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Militarinone A, a Neurotrophic Pyridone Alkaloid from *Paecilomyces militaris*[1]

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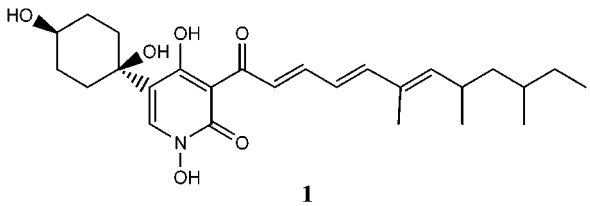
ABSTRACT



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A new pyridone alkaloid, militarinone A (1), was isolated by bioassay-guided fractionation from the mycelium of the entomogenous fungus *Paecilomyces militaris*. Its structure was established by extensive spectroscopic analysis. The compound features an unprecedented side chain and a 1,4-substituted cyclohexyl moiety not previously encountered in microbial metabolites. Militarinone A had a pronounced neurotrophic effect in PC-12 cells at 10 μM concentrations.

The imperfect fungus *Paecilomyces militaris* (L.:Fr.) Link[2] belongs to the taxonomically diverse group of entomogenous fungi. These microorganisms are intimately or even obligately associated with insects. In some cases, the host-insect relationship is known to involve bioactive secondary metabolites. Inspired by the medicinal uses of selected insect pathogenic fungi such as *Cordyceps sinensis* in the Traditional Chinese Medicine[3] and by the lack of systematic investigations on the secondary metabolite profiles of entomogenous fungi, we recently embarked on a screening of such deuteromycetes for CNS-related bioactivites.[4] A mycelial extract of *P. militaris* strain RCEF 0095 exhibited neuritogenic activity on PC-12 cells when tested at 100 μg/mL. Bioassay-guided fractionation revealed the presence of several active pigments and led to the isolation of the principal bioactive metabolite militarinone A (**1**),[5] a novel pyridone alkaloid. The communication reports on the isolation, structure elucidation, and preliminary biological evaluation of **1**.

Precultures of *P. militaris* RCEF 0095 were grown for 10−15 days at 20 °C on SDAY containing 4% sucrose, 1% peptone, 1% yeast extract, and 2% agar. Fermentation was carried out in still cultures at 25 °C for 20 days in 33 Erlenmeyer flasks (500 mL) containing 150 mL medium each (2% glucose, 2% neopeptone, 0.5% glycine, 0.2% K_2HPO_4, 0.1% $MgPO_4 \cdot 7H_2O$). After removal of broth, the mycelia were freeze-dried prior to extraction with MeOH. TLC on silica gel with $CHCl_3$/MeOH/H_2O (63:35:5) revealed a complex metabolite pattern, among these several bright yellow pigments. The MeOH extract (14.45 g) was treated with H_2O to eliminate water-soluble materials. The residue

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(1) Dedicated to Prof. Dr. W. Fleischhacker on the occasion of his 70th birthday.

(2) *P. militaris* strain RCEF 0095 was isolated from a Lepidopteran pupa collected in Anhui province, China, Collection No. YLP970727-02, and identified by Z. Li.

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(5) (−)*cis*-5-(1,4-Dihydroxycyclohexyl)-1,4-dihydroxy-3-[(2*E*,4*E*,6*E*)-6,8,10-trimethyl-2,4,6-dodecatrienoyl]-2(1*H*)-pyridinone.

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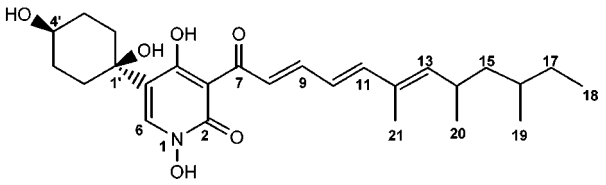
(1.95 g) was partitioned in the two-phase system hexane/EtOAc/MeOH/1% AcOH (1:1:1:1).

Bioassay-guided fractionation of the lower phase (0.33 g) with preparative low-pressure chromatography (Lobar Li-Chroprep RP-18, 310 × 25 mm, 40−63 μm, MeOH/1% AcOH, 82:18) and gel filtration on Sephadex LH20 (MeOH) led to the isolation of militarinone A (**1**, 9.6 mg, 0.068%).



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The molecular formula of compound **1** was established as $C_{26}H_{37}NO_6$ by HREIMS.[6] It showed a UV−vis spectrum with an absorption maximum at 388.5 nm (MeOH), which underwent a hypsochromic shift to 337 nm upon addition of dilute NaOH. The ^{1}H spectrum in MeOH-d_4 displayed signals for 33 nonexchangeable protons, whereas the ^{1}H NMR spectrum in DMSO-d_6 revealed an additional four exchangeable protons, appearing as a sharp singlet at 4.44 ppm and three broad signals at 4.95, 11.53, and 17.33 ppm. Comparison of ^{13}C NMR, DEPT, and HSQC spectra revealed the presence of 26 carbon resonances, which were attributable to four methyl groups and six methylene, nine methine, and seven quaternary carbons. A noticeable feature was the appearance of four methylene carbons as two chemically equivalent pairs.

The gross structure of **1** was established by combined analysis of its COSY, HSQC, and HMBC spectra, and the terminal portion of the side chain with its overlapping methylene and methine resonances in the ^{1}H NMR spectrum was corroborated with HSQC-TOCSY and selective TOCSY experiments. Four spin systems belonging to partial structures **a**−**c** (Figure 1) were assembled on the basis of the COSY spectrum. The geometry of the two *E*-configured double bonds in fragment **a** was deduced from the coupling constants (Table 1). Partial structure **d** was characterized by the presence of equivalent signal pairs for the methylene protons at C-2′ and C-6′ as well as at C-3′ and C-5′, respectively, suggesting a 1,4-substituted cyclohexane ring. The quintet-like appearance of H-4′ resulted from the vicinal coupling with four quasi-equivalent protons. The fragments **a** and **b** in the side chain of **1** were linked together with the aid of diagnostic HMBC correlations (Figure 1), in particular via three-bond connectivities between H-10 and C-12 as well as between H-13 and C-11.

(6) Compound **1**: yellow solid; R_f 0.35, silica gel 60 F_{254}, $CHCl_3$/MeOH/H_2O (80:20:2); $[\alpha]^{25}_D$ −14.6° (*c* 1.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 388.5 (4.65), 261 (3.98) nm; UV (MeOH + HCl) λ_{max} 393, 258 nm; UV (MeOH + NaOH) λ_{max} 337, 258 (sh) nm; NMR (see Table 1); ESIMS (positive ion mode) *m*/*z* 482 $[M + Na]^+$, 464 $[M + Na - H_2O]^+$, 460 $[M + H]^+$, 442 $[M + H - H_2O]^+$, 424 $[M + H - 2H_2O]^+$; ESIMS (negative ion mode) *m*/*z* 458 $[M - H]^-$; APCIMS (negative ion mode) *m*/*z* 458 $[M - H]^-$, 442 $[M - H - O]^-$, 440 $[M - H - H_2O]^-$, 424 $[M - H - O - H_2O]^-$; HREIMS *m*/*z* 441.2466 $[M - H_2O]^+$, (calcd for $C_{26}H_{35}NO_5$ 441.2515).

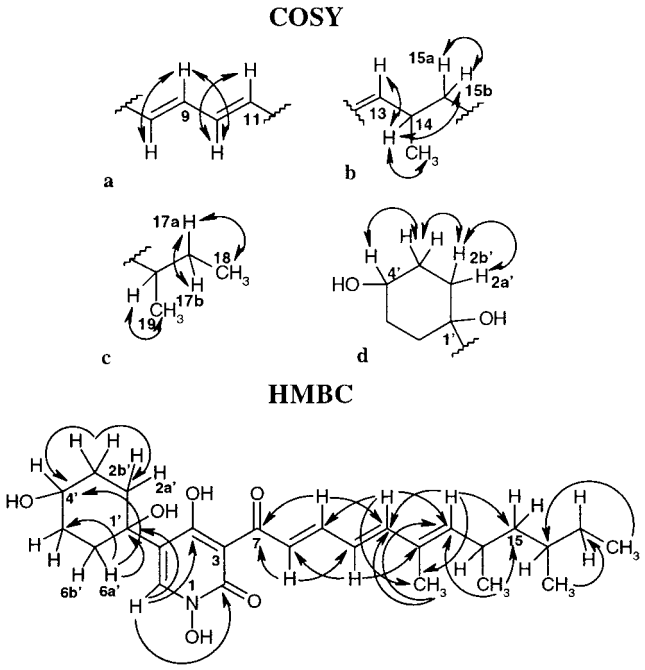

Figure 1. Selected COSY and HMBC correlations of **1**.

The linkage between substructures **b** and **c** was established with the aid of HSQC−TOCSY and selective TOCSY experiments. Irradiation at 5.56 (H-13) and 2.70 ppm (H-14) showed all neighboring proton signals from H-13 up to H-21, whereas irradiation of the terminal methyl signals at 0.86 ppm revealed H-15, H-16, and H-17 as closely positioned coupling partners. The *E*-configuration of the C-12/C-13 double bond was established on the basis of a NOESY spectrum (Figure 2), which also confirmed the previous structural assignments for the side chain.

The HO-4′ group in fragment **d** was corroborated by treatment of **1** in $CDCl_3$ with trichloroacetylisocyanate,

Table 1. NMR Data of **1**[a]

pos	δC	δH	mult	(*J* in Hz)	pos	δC	δH	mult	(*J* in Hz)
2	160.2				**15a**	45.9	1.16	m	
3	107.5				**15b**		1.35[b]	m	
4	174.7				**16**	33.7	1.30[b]	m	
5	119.6				**17a**	31.2	1.16	m	
6	139.2	8.02	s		**17b**		1.35[b]	m	
7	195.4				**18**	11.7	0.87[b]	t	(7.4)
8	128.4	7.96	d	(14.9)	**19**	19.5	0.85[b]	d	(6.9)
9	147.5	7.64	dd	(14.9, 11.2)	**20**	21.6	0.98	d	(6.6)
10	126.4	6.46	dd	(15.1, 11.2)	**21**	12.6	1.85	br	(0.8)
11	149.7	6.77	d	(15.1)	**1′**	71.7			
12	134.2				**2a′, 6a′**	34.7	1.60	m	
13	147.7	5.56	d	(9.8)	**2b′, 6b′**		2.42	m	
14	32	2.70	m		**3′, 5′**	31.5	1.76	m	
					4′	70.7	3.62	∼quintet	

[a] ^{1}H and ^{13}C NMR (400.13 and 100.63 MHz, respectively) spectra were recorded in CD_3OD; chemical shifts were referred to the residual solvent signal (δ_H = 3.3, δ_C = 49.0). [b] Partially overlapped by other resonances.

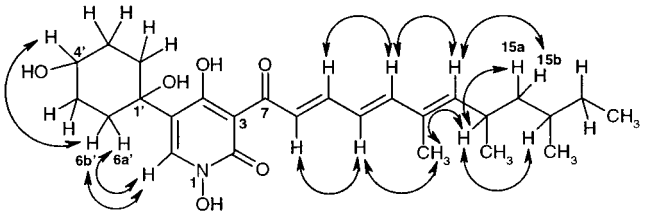

Figure 2. Relevant NOE contacts of 1.

resulting in a characteristic deshielding of the H-4′ signal by approximately 1 ppm. Because no other ^{1}H resonances were deshielded upon the derivatization, the remaining three exchangeable OH groups had to be attached to quaternary carbons or nitrogens. Diagnostic HMBC connectivities (Figure 1) in the cyclohexyl ring and selective TOCSY experiments corroborated its structure.

The remaining ^{1}H and ^{13}C NMR signals were attributable to an sp^2 methine, four quaternary carbons, and two hydroxy groups. In addition, a nitrogen could be inferred on the basis of the HREIMS. Taken together, these features were indicative of a highly substituted six-membered heterocyclic ring system, in particular an α-1,4-dihydroxy-pyridone moiety. The spectral data were indeed in good agreement with those of *N*-hydroxy-pyridone moieties of fungal metabolites such as tenellin, bassianin,[7] and fischerin.[8] According to the ^{13}C NMR chemical shifts (174.7, 107.5, and 195.4 ppm) and the low-field HO-4 resonance (17.33 ppm) in DMSO-d_6, the structural fragment C-4, C-3, C-7 constituted the enol form of a β-diketone system with strong electron delocalization in the chelate ring. HMBC correlations of H-6 with C-2 and C-4 further supported the pyridone moiety. The C-3 linkage of the pyridone unit with the side chain was hence through a carbonyl bridge (C-7; 195.4 ppm). The attachement position of the 1′,4′-dihydroxy-cyclohexyl ring at C-5 was established via the three-bond correlation between H-6 and C-1′ and supported by a strong NOESY interaction between the vinylic H-6 and the diastereotopic methylene pairs at C-2′/C-6′.

Militarinone (**1**) has four stereocenters occurring in two distant portions of the molecule (C-14 and C-16, C-1′ and C-4′). For the cyclohexyl moiety, the relative stereochemistry and preferred conformation could be assigned on the basis of diagnostic NOE contacts. Given the substitution pattern of the ring, it was reasonable to assume that the bulky pyridone moiety would prefer an equatorial orientation, leaving HO-1′ in the axial position. The NOE contact between H-4′ and H-2b′/H-6b′ indicated that the ring had a chair conformation with HO-4′ adopting an equatorial orientation. This finding was corroborated by calculation of the energy-minimized conformation for **1** and by comparison of ^{13}C signals with data of stereoisomeric 1,4-dihydroxy-cyclohexyl moieties reported as substructures of certain plant metabolites.[9−11]

Pyridone alkaloids are a very small group of microbial secondary metabolites. Their biosynthesis involves condensation of phenylalanine and polyketide precursors, resulting in tetramic acid intermediates that subsequently undergo rearrangement and ring expansion.[12,13] Militarinone A features an unprecedented side chain and a *cis*-(1,4-dihydroxy-cylohexyl) moiety not previously encountered in microbial metabolites. We assume that the latter is formed from the phenyl remainder of a putative precursor via oxidation and subsequent reduction steps.[14]

Various concentrations of **1** were evaluated for their potential to stimulate neuronal differentiation of PC-12 cells, a well-established model for the study of neurotrophins.[15] Whereas 25 ng/mL of the positive control nerve growth factor (NGF) induced pronounced neurite sprouting after 10 h, the effects of militarinone A reached a maximum after 24 h. At 33 μM and 10 μM concentrations, **1** induced pronounced spikes in approximately 80 and 70% of cell aggregates, respectively.[16] Compound **1** showed no significant cytotoxicity in PC-12 cells at concentrations up to 100 μM.[17]

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, COSY, HMBC, HMQC, and NOESY spectra of **1**, and phase contrast microscopic pictures of effects of control, NGF, and **1** in PC-12 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) The assay was performed according to an established protocol,[15] with some modifications. Cells at a density of 10^5 cells/ml in DMEM supplemented with 15% serum were transferred to collagen-coated 24-well plates and incubated with samples (37 °C, 5% CO_2). Cell differentiation and spike induction were visualized after 24 h by phase contrast microscopy and effects scored with "1", if appendices appeared as long as cell body, and "2" and "3" if they were two or three times longer. Nerve growth factor (NGF) at 25 ng/mL was used as a positive control. Approximately 80% of cell aggregates were evaluated as "3" when tested with **1** at 33 μM concentration. 70% were either "2" or "3" at 10 μM, and 30% were differentiated into a neuron-like shape with spikes as long as their cell body at 3.3 μM.
(17) Cytotoxicity was determined after 24 h of exposure using an LDH kit (Roche Diagnostics), relative to the vehicle as a negative control and Triton X 100-lysed cells as positive control.