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# Novel neuroprotective hydroquinones with a vinyl alkyne from the fungus, *Pestalotiopsis microspora*

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## Abstract

New hydroquinone derivatives bearing a vinyl alkyne, pestalotioquinols A and B, were isolated from a fungal culture broth of *Pestalotiopsis microspora*. The structures of these novel compounds were determined by interpretation of spectroscopic data (1D/2D NMR, MS, and IR), and the absolute configuration of the stereogenic center of pestalotioquinol A was assigned using the modified Mosher's method. Nerve growth factor-differentiated neuronal PC12 cells were pretreated with pestalotioquinols A and B and removed from the medium, and then treated with a generator of peroxynitrite (ONOO<sup>-</sup>), a reactive nitrogen species, to induce cell death. The cytotoxicity of the treated cells was assessed by measuring lactate dehydrogenase leakage. As a result, 1–3 μM pretreatment of pestalotioquinols A and B rescued neuronal PC12 cells from peroxynitrite-induced cytotoxicity and the protective activity was sustained after removing each compound from the medium. These results demonstrate that pestalotioquinol derivatives are a new class of hydroquinones possessing a vinyl alkyne and exhibiting relatively high neuroprotective effects.

## Introduction

Peroxynitrite (ONOO<sup>-</sup>) is a strong reactive nitrogen species produced by the reaction of nitric oxide (•NO) and superoxide anion (•O<sub>2</sub><sup>-</sup>). Its high reactivity allows peroxynitrite to oxidize a number of various cellular components such as lipids, proteins and DNA, and stimulate cell death by a variety of mechanisms. Peroxynitrite generation could be the cause of a number of pathological conditions ranging from atherosclerosis to inflammatory, autoimmune, heart and neurodegenerative diseases [1]. Protective agents

against peroxynitrite-induced cell injury would lead to the treatment of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [2, 3].

In the past, we have focused on metabolites of fungi isolated from sands, soils, seaweed, mosses and other plants to obtain bioactive natural products. Extracts of fungal culture broth were purified by column chromatography to construct a chemical library, and a library screening resulted in the discovery of several bioactive compounds [4–6]. Of these compounds, neoechinulin A isolated from *Eurotium rubrum* Hiji025 was found to protect neuronal PC12 cells from cytotoxicity induced by the peroxynitrite generator 3-morpholinosydnonimine (SIN-1) [7, 8]. In addition, neoechinulin A improves memory functions in lipopolysaccharide-treated mice, and also exerts antidepressant-like effects [9]. Interestingly, neoechinulin A preferentially confers neuroprotection against nitrosative stresses caused by NO or NO-derived reactive species (i.e., SIN-1), but not against oxidative stresses (i.e., H<sub>2</sub>O<sub>2</sub>), and the neuroprotective activity of neoechinulin A was sustained after removing the compound from the medium [7, 10]. Although neoechinulin A possessed unique properties, a high concentration of neoechinulin A was required to exhibit the protective activity. Herein, we aimed to discover compounds with high neuroprotective activity

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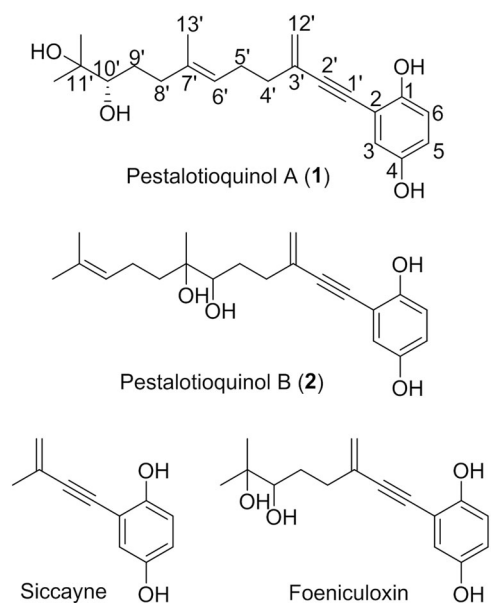
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**Fig. 1** Structures of pestalotioquinols A (1) and B (2)

from metabolites of fungi. In this screening program, neuronal PC12 cells were pretreated with compounds, removed from the medium, and then treated with SIN-1 in the absence of compounds to exclude antioxidants or direct scavengers of peroxynitrite as hits for neuroprotective compounds. The screening using the library containing fractions partially purified from the culture broths of fungi demonstrated that fractions of the culture extract of *P. microspora* rendered PC12 cells resistant to SIN-1-induced cytotoxicity (Figure S1). Activity-guided purification led to the discovery of novel hydroquinone derivatives designated pestalotioquinols A (1) and B (2) (Fig. 1). We herein report the isolation, structural elucidation, and neuroprotective effects of compounds 1 and 2.

## Results and discussion

Repeated separations of a culture extract from *P. microspora* using silica gel yielded compounds 1 and 2. The molecular formula of  $C_{21}H_{28}O_4$  for compound 1 was determined by HRMS (FAB). The IR spectrum showed a band typical of a hydroxy group ( $3344\text{ cm}^{-1}$ ) and a weak band typical of an alkyne group ( $2196\text{ cm}^{-1}$ ). As shown in Table 1, the  $^{13}\text{C}$  NMR and DEPT spectroscopic data suggested the presence of eight quaternary carbons, five methine carbons, five methylene carbons and three methyl carbons. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR and HMBC spectra established the presence of a monosubstituted hydroquinone, as shown in Table 1 and Fig. 2. HMBC correlations from protons of two terminal methyl groups (Me-11') to oxygenated carbons C-10' ( $\delta$  78.4) and C-11' ( $\delta$  73.5), and

consecutive  $^1\text{H}$ - $^1\text{H}$  COSY correlations from H-8' to H-10' revealed a partial structure of  $\text{CH}_3\text{-C}(\text{CH}_3)(\text{OH})\text{-CH}(\text{OH})\text{-CH}_2\text{-CH}_2\text{-}$ . The partial structure was found to be connected to an isoprene unit on the basis of  $^1\text{H}$ - $^1\text{H}$  COSY correlations and HMBC correlations from H-13' to C-8' and two olefinic carbons C-6' ( $\delta$  123.9) and C-7' ( $\delta$  135.8). HMBC correlations from H-3 to C-1' ( $\delta$  83.7), and from exomethylene protons of H-12' to C-3' and C-2' ( $\delta$  96.1) suggested the presence of a vinyl-acetylene moiety that was connected to the monosubstituted hydroquinone. The *E*-geometry of the C6' = C7' double bond was confirmed by NOESY correlations between H-5' and H-13', and H-6' and H-8' (Fig. 2). Thus, the structure of compound 1 was determined (Fig. 1), and it was named pestalotioquinol A.

To determine the absolute configuration at C-10', Mosher's (MTPA) ester of 1 was synthesized (Scheme 1). Compound 1 was reacted with methyl iodide in the presence of  $\text{K}_2\text{CO}_3$  to give dimethyl ether 3. Esterification of 3 was performed with (*R*)- or (*S*)-MTPA chlorides, affording (*S*)- or (*R*)-MTPA ester 4 and 5.  $\Delta\delta$  values ( $\delta_{(S)} - \delta_{(R)}$ ) indicated that the absolute configuration at C-10' was *S* (Fig. 3).

Compound 2 was found to have the same molecular formula of  $C_{21}H_{28}O_4$  as compound 1 by HRMS (FAB). As shown in Table 1, the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra suggested that the structure of compound 2 was similar to compound 1, except for their side chain from C-6' to C-11'. HMBC correlations from protons of two terminal methyl groups to olefinic carbons C-10' ( $\delta$  124.3) and C-11' ( $\delta$  132.3), and consecutive  $^1\text{H}$ - $^1\text{H}$  COSY correlations from H-8' to H-10' established an isoprene unit (Fig. 2). Two olefinic carbon signals of C-6' and C-7' in compound 1 was revealed to be replaced by those of C-6' ( $\delta$  76.9) and C-7' ( $\delta$  75.0) in compound 2 on the basis of HMBC correlations from H-13' to C-6' and C-7', which suggested the presence of a diol at this position in agreement with the HRMS results. Thus, the structure of compound 2 (pestalotioquinol B) was elucidated (Fig. 1) and was further confirmed by  $^1\text{H}$ - $^1\text{H}$  COSY, HMQC and HMBC experiments.

Several hydroquinone derivatives bearing a vinyl acetylene moiety have been isolated from fungi such as *Helminthosporium siccans*, *Phomopsis foeniculi* and *Sterium frustulosum* [11–14]. Siccayne is one of the most studied antibiotic hydroquinone derivatives with an isopentenyl side chain (Fig. 1) [11, 15–17]. Foeniculoxin is a closely related derivative of pestalotioquinols A and B [13]. This compound is a phytotoxic metabolite produced by *P. foeniculi* and possesses a geranyl side chain (Fig. 1). Pestalotioquinols A and B are the first hydroquinone derivatives with a vinyl alkyne that possesses a side chain composed of three isoprene units.

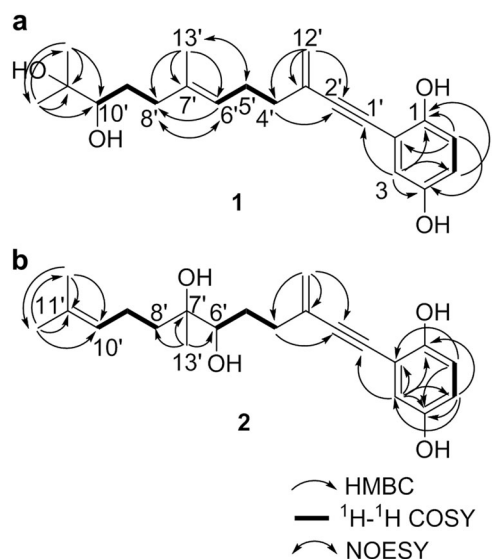
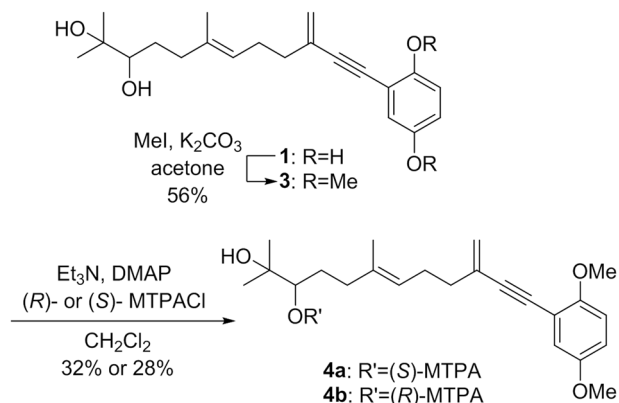
Pestalotioquinols A (1) and B (2) were evaluated for their neuroprotective effects on NGF-differentiated neuronal PC12 according to a procedure described previously [7]. In

**Table 1**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data for compounds **1** and **2**

Pos.	<b>1</b> <sup>a</sup>				<b>2</b> <sup>a</sup>			
	$\delta_{\text{C}}$	type	$\delta_{\text{H}}$	mult ( <i>J</i> in Hz)	$\delta_{\text{C}}$	type	$\delta_{\text{H}}$	mult ( <i>J</i> in Hz)
1	150.3	C			151.2	C		
2	109.9	C			109.8	C		
3	117.6	CH	6.82	d (2.8)	117.4	CH	6.79	d (3.0)
4	149.2	C			148.6	C		
5	118.0	CH	6.73	dd (2.8, 8.8)	118.0	CH	6.74	dd (3.0, 8.7)
6	115.7	CH	6.77	d (8.8)	115.9	CH	6.81	d (8.7)
1'	83.7	C			83.8	C		
2'	96.1	C			96.3	C		
3'	130.9	C			130.6	C		
4'	36.9	CH <sub>2</sub>	2.29	m	34.0	CH <sub>2</sub>	2.46	m
5'	26.8	CH <sub>2</sub>	2.29	m	29.9	CH <sub>2</sub>	1.65	m
							1.82	m
6'	123.9	CH	5.23	m	76.9	CH	3.53	dd (1.8, 10.7)
7'	135.8	C			75.0	C		
8'	36.7	CH <sub>2</sub>	2.06	m	36.1	CH <sub>2</sub>	1.43	m
			2.26	m			1.65	m
9'	29.3	CH <sub>2</sub>	1.42	m	22.1	CH <sub>2</sub>	2.11	m
			1.61	m				
10'	78.4	CH	3.38	d (10.3)	124.3	CH	5.13	m
11'	73.5	C			132.3	C		
Me-11'	23.2	CH <sub>3</sub>	1.15	s	25.7	CH <sub>3</sub>	1.68	s
	26.1	CH <sub>3</sub>	1.19	s	17.7	CH <sub>3</sub>	1.62	s
12'	122.4	CH <sub>2</sub>	5.43	d (1.5)	122.7	CH <sub>2</sub>	5.48	d (1.4)
			5.33	s			5.40	d (1.2)
13'	16.0	CH <sub>3</sub>	1.61	s	23.5	CH <sub>3</sub>	1.21	s

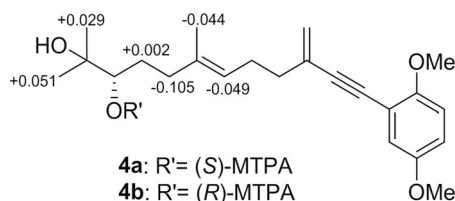
<sup>a</sup>NMR spectra were measured in CDCl<sub>3</sub> solvent

this assay, neuronal PC12 was treated with compounds **1**

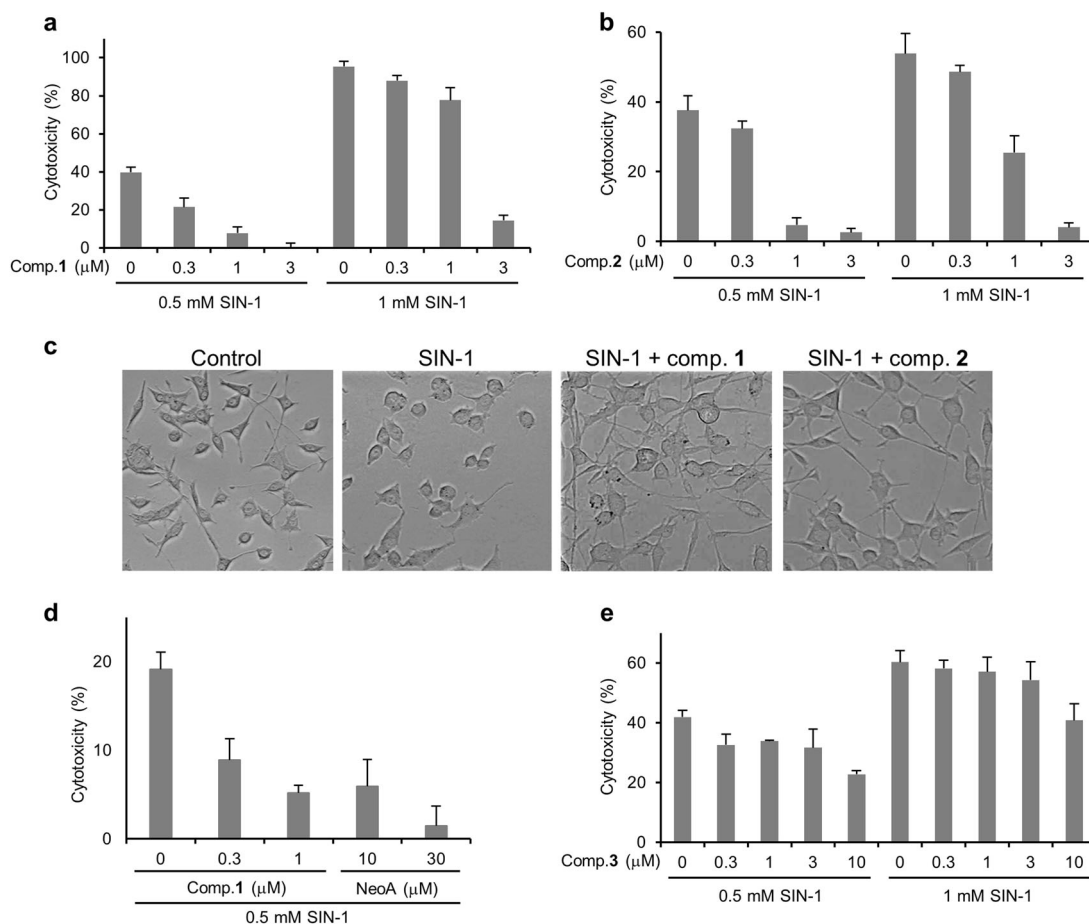
**Fig. 2** Key  $^1\text{H}$ - $^1\text{H}$  COSY, HMBC and NOESY correlations for **1** (a) and **2** (b)**Scheme 1** Preparation of dimethyl ether **3**, and (*S*)- and (*R*)-MTPA ester **4a** and **4b**

and **2**, and then the compounds were removed from the medium. The pretreated cells were incubated with the peroxynitrite generator SIN-1 in the absence of the compounds, and the level of cytotoxicity was then assessed by measuring the leakage of lactate dehydrogenase (LDH) (Fig. 4a,

b). As a result, both compounds were found to render PC12 cells resistant to SIN-1. Pretreatment with 1  $\mu\text{M}$  of compounds **1** and **2** decreased the cytotoxicity of PC12 cells induced by 0.5 mM SIN-1. Morphologies of neuron-like PC12 treated with compounds **1** and **2** were confirmed by a microscope (Fig. 4c). Compound **1** showed a stronger neuroprotective effect than neoechinulin A, a fungus-derived neuroprotective compound reported previously (Fig. 4d) [7, 8].



**Fig. 3**  $\Delta\delta$  values for the MTPA ester **4a** and **4b**



**Fig. 4** Neuroprotective effects of compounds **1–3**. PC12 cells were differentiated by NGF for 3 days. Differentiated PC12 cells were pretreated with the indicated concentration of compounds **1**, **2** and **3** for 24 h. After removal of the compounds, cells were treated with 0.5 or 1.0 mM SIN-1 for 24 h. Cytotoxicity was evaluated by measuring released LDH. The values shown are the mean  $\pm$  intra-assay deviation expressed as the S.D. **a** and **b** Neuroprotective effect of

The hydroquinone moiety is known to have antioxidant properties, and several neuroprotective natural products containing a hydroquinone moiety have been reported [18, 19]. To examine the importance of the hydroquinone moiety of the pestalotioquinols for neuroprotection, we compared the protective effects afforded by pestalotioquinol A (**1**) and dimethyl ether **3** (Fig. 4a, e). Although 10  $\mu\text{M}$  of compound **3** decreased SIN-1-mediated cell death, the protective activity was weaker than that of **1**, which suggested the hydroquinone of **1** is likely important for its neuroprotective activity.

In conclusion, two new hydroquinone derivatives bearing a vinyl alkyne moiety, pestalotioquinols A and B, were isolated from a fungal culture broth of *P. microspora*. To our knowledge, there are no reports on hydroquinone derivatives with a vinyl alkyne that possesses a side chain composed of three isoprene units. Pretreatment with pestalotioquinols A and B protected

pestalotioquinols A (**1**) and B (**2**). **c** Photomicrographs of control cells, 0.5 mM SIN-1-treated cells, and 0.5 mM SIN-1 + 3  $\mu\text{M}$  compound **1** or compound **2**-treated cells. pestalotioquinols B (**2**). **d** Comparison of neuroprotective effects between pestalotioquinols A (**1**) and neoechinulin A (NeoA). **e** Neuroprotective effect of dimethyl ether of pestalotioquinol A (**3**)

neuronal PC12 cells from peroxydinitrite-induced cell death, and the protective activity was sustained after removing the compounds. In addition, our data indicated that the hydroquinone moiety of **1** is important for neuroprotective effects. Although a further mechanistic study of pestalotioquinols is required, the compounds may be developed as agents for treatment of reactive nitrogen species-related disorders such as Parkinson's disease and Alzheimer's disease in the future.

## Materials and methods

### General experimental procedure

Optical rotations were recorded on a JASCO P-2200 digital polarimeter (Jasco Corp., Tokyo, Japan) at room temperature. UV spectra were obtained on a UVmini-1240 spectrophotometer (Shimadzu corp., Kyoto, Japan). Infrared spectra (IR) were recorded on a JASCO FT/IR-4600 spectrophotometer (Jasco Corp.) and reported as wavenumbers ( $\text{cm}^{-1}$ ).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker 400 MHz spectrometer (Avance DRX-400; Bruker, Billerica, MA), using  $\text{CDCl}_3$  solution (with TMS for  $^1\text{H}$  NMR and  $\text{CDCl}_3$  for  $^{13}\text{C}$  NMR as an internal reference). Chemical shifts are expressed in  $\delta$  (ppm) relative to TMS or residual solvent resonance, and coupling constants ( $J$ ) are expressed in Hz. Mass spectra (MS) were obtained on a JEOL mass spectrometer (JMS-700; JEOL, Tokyo, Japan). Analytical TLC was carried out on precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany). Silica gel 60 N (Kanto Chemical, Tokyo, Japan) was used for silica gel column chromatography.

### Preparation of the fraction library of fungal metabolites

Leaves, mosses, soils and sands were collected in various areas in Japan and suspended in sterilized water. The suspension was spread onto potato dextrose agar (PDA) plates (Difco & BBL, Franklin Lakes, NJ, USA), and the plates were incubated for 1–2 weeks at 30 °C. Fungi growing on these plates were transferred onto individual PDA plates and cultured under the same conditions. Cultures were repeated 2–5 times to obtain pure mycelium strains. Each fungal stain isolated was cultured by transferring a small piece of agar from the culture plate into 2-l Erlenmeyer flasks containing potato dextrose broth (24 g) (Difco & BBL) in  $\text{H}_2\text{O}$  (1.0 l). The culture was grown under static conditions at room temperature and in the dark for 3–6 weeks. The culture broth was filtered through cheesecloth to remove fungal mycelia and the filtrate was then extracted three times with 300 ml of  $\text{CH}_2\text{Cl}_2$ . The combined organic layer was evaporated *in vacuo* to

obtain a crude extract. The crude extract was separated by silica gel column chromatography with  $\text{CHCl}_3$ -MeOH (99:1–90:10) to produce several fractions to construct a fraction library. The library containing approximately 700 fractions obtained from more than 100 fungi was used to measure neuroprotective activity.

### Isolation and of the fungus that produced the pestalotioquinols

The fungus that produced the pestalotioquinols reported here was isolated from leaves collected in Sagami-hara, Kanagawa, Japan, and identified as *P. microspora*. The Internal Transcribed Spacer (ITS) region, which was amplified by PCR and sequenced, showed 100% sequence identity with *P. microspora* (GenBank accession number JN861776).

### Extraction and purification of compounds

The fungal stain was cultured in 10 l of PDB and the culture was grown under static conditions at room temperature and in the dark for 21 days. The culture broth was then extracted using  $\text{CH}_2\text{Cl}_2$ , and the organic layer was evaporated *in vacuo* to obtain a crude extract (323 mg). This crude extract was separated by silica gel column chromatography (column size: 13 mm $\phi$ , fraction volume: 7–10 ml) with  $\text{CHCl}_3$ -MeOH (99:1–95:5, 250 ml) to give fractions 1–5. Fraction 3 was separated by silica gel column chromatography (column size: 13 mm $\phi$ , fraction volume: 7–10 ml) with hexane-EtOAc (4:1–0:1, 200 ml) to give compound **1** (59.3 mg). Fraction 2 was separated by silica gel column chromatography (column size: 10 mm $\phi$ , fraction volume: 4 ml) with hexane-EtOAc (4:1–0:1, 150 ml) to give fraction 2-1–2–7. Fraction 2-3 was separated by silica gel column chromatography (column size: 5 mm $\phi$ , fraction volume: 2 ml) with toluene-EtOAc (1:1–0:1, 40 ml) to give compound **2** (2.5 mg).

**Pestalotioquinol A (1):** Yellow oil;  $[\alpha]_{\text{D}}^{22} -10.8$  ( $c$  1.94,  $\text{CHCl}_3$ ); UV  $\lambda_{\text{max}}^{\text{MeOH}}$  nm ( $\epsilon$ ) 264 (13,900), 276 (13,500), 328 (8,400); IR  $\nu_{\text{max}}$  (film)  $\text{cm}^{-1}$  3344, 2977, 2196, 1617, 1495; HRMS (FAB)  $m/z$  367.1884 [ $\text{M} + \text{Na}]^+$  (calcd for  $\text{C}_{21}\text{H}_{28}\text{O}_4\text{Na}$ , 367.1885);  $^{13}\text{C}$  and  $^1\text{H}$  data, see Table 1.

**Pestalotioquinol B (2):** Yellow oil;  $[\alpha]_{\text{D}}^{22} -7.6$  ( $c$  0.125,  $\text{CHCl}_3$ ); UV  $\lambda_{\text{max}}^{\text{MeOH}}$  nm ( $\epsilon$ ) 265 (9,500), 275 (9,300) 327 (5,400); IR  $\nu_{\text{max}}$  (film)  $\text{cm}^{-1}$  3394, 2977, 2196, 1606, 1495; HRMS (FAB)  $m/z$  343.1908 [ $\text{M} - \text{H}]^-$  (calcd for  $\text{C}_{21}\text{H}_{27}\text{O}_4$ , 343.1909);  $^{13}\text{C}$  and  $^1\text{H}$  data, see Table 1.

### Methylation of pestalotioquinol A

$\text{K}_2\text{CO}_3$  (14.7 mg) and MeI (0.2 ml) were added to a solution of pestalotioquinol A (12.2 mg) in acetone (0.5 ml), and the mixture was stirred at room temperature for 48 h. The reaction mixture was diluted with EtOAc (20 ml) and washed with



H<sub>2</sub>O (3 × 10 ml). The crude product was purified by HPLC (PEGASIL ODS SP100, Senshu, Tokyo, Japan, 10 × 150 mm; solvent 50–100% MeOH, flow rate 3.0 ml min<sup>-1</sup>) to afford dimethylated product **3** (6.8 mg, 52%).

$[\alpha]_D^{21}$  -8.7 (*c* 0.275, CHCl<sub>3</sub>); UV  $\lambda_{\max}^{\text{MeOH}}$  nm ( $\epsilon$ ) 240 (9,900), 267 (10,500), 325 (7,100); IR  $\nu_{\max}$  (film) cm<sup>-1</sup> 3431, 3001, 2977, 2196, 1640, 1499, 1227; HRMS (FAB) *m/z* 395.2196 [M + Na]<sup>+</sup> (calcd for C<sub>23</sub>H<sub>32</sub>O<sub>4</sub>Na, 395.2198); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.95 (1 H, d, *J* = 2.8 Hz), 6.84 (1 H, dd, *J* = 2.9, 9.0 Hz), 6.80 (1 H, d, *J* = 9.0 Hz), 5.44 (1 H, m), 5.30 (1 H, d, *J* = 1.7 Hz), 5.25 (1 H, m), 3.84 (3 H, s), 3.77 (3 H, s), 3.36 (1 H, m), 2.37–2.21 (4 H, m), 2.14–2.06 (2 H, m), 1.67 (3 H, s), 1.63–1.56 (1 H, m), 1.46–1.37 (1 H, m), 1.19 (3 H, s), 1.15 (3 H, s); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  154.4, 153.2, 135.7, 131.5, 124.2, 121.3, 118.1, 115.5, 113.0, 112.1, 93.8, 85.6, 78.1, 73.0, 56.5, 55.8, 37.4, 36.7, 29.5, 26.6, 26.4, 23.3, 15.9

### Preparation of (*R*)- and (*S*)-MTPA esters of compound **3**

Triethylamine (0.01 ml), DMAP (2.3 mg) and (*R*)-MTPACl (0.01 ml) were added to a solution of compound **3** (2.0 mg) in CH<sub>2</sub>Cl<sub>2</sub> (1.0 ml), and the mixture was stirred at room temperature for 6 h. The reaction mixture was diluted with EtOAc (20 ml) and washed with H<sub>2</sub>O (3 × 10 ml). The crude product was purified by HPLC (PEGASIL ODS SP100, Senshu, Tokyo, Japan, 10 × 150 mm; solvent 50–100% MeOH, flow rate 3.0 ml min<sup>-1</sup>) to afford (*S*)-MTPA ester (**4a**) (1.0 mg, 32%). (*R*)-MTPA ester (**4b**) was prepared in a similar way using (*S*)-MTPACl.

### (*S*)-MTPA ester **4a**

HRMS (FAB) *m/z* 588.2700 [M]<sup>+</sup> (calcd for C<sub>33</sub>H<sub>39</sub>F<sub>3</sub>O<sub>6</sub>, 588.2699); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.63–7.58 (2 H, m, aromatic signals of MTPA), 7.42–7.40 (3 H, m, aromatic signals of MTPA), 6.95 (1 H, d, *J* = 2.7 Hz, H-3), 6.83 (1 H, dd, *J* = 2.7, 9.0 Hz, H-5), 6.79 (1 H, d, *J* = 9.0 Hz, H-6), 5.43 (1 H, d, *J* = 1.8 Hz, H-12'), 5.29 (1 H, s, H-12'), 5.09 (1 H, m, H-6'), 4.98 (1 H, dd, *J* = 2.0, 10.1 Hz, H-10'), 3.83 (3 H, s, OMe), 3.76 (3 H, s, OMe), 3.58 (3 H, s, OMe of MTPA), 2.30 (4 H, m, H-4' and H-5'), 1.88 (2 H, t, *J* = 7.4 Hz, H-8'), 1.69 (2 H, m, H-9'), 1.57 (3 H, s, H-13'), 1.22 (3 H, s, Me-11'), 1.16 (3 H, s, Me-11')

### (*R*)-MTPA ester **4b**

HRMS (FAB) *m/z* 588.2697 [M]<sup>+</sup> (calcd for C<sub>33</sub>H<sub>39</sub>F<sub>3</sub>O<sub>6</sub>, 588.2699); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.62–7.58 (2 H, m, aromatic signals of MTPA), 7.42–7.40 (3 H, m, aromatic signals of MTPA), 6.95 (1 H, d, *J* = 2.8 Hz, H-3), 6.84 (1 H, dd, *J* = 2.8, 9.0 Hz, H-5), 6.80 (1 H, d, *J* = 9.0 Hz, H-6), 5.43

(1 H, s, H-12'), 5.30 (1 H, s, H-12'), 5.14 (1 H, m, H-6'), 5.00–4.97 (1 H, m, H-10'), 3.83 (3 H, s, OMe), 3.76 (3 H, s, OMe), 3.58 (3 H, s, OMe of MTPA), 2.30 (4 H, m, H-4' and H-5'), 1.98 (2 H, m, H-8'), 1.69 (2 H, m, H-9'), 1.61 (3 H, s, H-13'), 1.17 (3 H, s, Me-11'), 1.13 (3 H, s, Me-11')

### Cell culture

PC12 cells, a rat pheochromocytoma cell line, were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 5% horse serum (HS), 1% penicillin streptomycin (PS) (Thermo Fisher Scientific) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

### Determination of neuroprotective effect of compounds

PC12 cells were seeded into poly-D-lysine coated 96-well plates (greiner bio-one, Kremsmünster, Australia) at a density of 5 × 10<sup>3</sup> cells/well in DMEM containing 10% FBS, 5% HS and 1% PS. On day 2, PC12 cells were differentiated with RPMI 1640 medium (Sigma-Aldrich) containing 50 ng ml<sup>-1</sup> nerve growth factor (NGF) (Alomone labs, Jerusalem, Israel), 10% FBS and 1% PS for 3 days. The differentiated cells were incubated with test compounds or their vehicle (0.1% DMSO) in RPMI 1640 medium containing 50 ng ml<sup>-1</sup> NGF, 10% FBS and 1% PS for 24 h. After the removal of test compounds, the pretreated cells were incubated with 0.5 mM or 1 mM SIN-1 (Dojindo, Kumamoto, Japan) for 24 h. Cytotoxicity was measured using a cytotoxicity LDH assay kit-WST (Dojindo) according to the manufacturer's protocol.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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