(+)-*N*-Deoxymilitarinone A, a Neuritogenic Pyridone Alkaloid from the Insect Pathogenic Fungus *Paecilomyces farinosus*[#]

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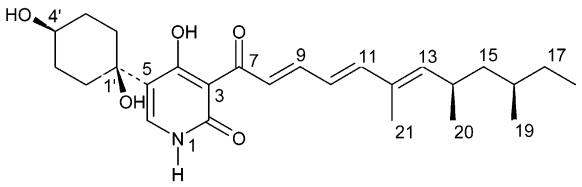
Received October 21, 2005

A new pyridone alkaloid, (+)-*N*-deoxymilitarinone A (**1**), was isolated from *Paecilomyces farinosus* RCEF 0097 along with the related metabolites, militarinone D and militarinone B. The sterol 22*E*,4*R*-ergosta-7,22-diene-3β,5α,6β,9α-tetraol was also identified. The structures were established by spectroscopic methods, in particular with the aid of extensive NMR experiments. Compound **1** induced neurite sprouting in PC 12 cells when tested at 33 and 100 μM concentrations. A cytotoxic effect was observed in human neurons (IMR-32) at a concentration of 100 μM.

Neurodegenerative disorders such as Alzheimer's disease are a major social and economic challenge for our aging society.[1] Various avenues toward disease modifying and preventative treatements are currently being explored.[2] In a search for natural products with potential neuroprotective activities, we have been screening for small molecules with neurotrophic properties.[3] Militarinone A, a pyridone alkaloid produced by the entomogenous deuteromycete *Paecilomyces militaris* RCEF 0095, showed distinct neuritogenic properties in PC12 cells.[4] Related alkaloids were subsequently isolated from the same strain and from *P. farinosus* RCEF 0101.[5,6] Recently, we demonstrated that the cellular differentiation induced by militarinone A involved activation of protein kinase B (Akt) and a delayed phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and of transcription factor cAMP responsive element binding protein (CREB). The nerve growth factor (NGF)-dependent activation of c-Jun amino terminal kinase (SAPK/JNK1) was potentiated.[7] To further explore the potential of pyridone alkaloids as neuritogenic molecules and for the purpose of structure−activity studies, we analyzed additional *Paecilomyces* extracts. An investigation of *P. farinosus* RCEF 0097 led to the isolation of compound **1** and three known substances.



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The MeOH mycelial extract of *P. farinosus* RCEF 0097 was suspended in water and centrifuged to remove water-soluble material, and the insoluble residue subjected to column chromatography on silica gel, RP-18, and gel chromatography on Sephadex LH-20.

(+)-*N*-Deoxymilitarinone A (**1**) was isolated as a yellow amorphous solid. Its molecular formula of $C_{26}H_{37}NO_5$ was deduced from the HRESIMS. The UV−vis spectrum exhibited absorption maxima at 391 and 253 nm (MeOH). ^{13}C NMR, APT, and HMQC experiments indicated the presence of 26 carbons, namely, four methyls, six methylenes, nine methines, and seven quaternary carbon signals. In the ^{1}H NMR spectrum obtained in MeOH-d_4, the most salient signals were for four methyl groups at δ 0.86 (d, H-19), 0.87 (t, H-18), 0.99 (d, H-20), and 1.85 (d, H-21), four conjugated olefinic protons at δ 6.48−7.94, a singlet at δ 7.67 (H-6), a doublet at δ 5.58 (H-13), and an oxygen-bearing methine at δ 3.64 (H-4′). Comparison of UV−vis and ^{1}H and ^{13}C NMR data with those of militarinone A[4] revealed a high degree of similarity. The mass difference of 16 amu indicated a difference in one oxygen atom in **1**. Confirmation was obtained by the ^{1}H NMR spectrum recorded in DMSO-d_6 instead of MeOH-d_4. The singlet at δ 7.67 changed to a doublet at δ 7.55, and an additional doublet of an exchangeable proton appeared at δ 11.4. The latter was assigned to a lactam NH group (NH-1). Compound **1** appeared to be an *N*-deoxy derivative of militarinone A. Its ^{1}H and ^{13}C NMR chemical shifts (Table 1) were assigned on the basis of 1D and 2D NMR spectra.

The *E*-geometry of the conjugated double bonds in the side chain of **1** was assigned on the basis of coupling constants (H-8 to H-11) and by 2D ROESY correlations between H-8 and H-10, H-10 and H-21, H-9 and H-11, and H-11 and H-13, respectively. ROESY interactions between diaxial protons H-4′ and H-2′a/H-6′a indicated that the 1,4-dihydroxyl cyclohexyl ring moiety was in a chair conformation with OH-4′ in an equatorial position. As for the substituents at C-1′, it was inferred that the bulky pyridone moiety was in the equatorial orientation as in militarinone A.[4] The relative configurations at C-14 and C-16 were deduced by comparison with experimental and calculated ^{13}C NMR shifts for *anti*- and *syn*-isomers of model compounds with comparable partial structures.[8−10] The shift difference between the C-20 and C-19 resonances was 2.1 ppm, and we, therefore, propose their *syn*-configuration. Interestingly, **1** and militarinone A differ in the sign of their optical rotations. A comparison of **1** with militarinone A, however, was not possible due to lack of material and low yields reported for N-oxidation of related pyridone alkaloids.[11] Taken together, the structure of (+)-*N*-deoxymilitarinone A (**1**) was established as (+)-*cis*-5-(1,4-dihydroxycyclohexyl)-4-dihydroxy-3-[(2*E*,4*E*,6E)-6,8,10-trimethyl-2,4,6-dodecatrienoyl]-2(1*H*)-pyridinone. Two known compounds were identified as militarinones D and B, respectively. Both compounds have been isolated recently from *P. militaris* RCEF 0095.[5] The forth compound was identified as 22*E*,4*R*-ergosta-7,-22-diene-3β,5α,6β,9-tetraol.[12]

Pyridone alkaloids form a small group of fungal metabolites that, apart from the militarinones[4,5] and farinosones,[6,13] includes bassianin

[#] Dedicated to Dr. Norman R. Farnsworth of the University of Illinois at Chicago for his pioneering work on bioactive natural products.

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10.1021/np050418g CCC: $33.50
Published on Web 02/02/2006

Table 1. ^{1}H and ^{13}C NMR Data and ROESY Correlations for Compound **1**[a]

pos.	δ_H	δ_C	ROESY
1	11.4 d (7.1)[b]		
2		164.7	
3		107.6	
4		178.8	
5		121.4	
6	7.67 s[a]; 7.55 d (7.1)[b]	139.2	
7		195.5	
8	7.94 d (15.3)	128.5	H-10
9	7.65 dd (15.3, 11.1)	147.5[c]	H-11
10	6.48 dd (15.1, 11.1)	126.6	H-8, H-21
11	6.78 d (15.1)	149.6	H-9, H-13
12		134.2	
13	5.58 d (9.8)	147.4[c]	H-11
14	2.70 m	32.1	H-19, H-21
15a	1.35 m	45.9	
15b	1.15 m		
16	1.26 m	33.8	
17a	1.32 m	31.3	
17b	1.17 m		
18	0.87 t (7.3)	11.7	
19	0.86 d (6.4)	19.5	
20	0.99 d (6.7)	21.6	
21	1.85 d (0.8)	12.7	H-10
1′		71.8	
2′a, 6′a	2.42 m	34.8	H-4′
2′b, 6′b	1.61 m		
3′, 5′	1.78 m	31.6	
4′	3.64 m	70.8	H-2′a

[a] Measured in MeOH-d_4. [b] Values observed in DMSO-d_6. [c] Signals may be reversed.

Table 2. Neuritogenic Activity of Compound **1**

		ratio of neurites (%)		
	concentration	A	B	C
1	100 μM	49	30	21
	33 μM	88	12	
NGF	10 ng/mL	30	16	54
MeOH	0.3%	99	1	

[a] A: round-shaped cells with no neurite outgrowth. [b] B: neurite length equal or less than cell body. [c] C: neurite length twice the cell diameter or longer.

and tenellin,[14] akanthomycin,[15] and cordypyridones A−D.[16] Instead of the more commonly occurring aromatic ring at C-5, compound **1** bears a cyclohexyl moiety, which has been previously found only in a few metabolites such as militarinone A,[4] fischerin, apiosporamide, oxysporidinone, and YM-215343.[17] The neuritogenic activity of **1** was tested in PC-12 cells at concentrations of 3.3, 10, 33, and 100 μM, respectively, according to an established protocol.[3,4] After incubation for 48 h, approximately 51% and 12% of cells exhibited neurite outgrowths at test concentrations of 100 and 33 μM, respectively (Table 2), while no changes in cell morphology were observed at 3.3 and 10 μM concentrations. The neuritogenic effect of **1** is thus weaker than that of militarinone A.[4] In addition, cell viability was assessed with human neurons (IMR-32 cells) at concentrations of 1, 10, and 100 μM (Figure S1, Supporting Information). Cell viability was not affected at concentrations of 1 and 10 μM, but was reduced to 22% at the highest concentration of 100 μM.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Polartronic E polarimeter (Schmidt and Haensch, Berlin) equipped with a 5 cm microcell. UV spectra were measured on a Beckman DU640 spectrophotometer. ^{1}H NMR, ^{1}H,^{1}H COSY, HMBC, HMQC, and ROESY 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 ^{13}C NMR and ^{1}H,^{13}C heterocorrelated 2D spectra. Samples were measured at 300 K in MeOH-d_4 or DMSO-d_6 with TMS as internal standard. LC-ESIMS spectra were determined in the positive- and negative-ion mode on a PE Biosystems API 165 single quadrupole instrument. The HRESIMS (negative-ion mode) spectrum was recorded on an API Qstar Pulsa mass spectrometer.

Fungal Strain and Preservation. The fungal strain was isolated from a Lepidopteran species collected in Yunnan Province, People's Republic of China, and was identified by Prof. Z. Li as *P. farinosus.* The voucher specimen (catalogued as strain RCEF 0097) was deposited at the Entomogenous Research Centre, Anhui Agricultural University.

Culture Conditions. The media and fermentation conditions have been described previously.[5,6]

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 (19.2 g). A portion (18.5 g) was suspended in 300 mL of water, ultrasonicated, and centrifuged at 10 °C and 8000 rpm for 20 min to remove water-soluble compounds. The insoluble residue was dried to yield a dark yellow solid (8.7 g), which was submitted onto a silica gel column (40−63 μm; 40 × 5 cm i.d.) with a stepwise gradient of $CHCl_3$−MeOH (99:1 to 50:50) to produce six fractions (1−6). Fraction 3 (365 mg) was separated on a Lobar LiChroprep RP-18 column (Merck, Darmstadt, 40−63 μm; 31 × 2.5 cm i.d.) with MeOH−0.1% aqueous TFA (90:10) as eluent. Three fractions (3.1−3.3) were collected. Fraction 3.2 (21.5 mg) was submitted to gel filtration on Sephadex LH-20 (50 × 1 cm, i.d.) with MeOH and to normal-phase chromatography on silica gel (40−63 μm; 10 × 1 cm, i.d.) with a gradient of $CHCl_3$−MeOH (15:1 to 5:1) to afford **1** (3.6 mg). Fraction 1 (1.5 g) contained a lipophilic yellow pigment, which was extracted by solid-phase extraction on a RP-18 cartridge (40−63 μm; 5 × 1.5 cm i.d.) eluted with 90−95% aqueous MeOH. The enriched pigment fraction obtained was further separated on a LiChroprep RP-18 column (40−63 μm; 31 × 2.5 cm i.d.) using a gradient of MeOH−0.1% aqueous TFA (70:30−95:5) for elution. Militarinone D (0.7 mg) was obtained after purification on Sephadex LH-20 (50 × 1 cm, i.d.) eluted with MeOH. Additional amounts (0.6 mg) of militarinone D were obtained from fraction 2 (802 mg) using the same isolation procedure as for fraction 1. Fraction 4 (470 mg) was separated on a LiChroprep RP-18 column (40−63 μm; 31 × 2.5 cm i.d.) eluted with MeOH−0.1% aqueous TFA (85:15). The pigment-containing portion (80 mg) was purified on Sephadex LH-20 (50 × 1 cm, i.d.) eluted with MeOH, to afford a pigment mixture (5.0 mg). Militarinone B (0.2 mg) was isolated by HPLC on a LiChrospher RP-18e column (5 μm, 125 × 4 mm i.d.) and acetonitrile−0.1% aqueous TFA (45:55). Fraction 3.1 (49 mg) was separated by semipreparative HPLC on a RP-18 LiChrosorb column (7 μm; 25 × 2.5 cm i.d.) with acetonitrile−0.1% aqueous TFA (85:15). A subfraction (6.0 mg) was submitted to preparative TLC on silica gel (40−63 μm; 1 mm layer thickness, 10 × 20 cm) and gel filtration on Sephadex LH-20 (50 × 1 cm, i.d.; MeOH) to afford 22*E*,4*R*-ergosta-7,22-diene-3β,5α,6β,9α-tetraol (1.0 mg).

(+)-*N*-Deoxymilitarinone A (1): yellow amorphous solid; $[\alpha]_D^{24}$ +33.3 (*c* 1.8, MeOH); UV (MeOH) λ_{max} (log ϵ) 253 (4.02), 391 (4.46) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; ESIMS (negative-ion mode) *m*/*z* 442 $[M - H]^-$; ESIMS (positive-ion mode) *m*/*z* 444 $[M + H]^+$, 426 $[M + H - H_2O]^+$; HRESIMS (negative-ion mode) *m*/*z* 442.2605 $[M - H]^-$ (calcd for $C_{26}H_{36}NO_5$, 442.2593).

The physicochemical data of militarinones D and B[4] and of 22*E*,4*R*-ergosta-7,22-diene-3β,5α,6β,9α-tetraol[11] were in accord with published values.

Assay for Neuritogenic Activity. The assay was carried out with PC-12 cells obtained from DSMZ (Braunschweig, Germany). Cell culture and pretreatment were as described previously.[3] An aliquot of cell suspension was plated into collagen-coated 24-well multiplates (1 mL, 1 × 10^5 cells/mL) and cultured for 4 h. Four concentrations (3.3, 10, 33, and 100 μM) of **1** in MeOH were added to the wells. The final MeOH concentration was 0.3%. NGF (10 ng/mL) and 0.3% MeOH were used as positive and negative controls, respectively. Each concentration was tested in duplicate. After addition of test compound and controls, cells were further incubated for 48 h. The wells were evaluated under a phase-contrast microscope. For each well, over 300 cells or cell aggregates in six random chosen areas were assessed and scored as described.[6]

Assay for Cytotoxic Activity. Cell viability in IMR-32 cells (DSMZ, Braunschweig, Germany) was evaluated with the MTT assay in 96-well microplates.[18] Cells were cultured in MEM/F12 media supplemented with 10% fetal bovine serum. The test concentrations were 1, 10, and 100 μM, respectively. Each concentration was tested six times as previously described.[6]

Acknowledgment. Y.X.C. is indebted to the Alexander von Humboldt Foundation for a research fellowship. The assistance of G.-U. Rüster, H. Graf, and S. Adler, in the research group for Pharmaceutical Biology, is acknowledged. We are grateful to J. Wange, Institute of Pharmacy, University of Jena, for measuring the optical rotation, and to H. L. Liang and Y. Wu, Kunming Institute of Botany, the Chinese Academy of Sciences, for the HRESIMS.

Supporting Information Available: Cell viability determination in human neurons (IMR-32). This material is available free of charge via the Internet at http://pubs.acs.org.

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NP050418G