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Neuritogenic Cerebrosides from an Edible Chinese Mushroom. Part 2†: Structures of Two Additional Termitomycesphins and Activity Enhancement of an Inactive Cerebroside by Hydroxylation

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Abstract—Termitomycesphins E and F, novel cerebrosides that are hydroxylated around the middle of the long-chain base (LCB), have been isolated from the edible Chinese mushroom *Termitomyces albuminosus* (Berk.) Heim. ('Jizong' in Chinese) together with termitomycesphins A–D, and shown to induce neuronal differentiation in rat PC12 cells. Their stereostructures have been determined based on their chemical derivatization and spectroscopic analysis. The major cerebroside obtained from the same mushroom was not hydroxylated around the middle of the LCB and was inactive against PC12 cells, suggesting the importance of the extra hydroxyl group on LCB. The Di- and tetrahydroxylation of this inactive cerebroside resulted in the enhancement of its neuritogenic activity.

Introduction

The nerve growth factor (NGF) is currently the best characterized neurotrophic factor essential for the survival and functioning of nerve cells.[2,3] Findings suggest that NGF can be effective for the treatment of dementia and cerebral paralysis.[4–7] Therefore, NGF was considered as a good candidate for the treatment of neurodegenerative diseases. However, it is very difficult to use NGF as a drug, because it is too large in size to cross the blood–brain barrier. Low-molecular-weight compounds exhibiting NGF-like activity could lead to promising therapeutic drugs to treat neurodegenerative diseases. The PC12 cell line derived from rat pheochromocytoma cells is well-known to express neuronal properties (elongation of neuronal processes) against NGF and is a useful model system for such studies.[8]

A previous search for such neuritogenic compounds from the Chinese mushroom *Termitomyces albuminosus* (Berk.) Heim. ('Jizong' in Chinese) was performed in our laboratory using the PC12 cell line system. This resulted in the isolation of four unusual cerebrosides, termitomycesphins A–D (**1**–**4**).[1] These cerebrosides all possessed a branch allylic alcohol system around the middle of the long-chain base (LCB). A further investigation of this mushroom resulted in the finding of two additional derivatives, termitomycesphins E (**5**) and F (**6**), accompanied by a known cerebroside **7** (Fig. 1). Furthermore, as part of the structure–activity relationship studies, the inactive cerebroside **7** was converted to the di- and tetrahydroxylated derivatives (**12ab**, **13ab,** and **13cd**) (Fig. 3), which turned out to become neuritogenic. In this paper, we describe the isolation and structural determination of two new termitomycesphins **5** and **6**, the conversion of the inactive cerebroside **7** into the active derivatives, and the evaluation of the neuritogenic activity of these natural and artificial cerebrosides.

Results and Discussion

The isolation of termitomycesphins A–D (**1**–**4**) was described in detail in a previous paper.[1] A further study of the other fractions from the last HPLC separation resulted in the isolation of termitomycesphins E (**5**) (0.00036%, base on dry mushroom) and F (**6**) (0.00057%). The examination of a less polar fraction than that of the termitomycesphins led to the isolation

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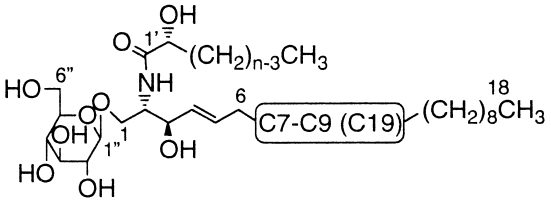
of a known cerebroside **7**[9,10] (0.042%) as a major cerebroside component.

Termitomycesphin F (**6**) has the molecular formula of $C_{43}H_{83}NO_{10}$, which was determined by HRESI-MS measurement [*m*/*z* 774.6087 $(M+H)^+$, $\Delta+0.8$ mmu]. The IR and NMR spectra of **6** indicated the presence of a fatty acid (large signal at δ_H 1.24), an amide (1648 and 1537 cm^{-1}), and a sugar moiety (complex NMR signals at δ_H 3.89–4.90) that are similar to those of the known termitomycesphins (**1**–**4**), indicating that **6** is a derivative of the termitomycesphins. The notable difference in the NMR spectra between **2** and **6** is that the signals due to the exomethylene (C9–C19) in **2** is replaced by a secondary methyl group (δ_{H19} 1.06 and 1.08, δ_{H9} 1.62 and 1.72; δ_{C19} 14.4 and 15.9, δ_{C9} 39.3 and 39.8) in **6**. The other NMR data are similar to those for **2** (Table 1). An analysis of the HMQC and DQFCOSY spectra allowed us to draw the following partial structures: β-glucoside moiety, C1–C10, C2′–C4′, and two terminal methyls connected to methylenes. In an HMBC spectrum, H2, N–H, and H2′ are correlated to the carbonyl carbon at δ 175.6, revealing the connectivity of the fatty acyl and LCB moieties. The correlations of H1/anomeric C1″ and anomeric H1″/C1 indicate the location of the β-glucose. Since the carbon signals assigned to C4, C5, and C7–C10 are split into two similar chemical shifts in the ratio of ca. 1:1, we presumed that **6** is an inseparable mixture of C8 or/and C9 epimers. Two carbon-chain lengths of the long-chain base (LCB) and fatty acid moieties of **6** were determined by the FAB-MS fragment analysis. It was reported that cerebrosides gave the characteristic fragment ions of $(LCB+H)^+$ and $(LCB+H-H_2O)^+$ in the positive mode FAB-MS measurement.[11] The FAB-MS spectrum of **6** displays the marked fragment ions at *m*/*z* 312 and 294 that could be due to $(LCB+H)^+$ and $(LCB+H-H_2O)^+$, respectively. An additional fragment peak at *m*/*z* 276 $(LCB+H-2H_2O)^+$ also arises from a further dehydration of the LCB moiety, revealing that **6** possesses the same carbon-chain length of LCB as that of **2** and, consequently, the same fatty acid moiety as **2** taking the molecular formula into consideration. The absolute stereochemistry of **6** except for the C8 and C9 positions was determined to be the same as that of **2**, because the hydrogenation product **8** (Fig. 3) from **6** showed an identical optical rotation $[\alpha]_D^{25}+9.3$ (*c* 0.14, MeOH)], FAB-MS, and ^1H NMR data to those for the hydrogenation product from **2**[1] $[\alpha]_D^{25}$ +10 (*c* 0.19, MeOH)].



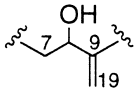
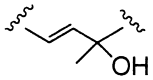
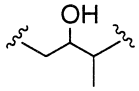
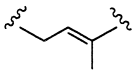
termitomycesphin	C7-C9 (C19)	n
A (**1**)		16
B (**2**)		18
C (**3**)		16
D (**4**)		18
E (**5**)		16
F (**6**)		18
known cerebroside (**7**)		16

Figure 1. Structures of termitomycesphins A–F (**1**–**6**) and the known cerebroside **7**.[9,10]

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR data for termitomycesphins E (**5**) and F (**6**) in pyridine-d_5[a]

Position	^1H (ppm) (*J* in Hz)	^{13}C (ppm)
LCB		
1a	4.22 dd (9.3, 3.5)	70.1 t
1b	4.68 dd (9.3, 4.5)	
2	4.80 m	54.6 d
3	4.77 m	72.3 d
4	6.05 dd (15.2, 2.8)	131.5, 131.6 d[b]
5	6.01 dd (15.2, 4.6)	132.9, 133.1 d[b]
6a	2.35, 2.37 m[b]	29.8 t
6b	2.55, 2.56 m[b]	
7a	1.66, 1.71 m[b]	34.0, 34.8 t [b]
7b	1.72, 1.80 m[b]	
8	3.68, 3.77 m[b]	73.7, 74.6 d[b]
9	1.62, 1.72 m[b]	39.3, 39.8 d[b]
10	1.30, 1.34 m[b]	32.6, 34.0 t[b]
11-17	1.24 m	22.8–32.0 t[c]
18	0.85 t (6.8)[d]	14.2 q
19	1.08, 1.06 d (7.4)[b]	15.9, 14.4 q[b]
2-NH	8.35 d (8.5)	
Acyl		
1′		175.6 s
2′	4.56 m	72.4 d
3′a	2.00 m	35.5 t
3′b	2.20 m	
4′a	1.68 m	25.9 t
4′b	1.80 m	
5′–15′ (or 17′)	1.24 m	22.8–32.0 t[c]
16′ (or 18′)	0.84 t (6.8)[d]	14.2 q
Sugar		
1″	4.90 d (7.6)	105.5 d
2″	4.01 dd (7.6, 7.1)	75.0 d
3″	4.19 m	78.4 d
4″	4.20 m	71.5 d
5″	3.89 m	78.4 d
6″a	4.34 dd (11.7, 5.2)	62.6 t
6″b	4.49 br d (11.7)	

[a]Both data for **5** and **6** are superimposable.
[b]1:1 ratio due to C-8 and/or C-9 epimers.
[c]δ = 22.8, 27.8, 27.9, 29.5, 29.7, 29.8, 30.4 and 32.0.
[d]Interchangeable.

The molecular formula of termitomycesphin E (**5**), $C_{41}H_{79}NO_{10}$, was determined to be less than that of **6** by C_2H_4 based on the HRESI-MS measurement [*m/z* 746.5764 $(M+H)^+$, Δ +1.8 mmu]. The ^1H and ^{13}C NMR spectra of **5** are superimposable on those of **6** (Table 1), indicating that **5** is a homologue of **6** possessing a shorter carbon chain. The positive FAB-MS spectra of both **6** and **5** displayed the same fragment ions at *m/z* 312 $(LCB+H)^+$, 294 $(LCB+H-H_2O)^+$, and 276 $(LCB+H-2H_2O)^+$, revealing the presence of the same LCB moiety in **5** and **6**. The absolute stereochemistry of **5** could be the same as that of **6** because of the superimposable NMR data and a similar optical rotation [**5**: $[\alpha]_D^{24}$ +2.8 (*c* 0.20, MeOH); 6: $[\alpha]_D^{24}$ +2.0 (*c* 0.15, MeOH)].

The gross structure of the known cerebroside **7** was determined by a comparison of the NMR and FAB-MS data with the reported ones.[9,10] The degradation of **7** was carried out to determine the stereochemistry and the two carbon chain lengths (Fig. 2). The hydrogenation of **7** gave the tetrahydro derivative, which, upon methanolysis, gave methyl D-glucopyranoside (2:1 mixture of α and β-anomers), the fatty acid methyl ester **9**, and free LCB **10**. The configuration of the methyl glucoside was determined to be the D form by a comparison of the optical rotation [$[\alpha]_D^{25}$ +74 (*c* 0.027, MeOH)] with the reported one.[12] The *R* configuration of the methyl ester **9** was determined by the comparison of the optical rotation [$[\alpha]_D^{25}$ −5 (*c* 0.11, $CHCl_3$)].[13] The long-chain base **10** was converted to the tribenzoyl derivative **11**. The CD spectrum and partial ^1H NMR data (Table 2) of **11** agreed with those reported for the benzoate of a (2*S*,3*R*)-LCB,[14] revealing the 2*S*,3*R* stereochemistry of **11**. The structure and absolute stereochemistry of **7** was thus determined to be the same as the reported ones as shown in Figure 1.[9,10]

The neuritogenic activities of **5**, **6**, and **7** were evaluated using the PC12 cell line system. Figure 5 shows the time course of the neuronal differentiation of the PC 12 cells induced by these compounds. Termitomycesphin E (**5**) possessing a C16-fatty acyl moiety showed a much higher activity than termitomycesphin F (**6**) possessing a longer (C18) fatty acyl moiety at the same concentration of 14 μM. Such a tendency was also observed for termitomycesphins A–D (**1**–**4**); **1** and **3** were more active than **2** and **4**.[1] On the other hand, the known cerebroside **7** showed almost no neuritogenic activity. These findings suggest that not only the fatty acyl-chain length but also the polarity of the molecules (the presence of the extra hydroxyl group around the middle of the LCB) play important roles in the neuritogenic activity of these cerebrosides (Fig. 3).

To obtain supporting evidence for the importance of the extra hydroxyl group around the middle of the LCB of the termitomycesphins, we tried to introduce hydroxyl groups into the LCB of the major inactive cerebroside **7**. Dihydroxylation of **7** with microencapsulated osmium tetraoxide (MC OsO_4) and *N*-methylmorpholine *N*-oxide (NMO) gave the 8,9-dihydro-8,9-dihydroxyl derivative **12ab** in 44% yield. The number and positions of the introduced hydroxyl groups were determined from the FAB-MS data [*m/z* 784 $(M+Na)^+$] and the following newly observed ^1H NMR signals assigned by a DQFCOSY spectrum (Table 3): 3.79 (m, H-8), 1.41 (s, H-19), and 5.71 (brs, 8-OH). The split peaks of C4–C5 (δ_C 133.0/132.9 and 131.7/131.6, respectively) observed in ^{13}C NMR spectrum of **12ab** indicated that **12ab** was a 1:1 mixture of diastereomers. The hydroxylation using an excess amount of MC OsO_4 gave the 4,5,8,9-tetrahydro-4,5,8,9-tetrahydroxy derivatives, **13ab** and **13cd**, in 66 and 20% yields, respectively, both of which turned out to be a 1:1 diastereomeric mixture from the HPLC analysis and ^1H NMR spectra. The introduction of the four hydroxyl groups in **13ab** and **13cd** was revealed by the FAB-MS data [*m/z* 818 $(M+Na)^+$] and the newly observed ^1H NMR signals due to three oxymethines and a branch methyl (Table 3): 4.65/4.67 (1H, m, H-5), 4.19 (1H, m, H-4), 3.85/3.89 (1H, m, H-8), 1.39/1.40 (3H, s, H-19) for **13ab**; 4.36/4.39 (1H, m, H-5), 4.04 (1H, m, H-4), 3.82/3.86 (1H, m, H-8), 1.41/1.42 (3H, s, H-19) for **13cd**. The difference between **13ab** and **13cd** can be attributed to the difference in the configuration at the C4–C5 stereogenic centers, because the differences in the NMR chemical shifts are mainly observed around H4 and H5. To determine the absolute stereochemistry at C4–C5 of **13ab**, it was converted to an acetonide and then finally to a tetraacetate triacetonide **14** (Fig. 4).

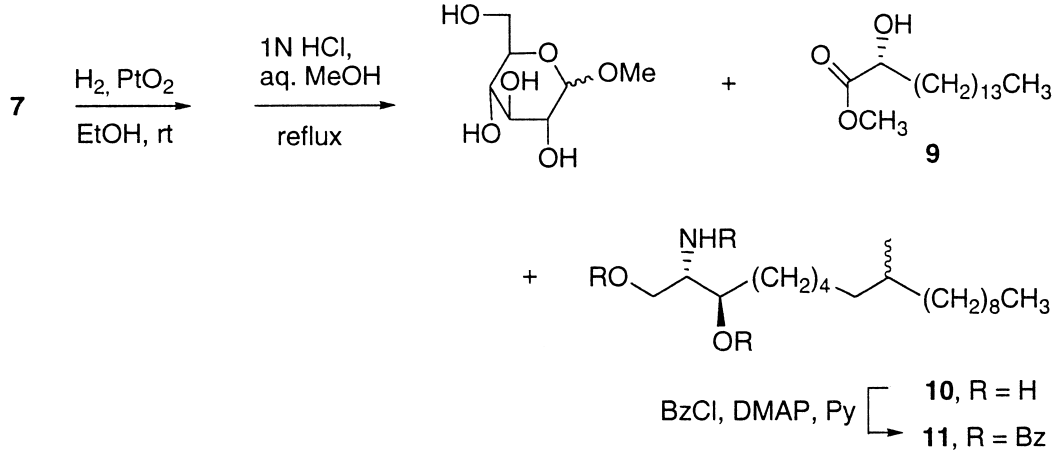
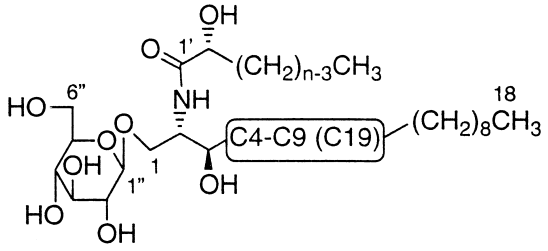


Figure 2. Degradation scheme of **7**.

The relative stereochemistry of C3–C5 in **14** was determined to be *anti–syn* on the basis of the coupling constants and NOE correlations as shown in Figure 4, suggesting that **14** adopted a twisted boat conformation.[15] Thus, the absolute configuration of C4–C5 of **13ab** was determined to be 4*R*,5*S*, and C4–C5 of **13cd** was 4*S*,5*R*.

The neuritogenic activity of **12ab** and **13a–d** (Fig. 5) indicated that hydroxylation markedly endowed **7** with the activity (25% maximum neuronal differentiation at a concentration of 2.5 μM), though these showed cytotoxicity at a concentration higher than 2.5 μM. The times and concentrations required for reaching the maximum activity of **12ab** and **13a–d** are much shorter and lower than those of **5**, respectively. Interestingly, the time course of their activities (rapid increase and then gradual lowering) was quite similar to that of NGF (5 ng/mL) rather than that of termitomycesphin E (**5**) (gradual increase). We presume that a peptidic compound, NGF, and highly hydroxylated lipids such as **12ab** and **13a–d** are relatively susceptible to metabolic degradation in the cell-culture conditions.



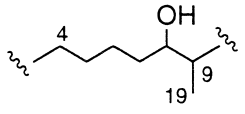
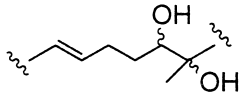
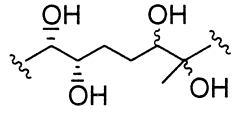
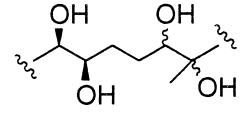
	C4-C9 (C19)	n
8		18
12ab		16
13ab		16
13cd		16

Figure 3. Structures of cerebroside derivatives.

Table 2. Selected ^{1}H NMR data ($CDCl_3$, 400 MHz) and CD data (MeOH) for tribenzoate **11**

^{1}H NMR						CD	
J_{NH-H2}	J_{H2-H3}	δ_{NH}	δ_{H3}	δ_{H2}	δ_{H1}	λ (Δε)	
7.9	3.9	7.09	5.38	4.88	4.61/4.67	238 (−7.8)	223 (+5.8)

Coupling constants *J* are in Hz, chemical shifts δ are in ppm, and wave length λ in nm.

Experimental

General procedures

Preparative HPLC was performed using JASCO PU-980 pumps (Japan). Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-7000S. UV spectra were recorded on a JASCO Ubest-50UV/VIS spectrometer. CD spectra were recorded with a JASCO J-720 spectrometer. FAB-MS spectra were recorded on a JEOL DX-705L (Japan) mass spectrometer, using *m*-nitrobenzyl alcohol as the matrix in the positive mode. High-resolution FAB-MS spectra were recorded on a JEOL Mstation JMS-700 mass spectrometer, using a mixture of the glycerol/thioglycerol/*m*-nitrobenzyl alcohol (3:1:1) + NaI as the matrix and PEG 600 or PEG 1000 as the calibration standard in the positive mode. HRESI-MS spectra were recorded on a MDS SCIEX (Canada) QSTAR mass spectrometer using a mixture of CsI and the sex pheromone inhibitor iPD1 (MW 829) (Bachem, Switzerland) as the calibration standards in the positive mode. NMR spectra were recorded on a Bruker AMX-600 or a Bruker ARX-400 spectrometer. The NMR chemical shifts in δ (ppm) were referenced to the solvent peak of δ_C 39.7 for DMSO-d_6, δ_H 7.26 for $CDCl_3$ (residual $CHCl_3$), and δ_H 7.19 and δ_C 123.4 for pyridine-d_5.

Isolation

The extraction and purification procedures were described in detail in a previous paper.[1] The BuOH fraction from the EtOH extract of the mushroom *T. albuminosus* was chromatographed on ODS to give four fractions. The HPLC purification of the third fraction yielded termitomycesphin E (**5**) (15.7 mg, 0.00036%, t_R = 120 min) and termitomycesphin F (**6**) (24.7 mg, 0.00057%, t_R = 205 min) as well as **1**–**4**.[1] A portion (100 mg) of the fourth fraction (8.9 g), which was eluted with MeOH, was purified by HPLC [Develosil ODS-10 (ϕ 20×250 mm; Nomura Chemical, Japan), solvent: MeOH/H_2O (98:2), flow rate: 8 mL/min, two injections, detection at 205 nm] to yield the known cerebroside **7** (20.4 mg, 0.042% calcd yield, t_R = 36 min).

Termitomycesphin E (**5**): a colorless powder; $[\alpha]_D^{24}$ +2.8 (*c* 0.20, MeOH); IR (KBr) 3375, 2923, 2853, 1648, 1537, 1468, and 1079 cm^{-1}; HRESI-MS *m*/*z* 746.5764, calcd for $C_{41}H_{80}NO_{10}$ (M + H) 746.5782; ^{1}H and ^{13}C NMR in Table 1.

Termitomycesphin F (**6**): a colorless powder; $[\alpha]_D^{24}$ +2.0 (*c* 0.15, MeOH); IR (KBr) 3375, 2921, 2853, 1648, 1538, 1468, and 1079 cm^{-1}; HRESI-MS *m*/*z* 774.6087, calcd for $C_{43}H_{84}NO_{10}$ (M + H) 774.6095; ^{1}H and ^{13}C NMR were superimposable on those of **5** (Table 1).

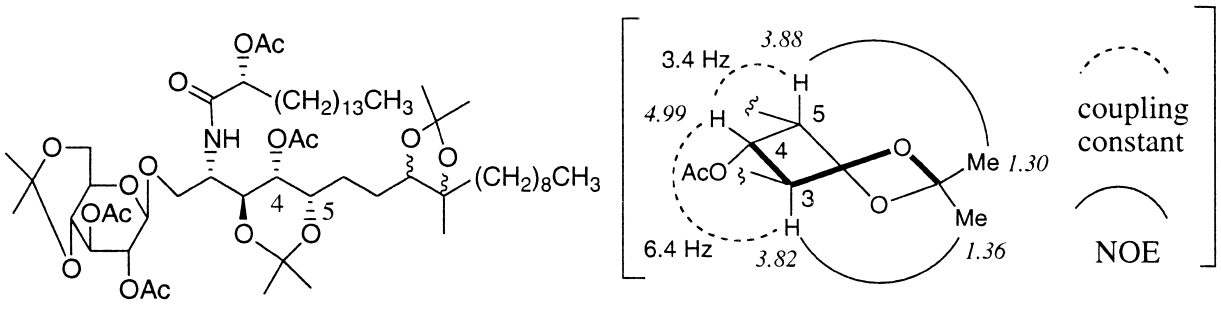


Figure 4. NMR analysis of tetraacetate triacetonide derivative **14** obtained from **13ab** to determine the absolute configuration of C4–C5 of **13ab**. Partial chemical shifts (in ppm) are in *italics*.

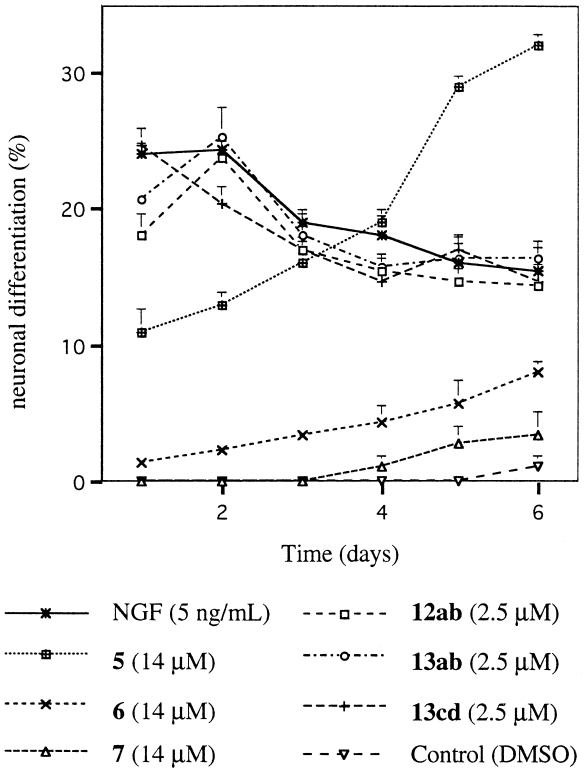


Figure 5. Time course of neuronal differentiation induced in PC12 cells by termitomycesphins E and F (**5** and **6**) and other cerebroside derivatives in comparison with nerve growth factor (NGF) as a positive control. The percentages of the cells with longer processes than the diameter of the cell body were plotted as neuronal differentiation.

Known cerebroside **7**: a colorless powder; $[\alpha]_D^{25}$ +4.5 (*c* 0.46, MeOH); IR (KBr) 3371, 2921, 2853, 1643, 1536, 1468, and 1081 cm^{-1}; FAB-MS *m/z* 750 $(M+Na)^+$; ^{13}C NMR (DMSO + 2% D_2O): δ 173.91, 135.08, 131.26, 131.05, 123.61, 103.56, 77.00, 76.63 (76.62, CH–OD), 73.49 (73.46), 71.20 (71.17), 70.81 (70.71), 70.25 (70.20), 68.86, 61.33 (61.21), 53.04 (53.00, CH–ND–), 39.90, 34.53, 32.23, 31.36, 29.10, 28.98, 28.74, 27.49, 27.38, 24.61, 22.14, 15.83, and 13.95.

Hydrogenation of 6

To a solution of termitomycesphin F (**6**, 5.3 mg) in EtOH (1 mL) were added PtO_2 (1.0 mg) and the mixture was stirred under a hydrogen atmosphere at room temperature for 4 h. The reaction mixture was filtered, and the filtrate was concentrated to give 5.2 mg of tetrahydro derivative **8**: $[\alpha]_D^{25}$ +9.3 (*c* 0.14, MeOH), FAB-MS *m/z* 798 $(M+Na)^+$, ^1H NMR data were the same as that from **2**.[1]

Degradation of 7

The known cerebroside **7** (5.4 mg) was hydrogenated under the above conditions, and the crude product was subjected to acidic methanolysis in the same conditions as those for **2** and **4**,[1] giving methyl α- and β-D-glucopyranoside (0.4 mg) $[\alpha]_D^{25}$ +74 (*c* 0.027, MeOH)], LCB **10**, and fatty acid methyl ester **9** (1.4 mg): colorless powder, $[\alpha]_D^{24}$ −5 (*c* 0.11, CHCl3) [lit.[13] $[\alpha]_D^{25}$ −3.6 ($CHCl_3$)], FAB-MS *m/z* 287 $(M+H)^+$. LCB **10** from the EtOAc fraction was benzoylated,[1,14] and the product was purified by preparative TLC (silica gel, EtOAc/hexane = 1:9, developed twice, R_f = 0.18) to give (2*S*,3*R*)-2-benzoylamido-1,3-dibenzoyloxy-9-methyloctadecane (**11**) (0.7 mg) as a colorless powder: HRFAB-MS *m/z* 628.4061, calcd for $C_{40}H_{54}NO_5$ $(M+H)$ 628.4002. ^1H NMR (400 MHz, $CDCl_3$) δ 8.02 (2H, m), 7.95 (2H, m), 7.77 (2H, m), 7.35–7.58 (9H, m), 7.08 (1H, d, *J* = 8.6 Hz), 5.38 (1H, m), 4.88 (1H, m), 4.63 (2H, m), 1.05–1.95 (27H, m, methylenes and one methine), 0.87 (3H, t, *J* = 6.8 Hz), and 0.80 (3H, d, *J* = 6.5 Hz). The concentration of **11** was calculated from an ε value of 32000 at 228 nm.[14]

Hydroxylation of 7 to 12ab and 13a–d

A mixture of **7** (9.5 mg), MC OsO_4 (4.0 mg, Wako, Japan), and NMO (10.0 mg) in H_2O/acetone/acetonitrile (1:1:1, 60 μL) was stirred for 48 h at 50 °C. The catalyst was separated by filtration and washed with MeOH. The combined filtrates were concentrated to dryness, which was purified by preparative TLC (silica gel, $CHCl_3$/MeOH = 4:1, developed twice, R_f = 0.42) to afford **12ab** (4.2 mg, 44%): colorless powder, $[\alpha]_D^{23}$ +5.5 (*c* 0.24, MeOH), IR (KBr) 3376, 2923, 2853, 1642, 1542, 1467, and 1080 cm^{-1}; HRFAB-MS *m/z* 784.5500, calcd for $C_{41}H_{79}NO_{11}Na$ $(M+Na)$ 784.5550. ^1H NMR in Table 3; ^{13}C NMR (pyridine-d_5, 100 MHz) δ 175.6, 133.0/132.9 (1:1), 131.7/131.6 (1:1), 105.5, 78.4, 78.3, 77.3/77.2 (1:1), 75.0, 74.4, 72.4, 72.3, 71.5, 70.0, 62.6,

Table 3. ^{1}H NMR data of dihydroxylated and tetrahydroxylated cerebrosides (600 MHz, in pyridine-d_5)

	12ab	13ab (13a/13b)[a]	13cd (13c/13d)[a]
LCB			
1a	4.24 dd (10.9, 3.1)	4.51 m	4.20 m
1b	4.66 m	4.67 m	4.77 m
2	4.78 m	5.31 m	4.88 m
3	4.78 m	4.68 m	4.41 m
4	6.11 m	4.19 m	4.04 m
5	6.06 m	4.65/4.67 m	4.36/4.39 m
6a	2.39 m	2.29/2.18 m	2.05/2.19 m
6b	2.70 m	2.54/2.61 m	2.54/2.38 m
7a	1.78 m	1.89/2.04 m	1.85/2.05 m
7b	1.90 m	2.29/2.14 m	2.33/2.13 m
8	3.79 m	3.85/3.89 m	3.82/3.86 m
10–17	1.25 br s, 1.63–1.90 m	1.23–1.30 m, 1.58–2.62 m	1.20–1.32 m, 1.62–2.55 m
18	0.86 t (6.7)[b]	0.85 t (7.0)[b]	0.86 t (6.9)[b]
19	1.41 s	1.39/1.40 s	1.41/1.42 s
NH	8.33 d (8.2)	8.51/8.52 d (8.6)	8.63 d (8.8)
Acyl			
2′	4.56 m	4.51 m	4.55 m
3′a	2.00 m	1.88 m	1.93 m
3′b	2.20 m	2.13 m	2.15 m
4′a	1.63–1.90 m	1.62 m	1.67 m
4′b	1.63–1.90 m	1.71 m	1.74 m
5′–15′	1.25 br s	1.23–1.30 m	1.20–1.32 m
16′	0.85 t (5.7)[b]	0.86 t (7.0)[b]	0.87 t (6.9)[b]
Sugar			
1″	4.89 d (7.9)	4.91/4.92 d (7.9)	4.86 d (7.8)
2″	4.00 dd (8.0, 7.7)	3.95 dd (7.9, 7.5)	3.98 m
3″	4.17 m	4.16 m	4.15 m
4″	4.19 m	4.15 m	4.15 m
5″	3.89 m	3.82 m	3.88 m
6″a	4.34 dd (11.4, 4.3)	4.30 m	4.32 m
6″b	4.49 br d (11.4)	4.43 d (11.5)	4.49 d (12.5)

[a]A 1:1 mixture of diastereomers. Measured at 310 K.
[b]Interchangeable.

54.6, 39.2, 35.5, 32.0, 31.8/31.7 (1:1), 30.9, 30.4/30.3 (1:1), 30.0, 29.9, 29.5, 25.9, 24.0, 22.8, 22.4, and 14.2.

A large excess of MC OsO_4 (12.0 mg) was used to oxidize 10.0 mg of **7**. The mixture was stirred for 24 h at 50 °C and then filtered. The filtrate was evaporated and the residue was dissolved in MeOH and passed through a TOYOPAK ODS-M cartridge (TOSOH, Japan), which was then washed with MeOH. The filtrate was concentrated and subjected to preparative HPLC [Develosil ODS-10 (ϕ 20×250 mm), solvent: CH_3CN/H_2O/MeOH (75:15:10), flow rate: 8 mL/min] to give two mixtures of tetrahydroxylated derivatives **13ab** (6.6 mg, 66%, t_R = 50–66 min) and **13cd** (2.0 mg, 20%, t_R = 68–73 min). **13ab**: colorless powder, $[\alpha]_D^{23}$ −1.2 (*c* 0.41, MeOH), IR (KBr) 3398, 2926, 2853, 1624, 1541, 1467, and 1084 cm^{-1}; HRFAB-MS *m*/*z* 818.5565. calcd for $C_{41}H_{81}NO_{13}Na$ (M + Na) 818.5605. ^{1}H NMR in Table 3. **13cd**: colorless powder, $[\alpha]_D^{23}$ +12 (*c* 0.094, MeOH), IR (KBr): 3384, 2922, 2852, 1647, 1541, 1467, and 1079 cm^{-1}; HRFAB-MS *m*/*z* 818.5565, calcd for $C_{41}H_{81}NO_{13}Na$ (M + Na) 818.5605. ^{1}H NMR in Table 3.

Conversion of 13ab to 14

A solution of **13ab** (2.0 mg) in a mixture of acetone (0.5 mL), 2,2-dimethoxypropane (0.5 mL), and pyridinium *p*-toluene sulfonate (PPTS) (1.0 mg) was stirred at room temperature for 2 h. The reaction was quenched by adding saturated $NaHCO_3$ solution (2.5 mL). The mixture was extracted three times with ether (2.5 mL). The ether layers were combined, concentrated, and the residue was purified by preparative TLC (silica gel, acetone/hexane = 4:6, R_f = 0.3) to afford a mixture of triacetonides (0.7 mg). The second trial using **13ab** (2.0 mg), acetone (1.5 mL), 2,2-dimethoxypropane (1.5 mL), and PPTS (1.0 mg) at 0 °C for 1 h then room temperature for 4 h afforded a mixture of triacetonides (0.7 mg). The combined mixtures (1.4 mg) of triacetonides were subjected to HPLC [Develosil ODS-HG-5 (ϕ 10×250 mm), solvent: MeOH/H_2O (93:7), flow rate: 2.5 mL/min] and four peaks were collected (t_R = 94, 98, 101 and 116 min). The first fraction (0.3 mg, t_R = 94 min), FAB-MS *m*/*z* 938 $(M + Na)^+$, was acetylated with a mixture of dried pyridine (0.5 mL) and acetic anhydride (0.5 mL) at room temperature for 5 h to afford **14** (0.5 mg); HRFAB-MS *m*/*z* 1106.6895, calcd for $C_{58}H_{101}NO_{17}Na$ (M + Na) 1106.6967. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.30 (1H, d, *J* = 8.9 Hz, NH), 5.14 (1H, m, H-2′), 5.12 (1H, m, H-3″), 4.99 (1H, dd, *J* = 3.8, 2.2 Hz, H-4), 4.89 (1H, dd, *J* = 9.3, 8.0 Hz, H-2″), 4.50 (1H, d, *J* = 7.8 mz, H-1″), 4.19 (1H, m, H-2), 3.96 (1H, m, H-1b), 3.93 (1H, m, H-6″b), 3.88 (1H, m, H-5), 3.82 (1H, dd, *J* = 7.5, 6.4 Hz, H-3), 3.72 (1H, m, H-6″a), 3.69 (1H, m, H-4″), 3.67 (1H, m, H-1a), 3.34 (1H, m, H-5″), 2.18, 2.12, 2.04 and 2.03 (12H, s, 4 methyls from –$COCH_3$), 1.66 (2H, m, H-7), 1.65 (1H, m, H-6b), 1.45 (1H, m, H-6a), 1.46, 1.40, 1.38, 1.36 (3H each, s each, 4 methyls from acetonide), 1.30 (6H, s, 2 methyls from acetonide), 1.04–2.23 (40H, m, methylenes), 1.88 (1H,

m, H-3′b), 1.72 (1H, m, H-3′a), and 0.86 (3H, t, $J = 6.8$ Hz).

Bioassay method

Completely the same bioassay method was reported in our previous paper.[1]

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