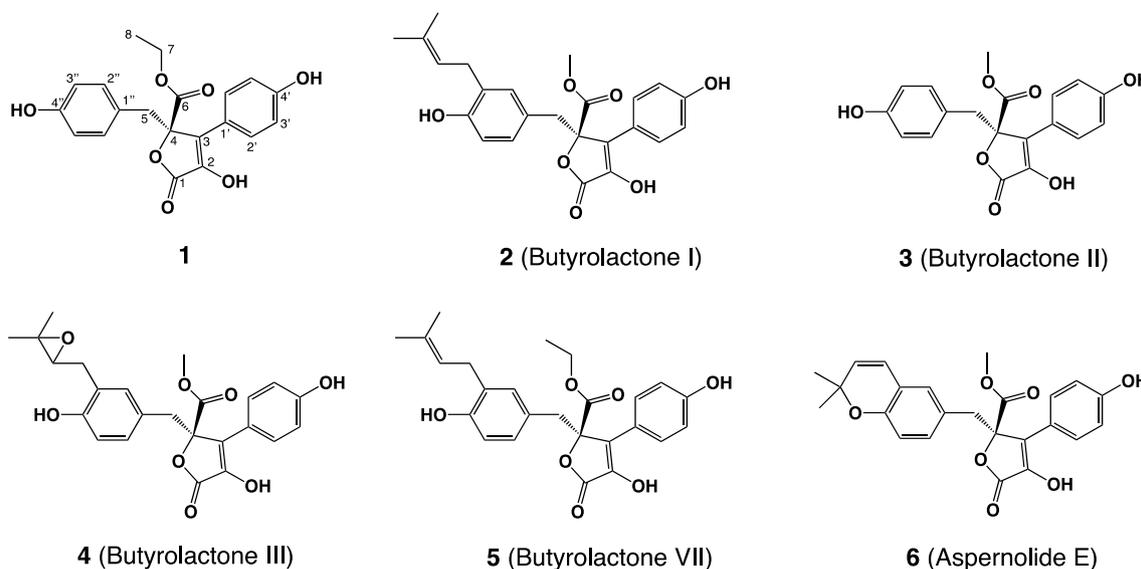


Fig. 1 Chemical structures of isolated butenolide derivatives from the cultured *A. terreus*

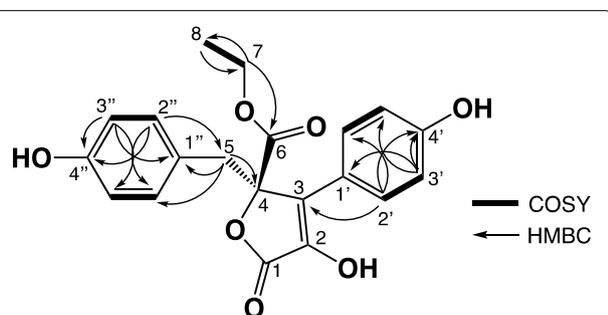


Fig. 2 2D NMR correlations used for structure determination of **1**

the NMR spectra of **1** with those of previously reported butenolide derivatives from *A. terreus* suggested that the structure of **1** is similar to that of butyrolactone II [10, 14]. However, the methoxy signals were absent in the ^1H and ^{13}C NMR spectra of **1**. COSY correlation between oxygenated methylene (H_2 -7: δ_{H} 4.25) and methyl (H_3 -8: δ_{H} 1.21) protons, and HMBC correlation from H_2 -7 (δ_{H} 4.25) to C-6 (δ_{C} 169.8) indicated that the methoxy group in butyrolactone II was replaced with an ethoxy group in **1**, completing the planar structure of **1**. The compound **1** possessed one chiral carbon (C-4), and thus the absolute configuration of C-4 was determined by comparing the specific rotation with those of structurally related butenolide derivatives possessing the same chiral carbon. A positive specific rotation ($+49^\circ$) observed for **1** indicated the absolute configuration of **1** to be the same as those of other known butenolide derivatives such as butyrolactones I ($+100^\circ$), II ($+85^\circ$) and III ($+80^\circ$) [8, 10].

Biological activities of isolated compounds

All the isolated butenolide derivatives were evaluated for their radical scavenging activities as well as protective activity against glutamate induced excitotoxicity. First, radical scavenging activity was tested using DPPH and $\text{ABTS}^{\cdot+}$ free radicals, and expressed as the amount of compound necessary to decrease the initial radical concentration by 50% (EC_{50} in μM). All tested compounds displayed moderate scavenging activities in both assays with EC_{50} values of 20–70 μM against the DPPH radical, and 4–25 μM against the $\text{ABTS}^{\cdot+}$ radical (Table 2). It has been previously reported that

Table 2 Protective activity against glutamate-induced excitotoxicity in N18-RE-105 cells, and scavenging activities against DPPH and $\text{ABTS}^{\cdot+}$ free radicals for compounds 1–6

	N18-RE-105 EC_{50} in μM	$\text{ABTS}^{\cdot+}$	DPPH
1	NA	9.3	54.3
2	91.9	4.3	24.4
3	NA	11.5	68.5
4	NA	14.1	37.9
5	130.1	6.7	37.9
6	NA	23.4	58.4
Vitamin E	37.2		
BHA		9	36.5
Trolox		11.3	14.4

NA not active

the prenylation of flavonoids increase their antioxidant activities [3]. Therefore, higher radical scavenging activities observed for compounds **2** and **5** in both assays could be attributed to the presence of a prenyl moiety in their structures. Next, we evaluated the protective activity against glutamate induced excitotoxicity for all isolated compounds using N18-RE-105 cells. Among six compounds tested, only compounds **2** and **5** exhibited a protective activity with the EC₅₀ values of 91.9 μ M and 130.1 μ M, respectively. Although all six compounds were active in radical scavenging activity assay, only compounds **2** and **5** that contain a prenyl group on the benzene ring exhibited activity in the cell line assay. This is probably due to their higher radical scavenging activity compared to other compounds, and also an increase in lipophilicity as the attachment of a prenyl moiety would increase overall hydrophobicity of molecules.

Additional file

[Additional file 1.](#) Additional figures.

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Authors' contributions

HK carried out experiments. HK and JK, designed experiments, analyzed data and wrote the manuscript. JK funded experiments. Both authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated or analyzed during this study are included in this published article and its Additional file 1.

Competing interests

The authors declare that they have no competing interests.

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