Tricholomalides A−C, New Neurotrophic Diterpenes from the Mushroom *Tricholoma* sp.

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Three new diterpenes, tricholomalides A−C (**1**−**3**), were isolated from the methanol extract of the fruiting body of *Tricholoma* sp. Their structures were elucidated on the basis of their spectral data, and the absolute configuration of **1** was determined by CD spectrum. Tricholomalides **1**−**3** significantly induced neurite outgrowth in rat pheochromocytoma cells (PC-12) at concentrations of 100 μM.

Neurotrophic factors are essential for the functional maintenance and organization of neurons, since neurons cannot proliferate and regenerate as terminally differentiated cells. Among neurotrophic factors, such as nerve growth factor (NGF),[1] brain-derived neurotrophic factor (BDNF),[2] neurotrophin 3 (NT-3),[3] and glia-derived neurotrophic factor (GDNF),[4] NGF has been well investigated to show pleiotrophic effects such as the induction of neuronal differentiation, neural cell survival, and the prevention of apoptosis of neurons in both central and peripheral nervous systems.[1] Rat pheochromocytoma cells (PC-12) have been used as an in vitro model of neuronal differentiation. NGF induces differentiation of PC-12 cells to extend neurites and to develop the characteristics of sympathetic neurons.[5] Mushrooms are used as foods having nutritional and medicinal properties around the world, and biologically active compounds have been isolated from various species.[6,7] The mushroom genus *Tricholoma* (Tricholomaceae family) is distributed all over the world, and several phytochemical studies have been reported in the literature.[8−16] During a search for neutrophic NGF-like compounds from natural sources, we isolated new diterpenes named tricholomalides A−C (**1**−**3**) which induced neurite outgrowth in PC-12 cells from the fresh fruiting body of *Tricholama* sp. In addition, we have revised the absolute stereochemistry of trichoaurantianolides B and C (**4** and **5**) isolated from *T. aurantium*.[13]

Results and Discussion

The fresh fruiting body (2.7 kg) of *Tricholoma* sp. was extracted with MeOH. After evaporation, the extract was partitioned between water and EtOAc. The bioassay-monitored purification of the EtOAc layer afforded tricholomalides A (**1**, 2.1 mg, 7.8×10^{-5} %), B (**2**, 9.5 mg, 3.5×10^{-4} %), and C (**3**, 2.8 mg, 1.0×10^{-4} %).

Tricholomalide A (**1**) was obtained as a colorless oil whose elemental composition was found to be $C_{20}H_{28}O_5$ as revealed by HREIMS and ^{13}C NMR spectrum (Table 1). It indicated seven degrees of unsaturation. The IR spectrum of **1** showed the presence of hydroxyl (3460 cm^{-1}), γ-lactone (1765 cm^{-1}), and carbonyl (1731 cm^{-1}) groups. The ^1H NMR spectrum of **1** revealed two singlet methyl signals at δ 1.01 and 1.31, two doublet methyl signals at δ 0.96 (3H, d, J = 6.8 Hz) and 1.10 (3H, d, J = 6.4 Hz), and an exomethylene group at δ 5.15 (1H, br s) and 5.23 (1H, br s). The ^{13}C NMR spectrum of **1** displayed two carbonyl (δ 175.9 and 216.0), two olephinic [δ 109.4 (t) and 154.4 (s)], and four oxygen-bearing [δ 71.9 (t), 78.7 (d), 80.4 (s), 84.4 (d)] carbon signals. The structure of **1** was determined by analysis of its 2D NMR spectral data. After assignment of all proton signals to their intact coupling carbon signals with the HMQC spectrum, major fragments a−d (Figure 1) of the molecule were deduced from $^1H-^1H$ COSY measurement. Fragment a consisted of an isopropyl group [δ 0.96 (H_3-16), 1.10 (H_3-17), 1.80 (dqq, J = 7.0, 6.8, 6.4 Hz, H-15)] which successively attached to a methine [δ 2.25 (ddd, J = 9.0, 8.8, 7.0 Hz, C-14)] and to a methylene [δ 2.15 (ddd, J = 19.1, 9.0, 1.5 Hz, H-13) and 2.62 (dd, J = 19.1, 8.8 Hz, H-13)] carbon. HMBC correlations between H_3-18 and three carbons C-10, C-11, and C-14 and between H_2-13 and both C-11 and carbonyl C-12 led to the presence of a cyclopentanone ring. Fragment b, established from couplings observed between H_2-9 [δ 2.04 (dd, J = 15.2, 1.5 Hz) and 2.43 (dd, J = 15.2, 12.2 Hz)] and H-8 [δ 4.44 (dd, J = 12.2, 1.5 Hz)], was connected to the quaternary carbon C-10 forming the

Table 1. NMR Spectral Data for **1** in $CDCl_3$

	δ_H	δ_C	HMBC
1	3.81 (1H, d, 11.2)	78.7 d	C-2
2		80.4 s	
3		154.4 s	
4	4.05 (1H, dt, 13.2, 2.5)	71.9 t	
	4.50 (1H, br d, 13.2)		C-2
5		175.9 s	
6	2.23 (1H, d, 17.1)	35.1 t	C-5, C-8, C-19
	3.64 (1H, d, 17.1)		C-2, C-5, C-7, C-19
7		46.4 s	
8	4.44 (1H, dd, 12.2, 1.5)	84.4 d	C-5, C-19
9	2.04 (1H, dd, 15.2, 1.5)	38.5 t	C-7, C-8, C-10, C-11, C-18
	2.43 (1H, dd, 15.2, 12.2)		C-8
10		42.7 s	
11	2.49 (1H, br d, 11.2)	61.6 d	C-1, C-14, C-18
12		216.0 s	
13	2.15 (1H, ddd, 19.1, 9.0, 1.5)	40.2 t	C-12, C-14, C-15
	2.62 (1H, dd, 19.1, 8.8)		C-10, C-11, C-12
14	2.25 (1H, ddd, 9.0, 8.8, 7.0)	44.2 d	C-10, C-13, C-18
15	1.80 (1H, dqq, 7.0, 6.8, 6.4)	29.0 d	C-16, C-17
16	0.96 (3H, d, 6.8)	22.8 q	C-14, C-15, C-17
17	1.10 (3H, d, 6.4)	23.3 q	C-14, C-15, C-16
18	1.01 (3H, s)	24.4 q	C-9, C-10, C-11, C-14
19	1.31 (3H, s)	25.4 q	C-2, C-6, C-7, C-8
20	5.15 (1H, br s)	109.4 t	C-2, C-4
	5.23 (1H, br s)		C-2, C-4

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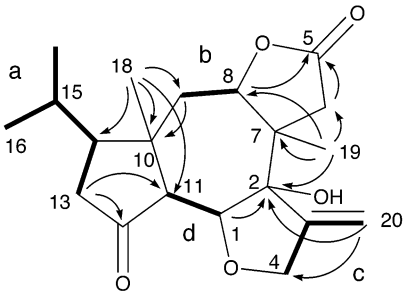
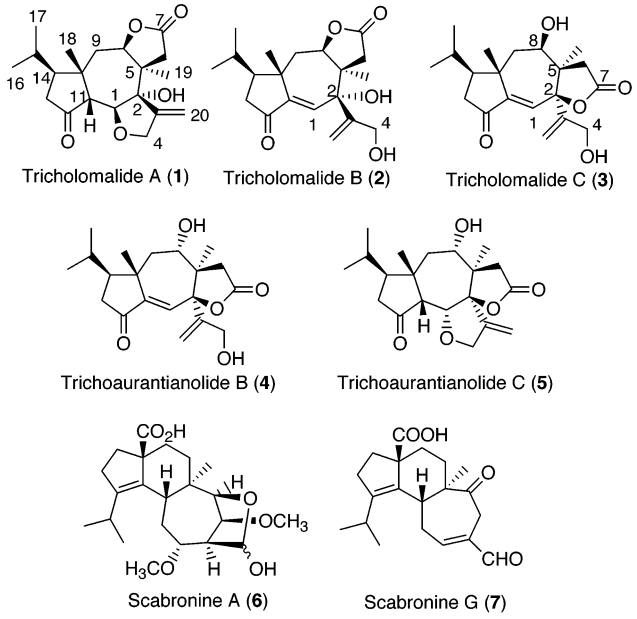


Figure 1. COSY (bold lines) and HMBC (arrows) correlations observed for **1**.



cyclopentanone ring, shown by HMBC cross-peaks H_3-18/C-9 and H-9/C-10 (Figure 1). HMBC correlations between methyl hydrogens H_3-19 and carbons C-2, C-6, C-7, and C-8 and between hydrogens H_2-6 and H-8 and a carbonyl carbon C-5 (δ 175.9) suggested a partial structure comprising a cyclopentanolide ring. The exomethylene signals [δ 5.15 and 5.23 (H_2-20)] showed correlations with methylene hydrogens H_2-4 [δ 4.05 (dt, J = 13.2, 2.5 Hz) and 4.50 (br d, J = 13.2 Hz)] in the COSY spectrum (fragment c) and HMBC correlations with two oxygen-bearing carbons C-2 (δ 80.4, s) and C-4 (δ 71.9, t). The presence of a seven-membered ring was implied by couplings observed for two methine hydrogens H-11 [δ 2.49 (br d, J = 11.2 Hz)] and H-1 [δ 3.81 (d, J = 11.2 Hz)] (fragment d) and an HMBC cross-peak between H-1 [δ 3.81 (d, J = 11.2 Hz)] and C-2 (δ 80.4, s). The carbon signal at δ 148.6 (q) was assignable to C-3,[12] and the remaining elements HO_2 were suggested to form a furan ring with a hydroxyl group attached to C-2. Thus, the gross structure of **1** was unambiguously established. The relative stereochemistry of **1** was established through its NOESY spectrum. NOE correlations between H-8 and three signals H-1, H-14, and H_3-19 and between H-1 and both H-14 and H_3-19 indicated they were oriented on the same side (Figure 2), while NOE correlations between H_3-18 and both H-11 and H-15 revealed that they were on the opposite side of H-1, H-8, H-14, and H_3-19. Thus, the *cis* junction was disclosed between the seven-membered ring and both the cyclopentane and lactone rings. NOE correlations between an exomethylene hydrogen (δ 5.23) and H-4 (δ 4.50) and between another exomethylene hydrogen (δ 5.15) and H_2-6 (δ 2.23 and 3.64)

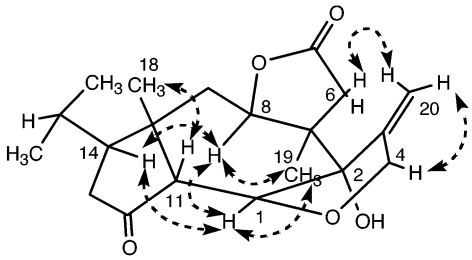


Figure 2. NOE correlations observed for **1**.

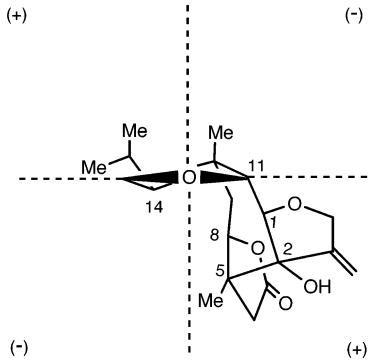
confirmed the relative stereochemistry at C-2 as shown in Figure 2. The absolute stereochemisty of **1** was substantiated by applying the octant rule to the positive Cotton effect observed at 302 nm ($\Delta\epsilon$ = +0.31) due to the cyclopentanone moiety in **1** (Figure 3).[17] The absolute configuration of the similar diterpene, trichoaurantianolide C (**5**), isolated from *T. aurantium*, has been reported by assigning the CD curve at 300 nm ($\Delta\epsilon$ = +0.14).[13] However, the positive Cotton effect due to the n−π* transition of the cyclopentanone unit revealed that trichoaurantianolide C (**5**) possesses the same absolute configuration of its cyclopentanone moiety as our tricholomalide A (**1**) (Figure 3). The structure of the former compound should thus be depicted as presented in structure **5**.

Tricholomalide B (**2**) possesses the same molecular formula as **1** and revealed ^{1}H and ^{13}C NMR spectra almost superimposable on those of **1**, except for the presence of a vinyl hydrogen at δ 6.96 (1H, s) in **2** instead of two methine hydrogens H-1 and H-11 in **1** (Table 2). Interpretation of the 2D spectra of **2** indicated that **2** was a congener of **1**; **2** contained the same substituent appearing in trichoaurantianolide B (**4**)[13] as a consequence of cleavage of an ether linkage between C-1 and C-4 in **1**. The relative stereochemistry of chiral carbons in **2** was established by NOE correlations between H-8 and both H-14 and H_3-19 and between H-15 and H_3-18 (Figure 4) except for C-2 because of the flexible feature of the cycloheptene ring. However, **2** was converted to **1** during storage at 4 °C in DMSO, confirmed by the ^{1}H NMR and CD spectra. Thereby, the absolute configuration of **2** was established as 2*S*, 7*S*, 8*S*, 10*R*, and 14*R*.

Tricholomalide C (**3**) also showed the same molecular formula as **1** and **2**. Analysis of 2D NMR data of **3** (Table 3), including COSY, HMQC, and HMBC spectra, resulted in the assignment of a structure similar to that of trichoaurantianolide B (**4**). NOESY cross-peaks, H-15/H_3-18; H-8/H-14 and H_3-19; H_3-19/H_2-4 and H-20 (δ 5.32) (Figure 5), suggested that **3** was the C-8 epimer of **4**. The *cisoid* α,β-unsaturated ketone system is common to both **2** and **3**. Their CD spectra revealed Cotton effects with the same signs for the respective peaks, 234 ($\Delta\epsilon$ −0.22) and 344 nm ($\Delta\epsilon$ +0.16) for **3** and 250 ($\Delta\epsilon$ −0.57) and 336 nm ($\Delta\epsilon$ +0.53) for **2**, which were closely associated with the same conformational and substitutional effects on *cisoid* α,β-unsaturated ketones[17] in **2** and **3**. Consequently, the structure of **3** was determined including the absolute stereochemistry.

The fact that trichoaurantianolide B (**4**) was converted to trichoaurantianolide B (**5**) by heating with *p*-TsOH in toluene[13] indicated that the absolute stereochemistry of trichoaurantianolide B should be as shown in **4**.

Tricholomalides A−C (**1**−**3**) extended neurite outgrowth in rat pheochromacytoma cells (PC-12) at a concentration of 100 μM, similar to NGF (Figure 6). In addition, diterpenes scabronines A (**6**)[18] and G (**7**),[19] isolated from the mushroom *Sarcodon scabrosus*, caused 1321N1 human astrocytoma cells to release neurotrophic substances, and



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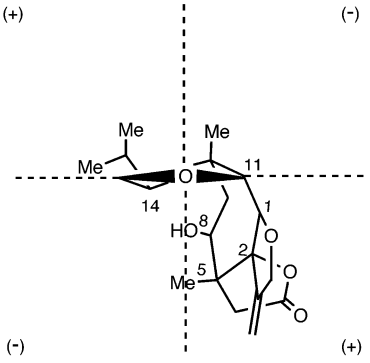

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Figure 3. Octant projections of **1** and **5**.

Table 2. NMR Spectral Data for **2** in $CDCl_3$

	δ_H	δ_C	HMBC
1	6.96 (s)	135.0 d	C-2, C-11
2		75.9 s	
3		148.6 s	
4	4.23 (d, 12.5)	65.9 t	
	4.30 (d, 12.5)		
5		176.2 s	
6	1.99 (d, 17.0)	35.1 t	C-5
	3.51 (d, 17.0)		
7		48.4 s	
8	3.95 (dd, 13.0, 2.5)	85.5 d	C-5
9	2.21 (dd, 14.0, 2.5)	36.8 t	
	2.79 (dd, 14.0, 13.0)		
10		44.3 s	
11		149.4 s	
12		206.4 s	
13	2.18 (dd, 19.0, 12.5)	39.4 t	C-11, C-12
	2.48 (dd, 19.0, 7.5)		C-11, C-12
14	1.80 (ddd, 12.5, 7.5, 7.0)	47.4 d	
15	1.87 (qq, 6.5, 6.5)	28.6 d	
16	1.10 (3H, d, 6.5)	24.2 q	
17	0.98 (3H, d, 6.5)	21.9 q	
18	1.19 (3H, s)	29.7 q	C-9, C-10, C-11, C-14
19	1.02 (3H, s)	25.6 q	C-2, C-6, C-7, C-8
20	5.43 (s)	119.6 t	C-2, C-4
	5.54 (s)		

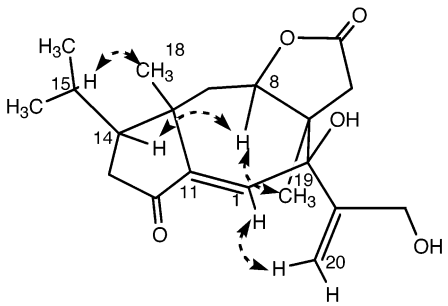

Figure 4. NOE correlations observed for **2**.

Table 3. NMR Spectral Data for **3** in $CDCl_3$

	δ_H	δ_C	HMBC
1	6.61 (s)	128.4 d	C-2, C-11
2		88.9 s	
3		148.2 s	
4	4.23 (2H, d, 13.0)	63.5 t	
5		176.7 s	
6	2.38 (d, 18.3)	34.4 t	C-5
	2.85 (d, 18.3)		C-5
7		50.2 s	
8	3.49 (dd, 11.7, 1.0)	72.4 d	
9	1.87 (dd, 15.0, 11.7)	39.7 t	C-11
	2.10 (dd, 15.0, 1.0)		C-11
10		44.7 s	
11		151.6 s	
12		194.6 s	
13	2.16 (dd, 18.9, 13.0)	41.4 t	C-11, C-12
	2.48 (dd, 18.9, 7.5)		C-11, C-12
14	1.73 (ddd, 13.0, 7.5, 7.1)	49.2 d	
15	1.86 (qqd, 7.1, 6.5, 6.5)	28.6 d	
16	1.09 (3H, d, 6.5)	24.1 q	
17	0.98 (3H, d, 6.5)	21.9 q	
18	1.18 (3H, s)	21.3 q	C-10, C-11, C-14
19	1.16 (3H, s)	21.8 q	C-2, C-6, C-7, C-8
20	5.32 (s)	117.9 t	C-2, C-3, C-4
	5.64 (s)		C-2, C-3, C-4

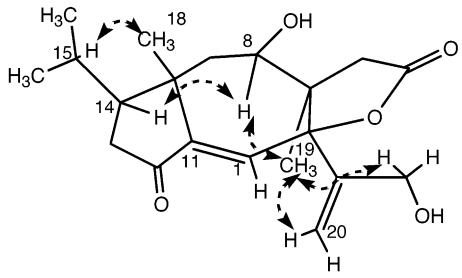

Figure 5. NOE correlations observed for **3**.

the conditioned medium with **6** or **7** stimulated neurite outgrowth in PC-12 cells.[19] However, neither **6** nor **7** directly caused neurite extension in PC-12. It is interesting to know why such difference occurred among these compounds having a similar carbon framework. Although ustalic acid, recently isolated from the same mushroom,[20] showed Na^+,K^+-ATPase inhibitory activity, tricholomalides A−C were nontoxic against HeLa cells at a concentration of 50 μg/mL. On the other hand, NGF may prevent neuronal death and activate neuronal function in the brain when peripherally administered. However, exogeneously administered NGF cannot cross the blood−brain barrier. Therefore, low-molecular-weight NGF-like substances such as tricholomalides or NGF inducers such as scabronines are useful for treatment of serious neuronal disorders such as Alzheimer's disease. Detailed pharmacological properties of tricholomalides will be reported elsewhere.

Experimental Section

General Experimental Conditions. Optical rotations were determined with a Horiba SEPA-300 high-sensitivity polarimeter. UV spectra were measured on a Shimadzu UV-1600 UV−visible spectrometer. IR spectra were recorded on a Shimadzu IR-460 infrared spectrophotometer. CD spectra were measured on a JASCO J-725 spectropolarimeter in MeOH. NMR spectra were recorded on a JEOL GSX500 NMR spectrometer in $CDCl_3$. Chemical shifts were referenced to the

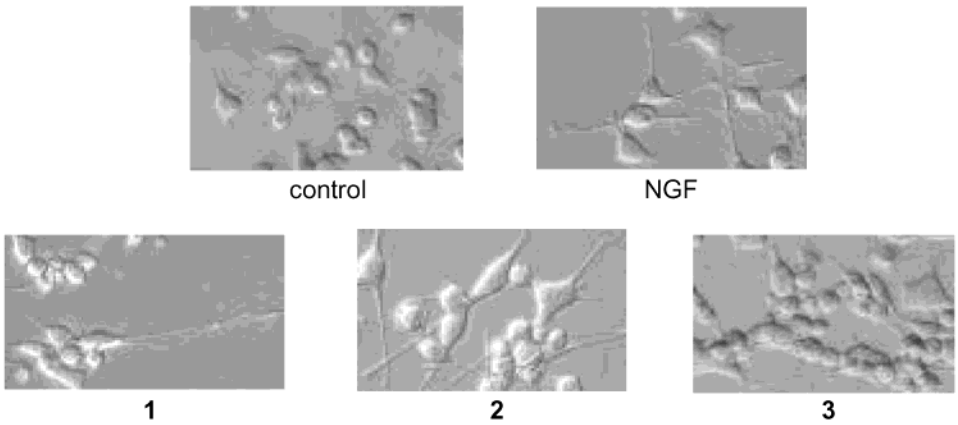

Figure 6. Effects of **1**, **2**, **3**, and NGF on neurite outgrowth in PC-12 cells. PC-12 cells were incubated for 2 days without drug (control) or with 50 ng/mL of NGF (NGF), 100 μM **1**, **2**, or **3**.

residual solvent peaks (δ_H 7.26 and δ_C 77.0). Mass spectra were measured on a JEOL SX-102 mass spectrometer.

Extraction and Isolation. The fresh fruiting body (2.7 kg) of *Tricholoma* sp. was soaked in MeOH. Evaporation of the solvent gave a yellowish-brown residue, which was subsequently partitioned between water and EtOAc. The organic layer (18.4 g) was subjected to silica gel column chromatography using $CHCl_3$/MeOH. The fractions (1.04 g) eluted with $CHCl_3$/MeOH (90:1 to 9:1) were combined and purified with silica gel column chromatography using hexane/EtOAc (2:1 to 1:2) as eluent. The fraction (102.0 mg) eluted with hexane/EtOAc (1:1) was purified with silica gel chromatography using $CHCl_3$/MeOH to afford tricholomalide B (**2**, 7.8 mg, 0.29%) and a mixture of tricholomalides A−C (**1**−**3**), followed by HPLC purification using hexane/EtOAc/MeOH (120:80:1) to yield **1** (2.1 mg, 7.8 × 10^{-5} %), **2** (1.7 mg, 3.5 × 10^{-4} %), and **3** (2.8 mg, 1.0 × 10^{-4} %).

Tricholomalide A (1): $[\alpha]_D^{25}$ −3.5° (*c* 0.0031, MeOH); UV (MeOH) λ_{max} (log ϵ) 242 nm (2.4); CD (MeOH) $\Delta\epsilon_{244}$ −0.19, $\Delta\epsilon_{302}$ +0.31; IR (film) ν_{max} 3460, 2955, 2930, 1765, 1731 cm^{-1}; NMR data, see Table 1; HREIMS *m*/*z* 348.1948 (calcd for $C_{20}H_{28}O_5$, $[M]^+$, 348.1929).

Tricholomalide B (2): $[\alpha]^{25}_D$ −53° (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ϵ) 248 nm (1.7); CD (MeOH) $\Delta\epsilon_{250}$ −0.57, $\Delta\epsilon_{336}$ +0.53; IR (film) ν_{max} 3430, 3023, 3017, 2936, 1780, 1723, 1640, 1200 cm^{-1}; NMR data, see Table 2; HREIMS *m*/*z* 348.1942 (calcd for $C_{20}H_{28}O_5$, $[M]^+$, 348.1929).

Tricholomalide C (3): $[\alpha]^{25}_D$ 0° (*c* 0.010, MeOH); UV λ_{max} (MeOH) (log ϵ) 246 nm (1.5); CD (MeOH) $\Delta\epsilon_{234}$ −0.22, $\Delta\epsilon_{344}$ +0.16; IR (film) ν_{max} 3420, 2975, 2950, 1770, 1720, 1260 cm^{-1}; NMR data, see Table 3; HREIMS *m*/*z* 348.1975 (calcd for $C_{20}H_{28}O_5$, $[M]^+$, 348.1929).

Neurite Outgrowth Assay. A neurite outgrowth assay was carried out with rat pheochromocytoma cells (PC-12).[17] PC-12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5% horse serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) in an incubator containing 5% CO_2 at 37 °C. PC-12 cells were seeded onto 24-well multiplates (1 × 10^5 cells/mL) and cultivated for a day. The medium was replaced with that containing 100 μM tricholomalides A−C or 5 ng/mL NGF as positive control, and then PC-12 cells were cultivated for 2 days and observed under a phase-contrast microscope.

Cytotoxicity Test. A cytotoxicity test was carried out with HeLa cells. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were seeded into 96-well microplates (3 × 10^3 cells/well) and precultured for a day. The medium was replaced with that containing test compounds at a concentration of 50 μg/mL and further cultured at 37 °C for 3 days. The medium was then replaced with 50 μL of MTT solution (0.2 mg/mL in medium) and incubated under the same conditions for 4 h. After addition of 200 μL of DMSO, the optical density was measured with a microplate reader at 570 nm.

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