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Farinosones A−C, Neurotrophic Alkaloidal Metabolites from the Entomogenous Deuteromycete *Paecilomyces farinosus*

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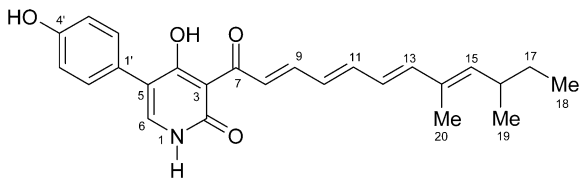
Two new yellow pigments, farinosones A (**1**) and B (**2**), were isolated from the mycelial extract of the entomogenous fungal strain *Paecilomyces farinosus* RCEF 0101, together with farinosone C (**3**), a new metabolite derived from an early step of pyridone alkaloid biosynthesis. The structures were determined by spectroscopic means, in particular by extensive NMR experiments. Compounds **1** and **3** induced neurite outgrowth in the PC-12 cell line at concentrations of 50 μM, while compound **2** was inactive. No cytotoxicity was observed for compounds **1**−**3** in PC-12 cells when tested at 50 μM concentration in the MTT assay.

Neurodegenerative disorders, in particular Alzheimer's disease (AD), increasingly affect our aging societies worldwide. Current drug therapies are symptomatic and merely retard the progress of the disease. Significant advances in the understanding of the molecular and cellular processes underlying AD[1,2] have unraveled a number of potential targets for intervention.[3,4] Among these, approaches centered around the functions of neurotrophic factors have been intensively explored over the past decade. Neurotrophic factors such as nerve growth factor (NGF) are endogenous glycoproteins that are essential for neuronal differentiation and survival.[5] Clinical interventions with neurotrophic factors, however, have been disappointing.[6,7] This has been, in part, due to the proteinaceous nature of the endogenous compounds, which pose formidable challenges for delivery into the brain. The search for orally bioavailable small organic molecules that could mimic neurotrophin action is thus an attractive alternative.[5,7,8]

On the basis of chemo-ecological considerations, we recently embarked on the investigation of entomopathogenic deuteromycetes as a source for new secondary metabolites with CNS-related bioactivities.[9,10] There is ample evidence for complex interactions between entomopathogenic fungi and their insect host,[11] including behavioral changes,[12] which may be caused by fungal metabolites. We isolated the pyridone alkaloid militarinone A from a mycelial extract of *Paecylomyces militaris* RCEF 0095 guided by the compound's distinct neurotrophic properties in PC-12 cells.[13] The compound activates signaling cascades such as MAP and SAP/cJun kinase pathways and potentiates the effect of NGF in PC-12 cells.[14] Given that structurally and biogenetically related militarinones B, C, and D showed cytotoxic rather than neurotrophic effects,[15] it seemed that subtle structural differences would account for the differing biological properties. In an attempt to gain a better understanding of structure−activity relationships, we embarked on a search for structurally related compounds from related entomogenous deuteromycetes. Here, we report on the isolation, structure elucidation, and bioactivity of two new pyridone alkaloids, farinosones A (**1**) and B (**2**), and the biogenetically related amide farinosone C (**3**) from *Paecylomyces farinosus* RCEF 0101.



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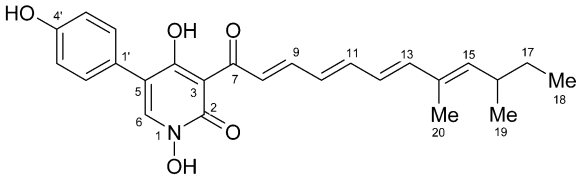

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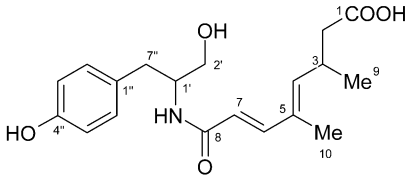

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Results and Discussion

The MeOH mycelial extract of *P. farinosus* RCEF 0101 was suspended in water and partitioned successively with petroleum ether and *n*-BuOH. A combination of normal-phase, reversed-phase, and gel chromatography of the *n*-BuOH and MeOH mycelial extracts afforded farinosone A (**1**) and C (**3**), and B (**2**), respectively. Farinosone A (**1**) was obtained as a bright yellow solid. A molecular formula of $C_{25}H_{27}NO_4$ was deduced from its HRESIMS spectrum. The UV data revealed absorption maxima at 415 and 249 nm. The NMR data (Table 1) were reminiscent of militarinone D from *P. militaris*.[15] ^{1}H and ^{13}C chemical shifts obtained from measurements in acetone-d_6 (Table 1), ^{1}H,^{1}H COSY, HMQC, and HMBC cross signals consistent with

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2**

	farinosone A (**1**)			farinosone B (**2**)	
		acetone-d_6	DMSO-d_6		DMSO-d_6
pos.	δ_C	δ_H (mult.; J_{HH} in Hz)	δ_H (mult.; J_{HH} in Hz)	δ_C	δ_H (mult.; J_{HH} in Hz)
1 (NH)			11.62 d (6.7)		
1-OH					11.66 s
2	162.8			157.7	
3	107.5			106.6	
4	179.3			173.4	
4-OH			17.64 s		17.09 s
5	114.8			111.1	
6	140.3	7.61 s	7.54 d (6.7)	140.7	8.14 s
7	194.8			193.3	
8	128.2	8.15 d (15.0)	7.99 d (15.3)	126.8	7.98 d (15.3)
9	145.7	7.65 dd (15.0, 12.0)	7.58 dd (15.3, 11.6)	145.5	7.62 dd (15.3, 11.8)
10	131.7	6.62 dd (12.0, 14.7)	6.61 dd (11.6, 14.6)	130.1	6.62 dd (11.8, 14.7)
11	144.6	6.92 dd (14.7, 11.0)	6.93 dd (14.6, 11.0)	144.5	6.96 dd (14.7, 10.8)
12	126.9	6.45 dd (11.0, 15.4)	6.41 dd (11.0, 15.3)	126.2	6.43 dd (10.8, 15.3)
13	144.0[a]	6.60 d (15.4)	6.57 d (15.3)	143.8	6.58 d (15.3)
14	134.2			132.8	
15	144.0[a]	5.51 d (9.9)	5.51 d (10.4)	143.4	5.52 d (10.0)
16	35.6	2.49 m	2.51 m[b]	33.9	2.44 m[b]
17a	31.3	1.31 m	1.25 m	29.5	1.25 m
17b		1.42 m	1.37 m		1.36 m
18	12.6	0.85 t (7.6)	0.81 t (7.6)	11.7	0.81 t (7.6)
19	21.1	0.98 d (6.6)	0.94 d (6.6)	20.1	0.94 d (6.5)
20	13.1	1.83 d (1.1)	1.77 s	12.5	1.77 s
1′	125.4			123.0	
2′,6′	131.3	7.35 d (8.6)	7.26 d (8.5)	130.5	7.29 d (8.5)
3′,5′	116.1	6.88 d (8.6)	6.77 d (8.5)	115.2	6.77 d (8.5)
4′	157.9			157.1	
4′-OH			9.45 s		9.49 s

[a] Assignments may be interchanged. [b] Overlapped signals.

the 4-hydroxyphenyl substituent attached to C-5 of the 4-hydroxy-2-pyridone heterocycle were found in the spectra. The ^{1}H spectrum measured in DMSO-d_6 displayed three exchangeable protons, which were attributed to 4′-OH (δ 9.45), 4-OH (δ 17.64), and an amide proton (δ 11.62), respectively. Thus, the difference between militarinone D and compound **1** was exclusively due to the side chain. A proton spin system of an olefin chain, consisting of two doublets of H-8 (δ 8.15) and H-13 (δ 6.60) and four doublets of doublets (H-9 to H-12), was readily detectable by ^{1}H,^{1}H COSY signals. The configuration of these three double bonds was established as *E,E,E* by large coupling constants of H-8 to H-13. HMBC correlations (Figure 1, Supporting Information) from H-8 and H-9 (δ 7.65) to C-7 (δ 194.8) indicated conjugation of the olefin chain with the bridge-carbonyl. Extension of the olefin chain with an another double bond was deduced from HMBC correlations of H-13, H-15 (δ 5.51), and the methyl signal of H_3C-20 (δ 1.83) with the quaternary olefinic C-14 (δ 134.2). The *E*-form of this additional double bond was indicated by a strong spatial interaction between H-12 (δ 6.45) and H_3C-20 which was observed in the 2D NOESY spectrum (Figure 1, Supporting Information). The doublet of H-15 was part of the proton spin system of the terminal side chain segment. Two methyl (δ 0.85, H-18; δ 0.98, H-19), a methylene (δ 1.31/1.42, H-17a/b), and a methine signal (δ 2.49, H-16) were readily attributed by ^{1}H,^{1}H COSY, HMBC, and HMQC data to an isobutyl unit attached to C-15. The stereochemistry at C-16 was not determined due to the small quantity of compound available. Hence, farinosone A (**1**) was identified as (8*E*,10*E*,12*E*,14*E*)-4-hydroxy-5-(4-hydroxyphenyl)-3-(8,10-dimethyldodeca-2,4,6,8-tetraenoyl)-1*H*-pyridin-2-one.

Farinosone B (**2**) was also obtained as a bright yellow solid. The molecular formula of $C_{25}H_{27}NO_5$ was established by the HRESIMS spectrum, which, compared to compound **1**, suggested an additional oxygen atom. The UV−vis spectrum of **2** was very similar to that of **1**. Some unexpected difficulties arose in the ^{1}H NMR measurement of **2**. In acetone-d_6, the proton NMR spectral signals appeared broad and unresolved. This problem, apparently due to the formation of intermolecular hydrogen bonds between analyte and the solvent, was overcome by measurement in DMSO-d_6. A comparison of the ^{1}H NMR data of **1** and **2** in DMSO-d_6 revealed very high resemblance of the two compounds. Two major differences were apparent, however. First, the doublet of H-6 (δ 7.54) in compound **1** appeared downfield at δ 8.14 and collapsed to a singlet in the spectrum of **2**. This resonance at δ 8.14 was unambiguously assigned to H-6 by HMBC correlations with C-2 (δ 157.7), C-4 (δ 173.4), and C-1′ (δ 123.0). Second, the signal of the amide proton (δ 11.62) of **1**, characterized by COSY correlation with H-6, was absent in the spectrum of **2**. Instead, a singlet emerged at similar frequency (δ 11.66) which did not show a COSY cross-peak with H-6 but exhibited HMBC correlations with C-2 and C-6 (δ 140.7). Thus, the signal was assignable to an N-OH proton. The *E*-forms of all olefinic double bonds of the side chain were deduced on the basis of their respective coupling constants and NOESY cross-peaks (Figure 2, Supporting Information). Again, the stereochemistry at C-16 was not determined due to scarcity of material. Farinosone B (**2**) was, therfore, identified as (8*E*,10*E*,12*E*,14*E*)-1,4-dihydroxy-5-(4-hydroxyphenyl)-3-(8,10-dimethyldodeca-2,4,6,8-tetraenoyl)-1*H*-pyridin-2-one. It should be noted that the N-hydroxy substituent in **2** led not only to solvent-dependent line shape of NMR spectra but also to a significantly altered chromatographic behavior compared to that of **1**. Strong peak tailing was observed for **2** on normal- and reversed-phase sorbents. Acetylation of **2** was successful, as evidenced by TLC. However, after purification of the peracetate over a Sephadex LH-20 column (eluent MeOH) the heavy tailing reappeared, suggesting that the acetyl moiety at N-OH was readily cleaved.

Farinosone C (**3**) was obtained as a white amorphous powder and gave a quasi-molecule ion peak $[M + K]^+$ at *m/z* 386.1434 by HRESIMS, corresponding to a molecular formula of $C_{19}H_{25}NO_5$. The HPLC-DAD spectrum of **3** was significantly different from **1** and **2** in that only a maximum absorption at 266 nm was observed in **3**. The structure of **3** was determined by a combination of 1D and extensive 2D NMR experiments (Figure 3, Supporting Information). The assignment started from a typical AA′BB′ spin system of a 1,4-disubstituted phenyl ring (δ 7.96, H-2″/6″, and 6.65, H-3″/5″, each integrating for two protons). ^{1}H, ^{13}C long-range heteronuclear bond correlations (HMBC) of a singlet of an exchangeable proton at δ 9.24 (OH-4″) with C-4″ (δ 155.5) and C-3″/5″ (δ 114.5) together with a cross-peak of H-2″/6″ with a methylene carbon (δ 35.3) suggested a 4-hydroxybenzyl unit. As established by a series of ^{1}H,^{1}H COSY signals, the benzylic protons (δ 2.52 and 2.72, H-7″a/b) were part of a proton spin system further consisting of a methine multiplet (δ 3.88, H-1′), another methylene group (δ 3.30, H-2′), and two exchangeable signals of a hydroxyl (δ 4.79, t) and a NH group (δ 7.91, d). The multiplicities as well as the ^{1}H,^{1}H COSY signals of the latter indicated linkage to the methylene (H_2-2′) and the methine protons (H-1′), respectively. HMBC connectivities, e.g., of H-7″, H-2′, and NH with C-1′ (δ 52.4), further supported the 2′-hydroxy-1′-amino-1′-benzyl substructure. Another HMBC correlation of the NH proton with a carbonyl (δ 165.1, C-8) provided evidence for an amide-linked moiety. Two doublets (δ 6.90, H-6, and 5.98, H-7) of the *trans*-olefinic bond were connected by ^{1}H,^{13}C long-range correlations to the amide carbonyl and a quaternary olefinic carbon assignable to C-5 (δ 131.2). Both olefinic carbons C-5 and C-4 (δ 142.7) were correlated by HMBC with a methyl singlet (δ_H 1.71), which, therefore, was attributed to H_3C-10. The olefinic doublet at δ 5.61, assignable to H-4, was linked through a ^{1}H,^{1}H COSY connection with the methine proton at δ 2.91 (H-3). Further ^{1}H,^{1}H COSY and HMBC cross signals were consistent with the remaining H_3C-9 (δ_H 0.97, δ_C 19.7) and the carboxymethylene group (δ 40.6/172.9) attached to C-3 (δ 29.4). 2D NOESY data, e.g., strong spatial interactions between H-3 ↔ H_3C-10 ↔ H-7 ↔ NH and H-4 ↔ H-6, confirmed the suggested structure, including *trans*-stereochemistry of the Δ_4 double bond in the side chain. The stereochemistries at C-3 and C-1′ could not yet be clarified due to scarcity of material. The structure of farinosone C was thus established as (4*E*,6*E*)-8-[2-hydroxy-1-(4-hydroxybenzyl)ethyl]amino-3,5-dimethyl-8-oxo-4,6-octadienoic acid.

Pyridone alkaloids of the 3-acyl-5-(4-hydroxyphenyl)-pyridin-2-one type presumably are formed by condensation of tyrosine or a tyrosine derivative with an activated polyketo acid.[16,17] Farinosones A (**1**) and B (**2**) are closely related to militarinones A and D,[15] bassianin, and tenellin,[18] as they differ only in the structure of the side chain. The pyridone moiety presumably forms by ring expansion of an acyltetramic acid, as can be assumed by the discovery of putative intermediates militarinones B and C co-occurring with militarinones A and D.[15] Farinosone C (**3**) is a metabolite that directly derives from the initial condensation step. The presence of a tyrosinol moiety in **3** supports our proposed biosynthetic pathway leading to pyridone alkaloids.[15] Due to the reduced carboxyl functionality, however, it is unable to undergo cyclization to form the pyridinone ring via a tetramic acid intermediate. The terminal carboxylic group is indicative of further biosynthetic modifications after the condensation to the amide.

Farinosones A (**1**) and C (**3**) induced a change in cell morphology and neurite outgrowth in PC-12 cells [19] when tested at a concentration of 20 and 50 μM. Treatment with 50 μM of **1** produced neurites with a length of one cell diameter in 60% of cells and longer neurites in 10% of cells analyzed. Farinosone C (**3**), at 50 μM, produced a characteristic flattened appearance in 60% and neurite outgrowth in 13% of cells (Figure 4 and Table, Supporting Information). Farinosone B (**2**) was not active. Given the structural similarity of **1** to militarinone A, the molecular mode of action is likely the same. The activity of the structurally much simpler **3** is intriguing. Whether the compound activates MAP and SAP/Jun kinase pathways similar to militarinone A [14] needs to be checked. PC-12 cell viability was assessed at concentrations up to 50 μM (Figure 5, Supporting Information). No cytotoxicity was observed for **1**−**3**. Farinosones A (**1**) and C (**3**), similar to NGF at 20 and 50 ng/mL, led to higher mitochondrial dehydrogenase activity, as determined by an increased conversion of MTT.

Experimental Section

General Experimental Procedures. Solvents and chemicals used were of analytical or HPLC grade. Optical rotations were determined on a Polartronic E polarimeter (Schmidt and Haensch, Berlin) equipped with a 5 cm microcell. UV spectra were recorded on a Beckman DU640 spectrophotometer. ^{1}H NMR, ^{1}H,^{1}H COSY, HMBC, HMQC, and NOESY spectra were recorded with a Bruker AVANCE DRX 500 spectrometer at 500.13 MHz using a 5 mm CryoProbe; ^{13}C chemical shifts were obtained from ^{1}H,^{13}C heterocorrelated 2D spectra. Samples were measured at 300 K in DMSO-d_6 or acetone-d_6 with TMS as internal standard. LC-ESIMS spectra were determined in the positive ion mode on a PE Biosystems API 165 single quadrupole instrument. HRESIMS (positive ion mode) spectra of compounds **1** and **2** were recorded on a Thermo Finnigan MAT 95 XL mass spectrometer; **3** was recorded on a Micromass Quattro II tandem quadrupole mass spectrometer (geometry quadrupole-hexapole-quadrupole). TLC were carried out on silica gel 60 F_{254} precoated Al sheets (Merck, Darmstadt) and RP-18 F_{254}s precoated Al sheets (Merck). Detection was at UV 254 and 365 nm, followed by spraying with Godin's reagent.[20] Chemical screening was carried out by analytical HPLC with a Hewlett-Packard 1100 HPLC series system consisting of a binary high-pressure mixing pump, autosampler, column oven, DAD, and a HP workstation. Separations were carried out on a LiChrospher RP-18e column (5 μm, 125 × 4 mm i.d.) with the following eluents: system 1: water−acetonitrile (45/55), 0.05% TFA; system 2: A = acetonitrile, B = 0.1% TFA in water, linear gradient from 20% A to 70% A in 15 min. The flow rate was 1 mL/min, and detection was at 254 and 400 nm. Preparative low-pressure reversed-phase LC was performed with a system consisting of a Knauer Wellchrom K-1001 pump, a 6-way valve with a 10 mL sample loop, a Knauer K-2500 UV detector, a Pharmacia RediFrac fraction collector, and a Lobar LiChrosorb RP-18 column (40−63 μm, 310 × 25 mm i.d.).

Fungal Strain and Preservation. The fungal strain was isolated from an unidentified Lepidopteran collected in Anhui Province, China, and identified by one of us (Z.L.) as *P. farinosus*. It has been catalogued as strain RCEF 0101 in the culture collection of the Entomogenous Research Center, Anhui Agricultural University. Long-term preservation of mycelial pieces was in sterile aqua dest. at 4 °C, in sterile 10% aqueous glycerol at −32 °C, in sterile 10% aqueous glycerol in liquid nitrogen, and on agar slants at 4 °C.

Culture Conditions. The mycelium preserved on agar slants with a medium containing 40 g/L sucrose, 10 g/L pepton, 10 g/L yeast extract, and 20 g/L agar was used for starter cultures, which were grown in Petri dishes for 10−15 days at 25 °C using the same medium. For fermentation, mycelial pieces were transferred into 56 Erlenmeyer flasks (500 mL each) with 150 mL of a liquid medium (20 g/L glucose, 20 g/L

Difco-neopeptone, 5 g/L glycine, 2 g/L K_2HPO_4, 1 g/L $MgPO_4 \times 7\ H_2O$, pH = 6.33). Still cultures were kept at 25 °C for 20 days.

Extraction and Isolation. Mycelium and broth were separated by filtration. The mycelium was freeze-dried to yield a solid residue, which was extracted with MeOH (3.6 L) for 24 h at room temperature under shaking. The extract was evaporated under reduced pressure and subsequently lyophilized to afford a crude extract (17.7 g). A portion (17.2 g) was suspended in 300 mL of H_2O and partitioned with petroleum ether (60−90 °C, 3 × 200 mL) and *n*-BuOH (3 × 100 mL), respectively. The combined *n*-BuOH extract (2.7 g) was submitted to column chromatography on silica gel (40−63 μm; 40 × 5.0 cm i.d.) using a step gradient $CHCl_3$−MeOH (100:0 → 0:100). Three fractions (1−3) were obtained. Fraction 1 (132 mg) containing bright yellow pigments was eluted with $CHCl_3$−MeOH (90:10). The fraction was dissolved in 97% aqueous MeOH and separated on a Merck LiChroprep RP-18 column (40−63 μm; 31 × 2.5 cm i.d) with MeOH−H_2O (97:3) as mobile phase. The flow rate was 4 mL/min, and detection at 254 nm and by visual observation. The pigment fraction collected was further purified by gel permeation chromatography on a Sephadex LH-20 column (50 × 1 cm i.d.) eluted with MeOH to afford **1** (3.0 mg). The petroleum ether extract (6.10 g) was dissolved in $CHCl_3$ and partitioned by 0.5% aqueous HCl. The intensely yellow interface portion (3.1 g) containing the pigments (due to their amphiphilic properties) was collected and combined with fraction 3 (1.0 g) eluted with $CHCl_3$−MeOH−H_2O (1:1:0.5) of the silica gel column above. This combined fraction was submitted to dry column chromatography on silica gel developed with $CHCl_3$−MeOH−H_2O (80:18:2). Five fractions (A−E) were collected. Fraction C (426 mg) was separated on a Sephadex LH-20 column (50 × 3.0 cm i.d.) with MeOH as eluent. Three fractions (a−c) were collected. Fraction b (196.8 mg) was further purified by LPLC on a LiChrosorb RP-18 column (40−63 μm, 30 × 2.5 cm i.d), using a step gradient MeOH/H_2O (70:30 → 75:25 → 80:20; 500 mL each) to afford **3** (19.8 mg). Workup of an aliquot of the MeOH mycelial extract (494 mg) by LPLC on RP-18 with MeOH−0.1% aqueous TFA (90:10) as mobile phase led to two pigmental fractions. Purification of the second fraction (10.0 mg) on Sephadex LH-20 (50 × 1.0 cm i.d.) with MeOH as eluent afforded **2** (2.1 mg).

Farinosone A (1): yellow amorphous powder; t_R 2.9 min (system 1); $[\alpha]_D^{24}$ −27.2° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 249 (4.24), 415 (4.34) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; ESIMS (pos. ion mode) *m/z* 406 $[M + H]^+$; HRESIMS (pos. ion mode) *m/z* 428.18458 $[M + Na]^+$ (calcd for $C_{25}H_{27}NaNO_4$, 428.18378).

Farinosone B (2): yellow amorphous powder; t_R 3.2 min (system 1); $[\alpha]_D^{24}$ −30.0° (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ϵ) 257 (3.64), 416 (3.88) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; ESIMS (pos. ion mode) *m/z* 422 $[M + H]^+$; HRESIMS (pos. ion mode) *m/z* 444.17987 $[M + Na]^+$ (calcd for $C_{25}H_{27}NaNO_5$, 444.17869).

Acetylation of 2. Acetic anhydride (500 μL) was added to a solution of **2** (1 mg) in anhydrous pyridine (200 μL). The mixture was stirred at room temperature overnight. Water (1 mL) was added to the solution on an ice bath; the reaction mixture was extracted by $CHCl_3$ (5 mL). After evaporation of the solvent, the residue was purified over Sephadex LH-20 (50 × 1 cm i.d.) with MeOH as mobile phase.

Farinosone C (3): white amorphous powder; t_R 4.5 min (system 2); $[\alpha]_D^{24}$ −20.0° (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ϵ) 266 (2.62) nm; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 2; ESIMS (pos. ion mode) *m/z* 348 $[M + H]^+$; HRESIMS (pos. ion mode) *m/z* 386.1434 $[M + K]^+$ (calcd for $C_{19}H_{25}KNO_5$, 386.1370).

Assay for Neuritogenic Activity. The assay was carried out with PC-12 cells[19] obtained from DSMZ (Braunschweig, Germany). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% horse serum, 0.5% fetal bovine serum, 1% penicillin, streptomycin (200 μg/mL), and 50 ng/mL NGF at 37 °C (5% CO_2). After 5 days, the medium was replaced by DMEM (10% HS, 5% FBS, 1% penicillin, streptomycin (200 μg/mL)). Cell aggregates were separated by passage through a 21-gauge needle, and aliquots of cell suspension were seeded into collagen-coated 24-well multiplates (1 mL, 1 × 10^5 cells/mL) and cultivated for 4 h. Afterward, MeOH solutions of **1** and **2**, and a DMSO solution in the case of **3**, were added to the wells. The final concentration of compounds was 20 and 50 μM, respectively, and the MeOH or DMSO concentration in the wells was 0.2%. NGF (50 ng/mL) served as positive control, and 0.2% MeOH or DMSO as negative controls, respectively. Cells treated with compounds, and positive and negative controls, were cultured for 48 h. Cells were assessed with a phase-contrast microscope. For each concentration, 300 cells in three randomly chosen areas of two wells were assessed for appearance and length of neurite outgrowths. The score used has been described earlier.[13]

Table 2. ^{1}H and ^{13}C NMR Data of Compound **3** in DMSO-d_6

pos.	δ_C	δ_H (mult.; J_{HH} in Hz)
1	172.9	
2a	40.6	2.18 dd (14.8, 7.5)
2b		2.25 dd (14.8, 6.7)
3	29.4	2.91 m
4	142.4	5.61 d (9.7)
5	131.2	
6	142.7	6.90 d (15.5)
7	120.3	5.98 d (15.5)
8	165.1	
9	19.7	0.97 d (6.9)
10	11.9	1.71 s
1′	52.4	3.88 m
2′	62.0	3.30[a]
2′-OH		4.79 t (5.6)
1″	129.3	
2″, 6″	129.7	7.96 d (8.5)
3″, 5″	114.5	6.65 d (8.5)
4″	155.5	
4″-OH		9.24 s
7″a	35.3	2.52[a]
7″b		2.72 dd (14.1, 5.6)
NH		7.91 d (8.4)

[a] Overlapped signals.

Cytotoxicity Assay. Cytotoxicity against PC-12 cells was determined with the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in 96-well microplates.[21] All assays were performed in quadruplicate. PC-12 cells were grown in DMEM (10% HS, 5% FBS, 1% penicillin, streptomycin (200 μg/mL)). Cell suspension (5 × 10^4 cells/mL) was seeded into each well of a 96-well flat-bottom microtiter plate (100 μL/well) and incubated at 37 °C for 1 h (5% CO_2). After incubation, MeOH solutions of **1** and **2** and DMSO solutions of **3** diluted in DMEM were added to give test concentrations of 50, 20, 10, 1, and 0.1 μM. Medium, 0.2% MeOH, 1% DMSO, and 10 and 50 ng/mL NGF were added into control wells. The cells were incubated for 2 days at 37 °C (5% CO_2). Then, 100 μL of medium was removed, and 11 μL of MTT solution (10 mg/mL) was added. After 4 h incubation, 100 μL of SDS solution (10% w/v) was added and the plate incubated overnight. The OD value was determined on a microplate reader at 544 nm. Data were expressed as mean ± SD (n = 4), with MeOH (0.2%) and DMSO (1%) as controls representing 100% cell viability.

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Supporting Information Available: Important HMBC and NOESY correlations of compounds **1**, **2**, and **3**, phase contrast

microscopic pictures of control, NGF, **1**, **2**, and **3** in PC-12 cells, scores of PC-12 assay, and cell viability determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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