Novel Tetramic Acids and Pyridone Alkaloids, Militarinones B, C, and D, from the Insect Pathogenic Fungus *Paecilomyces militaris*

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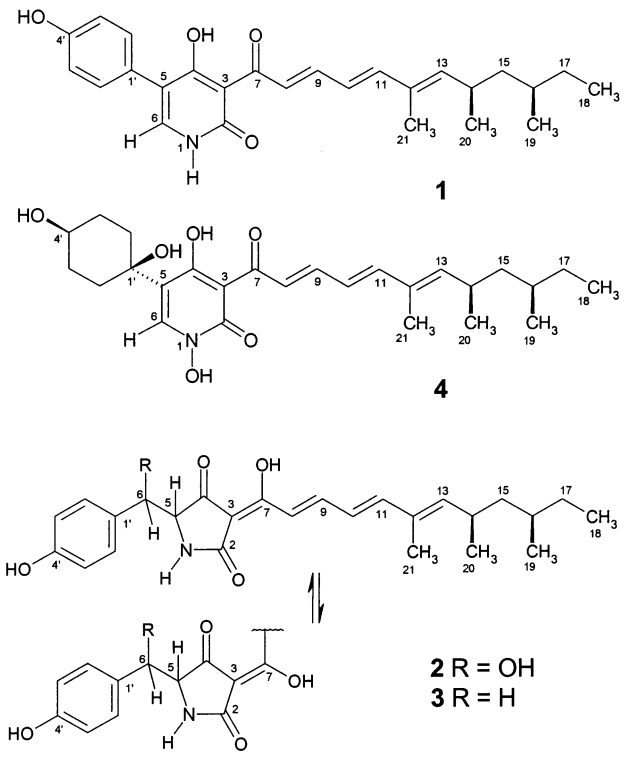
Received September 6, 2002

Three yellow pigments were isolated from a mycelial extract of the entomopathogenic fungus *Paecilomyces militaris*. With the aid of spectroscopic means, one compound was identified as a new pyridone alkaloid, militarinone D (**1**). The two other metabolites were characterized as two novel 3-acyl tetramic acids, militarinones B (**2**) and C (**3**). In contrast to the structurally related pyridone militarinone A (**4**), compounds **1**−**3** showed only negliable neuritogenic activity in PC-12 cells, whereas militarinone D (**1**) exhibited cytotoxicity. On the basis of a co-occurrence of 3-acyl tetramic acids and biogenetically related pyridone alkaloids in *P. militaris*, a revised biosynthetic pathway for pyridone alkaloids is proposed.

Insect pathogenic fungi are an ecologically highly specialized group of microorganisms. Some 700 entomopathogenic species are presently known, belonging to the families of Deuteromycetes, Ascomycetes, Zygomycetes, Oomycetes, Chrytridiomycetes, Trichomycetes, and Basidiomycetes.[1] They occur in all climate zones, with the exception of the arctic areas, and have specifically adapted to their particular environments. Species such as the Deuteromycetes *Metarhizium anisopliae* and *Beauveria bassiana* have been used since the late 19th century as biological insecticides.[2] *Cordyceps* (Ascomycetes, Clavicipitales), the teleomorphic stage of many entomopathogenic hypomycetes, is a widely occurring genus of tropical and subtropical regions and has found medicinal uses in East Asia.[3]

A limited number of insect pathogenic fungi have been investigated with respect to their secondary metabolite profiles. Pigments such as oosporein,[4] tenellin, and bassianin,[5,6] the cyclic depsipeptides destruxines,[7] beauvericin, bassianolides, and the beauveriolides,[8] and lactams such as pyridovericin[9] have been reported from some Deuteromycetes. Entomogenous fungi appear to interact with their host in complex ways. For example, distinct behavioral changes in the host have been described for some entomopathogens that seem to favor reproduction of the fungus.[10] The role of bioactive secondary metabolites in these fungus−insect interactions is presently known only for biocidal compounds such as the destruxins, efrapeptines, or hirsutellin A.[11,12] Nonetheless, the group of insect pathogenic fungi is of considerable promise as a source for bioactive natural products.

In a screening of entomopathogenic Deuteromycetes for various bioactivities of pharmaceutical interest, we recently identified several promising strains[13] and bioactive fungal metabolites.[14,15] Of particular interest was a mycelial extract of *Paecilomyces militaris* RCEF 0095 with pronounced neuritogenic activity in PC-12 cells. Activity-directed fractionation afforded a novel pyridone alkaloid, militarinone A (**4**), with distinct neurotrophic properties.[15] TLC analysis of the mycelial extract revealed the presence of several minor pigments.[13] We here report on the isolation and structure elucidation of three novel alkaloidal pigments, militarinones B−D (**1**−**3**). We also discuss their biogenetic relationship with the major pigment militarinone A (**4**) and propose a revised pathway for the biosynthesis of pyridone alkaloids.



Results and Discussion

The MeOH mycelial extract (25.9 g) of *Paecilomyces militaris* RCEF 0095 was treated with H_2O to eliminate H_2O-soluble substances. The MeOH-soluble portion of the residue (3.42 g) was submitted to gel chromatography on Sephadex LH 20. Nine pigment-containing fractions were collected. Purification of the minor fraction 9 by reversed-phase (RP) HPLC afforded a yellow pigment, militarinone D (**1**) (1.1 mg). The preceding fraction 7 contained two pigments. Militarinone B (**2**) (15.1 mg) and militarinone C (**3**) (7.9 mg) were isolated by RP low-pressure liquid chromatography (RP LPLC) and gel chromatography on Sephadex LH 20.

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10.1021/np020430y CCC: $25.00
Published on Web 02/21/2003

Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** in $CDCl_3$ and **2** and **3** in d_6-DMSO

	militarinone D (**1**)[a]				militarinone B (**2**)[b]				militarinone C (**3**)[b]			
pos	δC	δH		*J* in Hz	δC	δH		*J* in Hz	δC	δH		*J* in Hz
1-NH		9.6	br			8.93	br			8.75	br	
2	163.7				175.4				175.0			
3	106.7				100.4				~100			
4	178.3				192.8				194.7			
4-OH		17.95	s									
5	113.2				67.8	4.15	br		62.2	4.05	br	
6	137.5	7.33	s		72.5	4.86	d	2.3	35.8	2.82	m	
6-OH						5.59[c]	br					
7	195.9				172.2				172.5			
8	126.7	8.01	d	14.9	119.6	6.95	d	15.1	119.5	7.05	br	
9	147.1	7.68	dd	14.9, 11.3	144.5	7.38	dd	15.1, 11.2	144.5	7.42	dd	15.0, 11.4
10	125.5	6.45	dd	15.1, 11.3	125.1	6.47	dd	15.1, 11.2	125.1	6.48	dd	15.1, 11.4
11	148.8	6.69	d	15.1	148.4	6.81	d	15.1	148.4	6.82	d	15.1
12	132.7				132.7				132.7			
13	146.7	5.5	d	9.7	146.4	5.58[c]	d	9.3	146.3	5.58	d	9.7
14	30.9	2.63	m		30.3	2.64	m		30.3	2.63	m	
15a	44.7	1.28[c]	m		44.0	1.11	m		44.0	1.11	m	
15b		1.1[c]	m			1.25[c]	m			1.27[c]	m	
16	32.4	1.24[c]	m		31.9	1.18[c]	m		31.9	1.20[c]	m	
17a	30.0	1.28[c]	m		29.5	1.11	m		29.5	1.11	m	
17b		1.1[c]	m			1.25[c]	m			1.27[c]	m	
18	11.3	0.82 [c]	m		11.1	0.81[c]	m		11.1	0.81[c]	m	
19	19.1	0.8 [c]	m		18.8	0.79[c]	m		18.8	0.79[c]	m	
20	21.2	0.95	d	6.5	21.1	0.93	d	6.5	21.1	0.94	d	6.5
21	12.4	1.81	br		12.1	1.80	br		12.1	1.80	br	
1′	125.2				129.6				125.9			
2′, 6′	130.4	7.32	d	8.3	128.1	7.00	d	8.4	130.5	6.91	d	8.4
3′, 5′	115.4	6.87	d	8.3	114.2	6.59	d	8.4	114.8	6.60	d	8.4
4′	155.4				156.8				155.8			
4′-OH		4.86	br			9.19	s			9.15	s	

[a] ^{1}H and ^{13}C (500.13 MHz; 125.77 MHz) spectra were recorded in a microprobe tube in $CDCl_3$; chemical shifts were referred to the residual solvent signal. [b] ^{1}H and ^{13}C (300.13 MHz; 75.48 MHz) spectra were recorded in d_6-DMSO; chemical shifts were referred to the residual solvent signal. [c] Partially overlapped by other resonances.

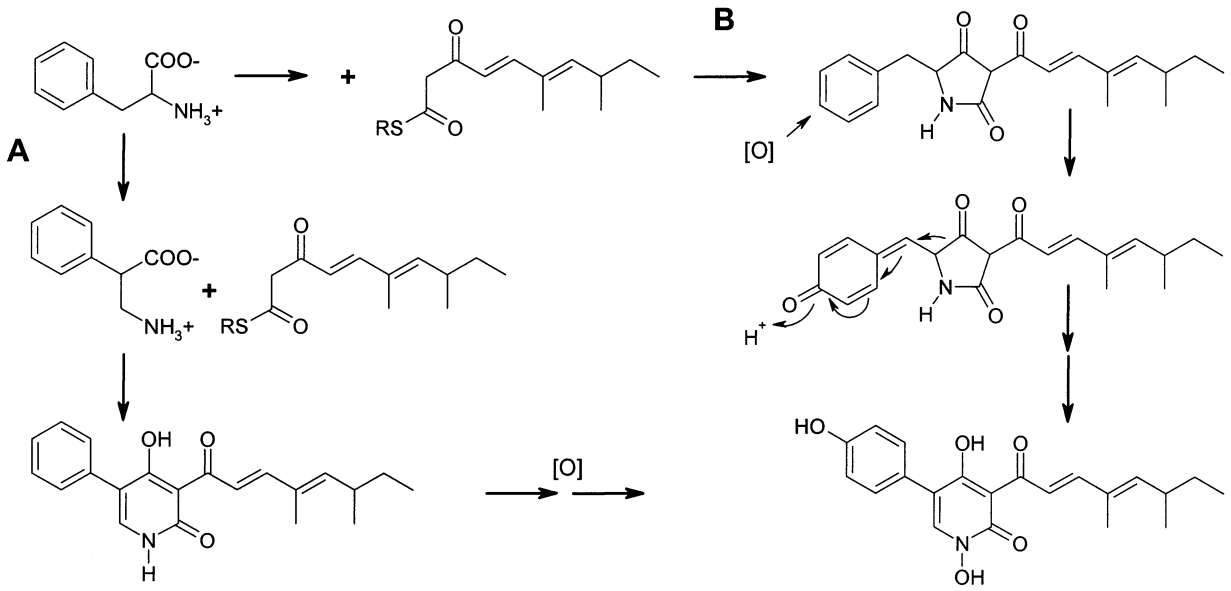

Figure 1. Biosynthetic pathways for pyridone alkaloids according to Cox and O'Hagan.[42]

Militarinone D (**1**) had a molecular formula of $C_{26}H_{31}NO_4$, as established by HRESIMS. The UV data of the compound were similar to those of militarinone A (**4**).[15] Addition of dilute NaOH induced a pronounced hypsochromic shift of the absorption maximum at 384 nm (MeOH) to 335 nm. The NMR spectra of **1** also revealed a number of structural similarities with militarinone A. The ^{1}H NMR spectrum of **1** (Table 1) showed resonances for 31 protons, including three exchangeable protons at δ 4.86, 9.60, and 17.95. The ^{13}C NMR, HMBC, and HMQC spectra confirmed the presence of 26 carbons (Table 1). The ^{13}C NMR signals were assigned to four methyl groups, two methylene, 12 methine, and eight quaternary carbons. The ^{13}C chemical shifts attributable to C-2 to C-21 were comparable to those of militarinone A.[15] The COSY spectrum revealed spin-systems assigned to five partial structures a−e, which were assembled with the aid of HMBC correlations (Figure 1, Supporting Information). Hence, the side chain of **1** was identical with that of **4**.

Partial structure d (Figure 1, Supporting Information) was a *p*-hydroxyphenyl ring. The ^{1}H NMR spectrum (Table 1) showed signals of a typical AA′BB′ spin system attributable to H-2′,6′ and H-3′,5′. The phenolic group at C-4′ was deduced from the HMBC correlations of H-2′,6′ (δ 7.32) to

C-4′ (δ 155.4) and corroborated by the upfield shifts of proton and carbon signals at the adjacent 3′- and 5′-positions.[6] The quaternary C-1′ carbon resonance (δ 125.2) was assigned via its distinct HMBC correlation with H-3′,5′ (δ 6.87). ^{13}C and ^{1}H signals of substructure d were in good agreement with data reported for other pyridone alkaloids.[9,16] The core ring (Figure 1, Supporting Information, substructure e) was identified as follows. Five remaing ^{13}C resonances, the presence of a nitrogen atom, and diagnostic three-bond correlations of an olefinic proton (H-6, δ 7.33) with an amide carbonyl C-2 (δ 163.7) and a quaternary carbon (C-4, δ 178.3) were consistent with a substituted pyridone ring.[9] The amide proton on N-1 (δ 9.60) showed a COSY cross-peak with H-6. The carbon shifts for C-4 (δ 178.3), C-3 (δ 106.7), and C-7 (δ 195.9) and a strongly deshielded HO-4 signal (δ 17.95) indicated the enol-form of a β-diketone system with strong electron delocalization in the chelate ring with the carbonyl oxygen at C-7.[15] Hence, the linkage of the side chain with C-3 of the pyridone moiety was through a carbonyl bridge. The carbonyl group at C-7 was further supported by HMBC correlations with H-8 and H-9 (Figure 1, Supporting Information). Militarinone D (**1**), therefore, was identified as 4-hydroxy-5-(4-hydroxyphenyl)-3-(6,8,10-trimethyldodeca-2,4,6-trienoyl)-1*H*-pyridin-2-one.

The molecular formulas of militarinones B (**2**) and C (**3**) were established by HRESIMS as $C_{26}H_{33}NO_5$ and $C_{26}H_{33}NO_4$, respectively. The UV−vis spectra of the two compounds suggested an identical chromophoric system, which markedly differed from that of **1** and **4**. In the spectra of **2** and **3**, the absorption maxima at 360 nm remained unchanged upon addition of dilute NaOH. Dilute HCl, however, induced a significant and reversible bathochromic shift of this band to 395−400 nm.

Some unexpected difficulties arose in the NMR measurements of **2**. In $CDCl_3$ and d_6-acetone, the ^{1}H spectral lines appeared at first broad and unresolved. Acceptable line widths were only recorded upon 10-fold dilution (1 mg/mL), strongly suggesting the occurrence of intermolecular association. Analysis of ^{13}C, DEPT, and HMQC spectra recorded in d_6-DMSO revealed the presence of four methyl, two methylene, 13 methine, and seven quaternary carbons. Two exchangeable protons were seen in the ^{1}H NMR spectrum measured in d_6-DMSO but not in $CDCl_3$. Given that the compound had a molecular formula of $C_{26}H_{33}NO_5$, one exchangeable proton was not detectable. ^{1}H and ^{13}C NMR shifts and COSY and HMBC correlations of the substructure C-10 to C-21 of **2** were comparable to those in militarinones A and D (**1**) (Table 1 and Figure 2, Supporting Information). The presence of a 4′-OH-substituted aromatic ring was corroborated by COSY and HMBC correlations (Figure 2, Supporting Information) and by comparison with data for **1**. Significant differences in chemical shifts and coupling pattern were found for the remaining signals, which had to belong to the core structure of the molecule. Notable features were the downfield positions of ^{1}H and ^{13}C signals at positions 8 and 9 and a HMBC correlation between H-9 (δ 7.38) and C-7 (δ 172.2). ^{1}H−^{1}H correlations confirmed the positions of H-8 and H-9 in the side chain. The signals of H-5 and H-6 were assigned with the aid of the HMQC experiment to the two remaining aliphatic methines. Diagnostic COSY correlations between H-5 and H-6, H-6 and H-2′/6′, and H-6 and the exchangeable HO-6 established the bridging position of C-6. The HO-6 was confirmed upon in situ derivatization of **2** with TCAI (trichloroacetylisocyanate) reagent. Comparison of ^{1}H NMR spectra (1 mg in $CDCl_3$) before and after addition of

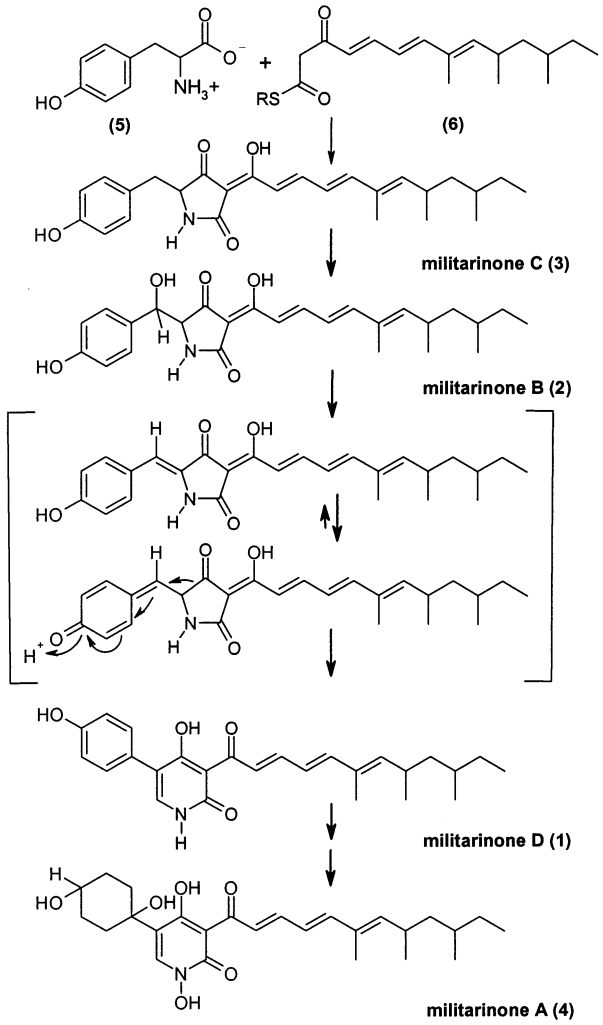

Figure 2. Proposed biogenetic relationship of militarinones.

TCAI revealed a characteristic downfield shift of 1.07 ppm upon derivatization. As for pyridone **1**, the quaternary carbons C-4 (δ 192.8), C-3 (δ 100.4), and C-7 (δ 172.2) in **2** were indicative of the enolic form of a β-diketone. Compared to **1**, the chemical shifts of C-4 and C-7 appeared inverted and, hence, suggested that the 7-OH tautomer was predominant. Diagnostic features in the HMBC spectrum were the ^{3}J coupling from H-9 to C-7 and a ^{2}J correlation between H-5 and C-4. The ^{13}C shifts attributable to the central heterocycle (C-2 to C-5) significantly differed from those of a pyridone ring but were in good accord with a tetramic acid moiety.[17,18] Militarinone B (**2**) was, therefore, identified as 5[hydroxy-(4-hydroxyphenyl)methyl]-3-(1-hydroxy-6,8,10-trimethyldodeca-2,4,6-trienylidene)pyrrolidine-2,4-dione.

The NMR spectra of militarinone C (**3**) indicated four methyl, three methylene, 12 methine, and seven quaternary carbons. Two exchangeable protons were detected in d_6-DMSO, compared to three in compound **2**. Major portions of the molecule were identical to militarinone B (**2**), such as the *p*-hydroxyphenyl moiety and the side chain linked to C-3. Diagnostic COSY and HMBC correlations observed for **3** are shown in Figure 3, Supporting Information. A notable feature for **3** was the presence of a methylene group at C-6 bearing two chemically equivalent protons (Table 1). They displayed diagnostic COSY cross-peaks with H-5 and H-2′/6′. HMBC correlations were observed between H-6 and C-2′/6′, H-6 and C-4, and H-2′/6′ and C-6. The NOESY spectrum of **3** showed several NOE

cross-peaks supporting the linkage of the pyrrolidine dione and the *p*-hydroxy benzyl rings as depicted (Figure 4, Supporting Information). Of particular importance were the NOESY contacts between H-2′/6′ and H-5 as well as H-6, between HO-4′ and H-3′/5′, and between H-2′/6′ and the amide proton. Additional NOE cross-peaks for the side chain corroborated the proposed structure, and **3** was thus identified as 5(4-hydroxybenzyl)-3-(1-hydroxy-6,8,10-trimethyldodeca-2,4,6-trienylidene)pyrrolidine-2,4-dione.

Militarinones B (**2**) and C (**3**) bear a conjugated side chain at C-7 and, hence, belong to the small group of polyenoyltetramic acids occurring as red or yellow pigments in *Penicillium* and *Streptomyces* strains and various slime molds.[19] ^{1}H and ^{13}C signals for the pyrrolidine dione moiety, for C-7, and the olefinic methines C-8 and C-9 were in good agreement with known compounds of this class, such as polycephalin C,[20] physarorubinic acid,[17] and fuligorubin A.[18,21] Furthermore, the pyrrolidin-2,4-dione ring is encountered in numerous other microbial natural products. Some of these reportedly have antibiotic, antiviral, and antiulcerative properties, whereas compounds such as α-cyclopiazonic acid, tenuazonic acid, equisetin, and erythroskyrine are mycotoxins.[19]

In solution, 3-acylated tetramic acids form rapidly interchanging internal tautomers arising through proton transfer along the intramolecular hydrogen bond, and external tautomers formed by rotation of the acyl side chain. The latter are detectable by NMR and may appear as distinct sets of signals.[19] NMR spectra of 3-acyl tetramic acid model compounds[22] and computational calculations[18,23,24] indicated a predominance of the *exo*-enol tautomers and a preference for the tautomeric form stabilized by an intramolecular hydrogen bond between OH-7 and the amide carbonyl at C-2. In the ^{1}H NMR of **2**, three pairs of signals with a 2:1 ratio were observed for H-1 (δ_1 8.93, δ_2 9.02), H-8 (δ_1 6.95, δ_2 6.7), and H-9 (δ_1 7.38, δ_2 7.25). Some ^{13}C signals of the pyrrolidin-2,4-dione ring and the side chain also appeared as signal pairs. However, conflicting NMR assignment in synthetic and natural 3-acyl tetramic acids[23,25,26] and a tautomer ratio that markedly differed from that of reported structures[18] precluded an empirical assignment of the NMR data to the *exo*- and *endo*-forms of **2**. The NMR spectra of **3** were also indicative of two tautomers, but the signals were not sufficiently resolved for a detailed analysis.

Militarinones A−D (**1**−**4**) have two to four stereocenters in two distant portions of the molecule. The relative configuration at C-14 and C-16 in **1**−**3** was deduced on the basis of experimental and calculated NMR shifts for *anti*- and *syn*-isomers of comparable partial structures.[27−29] In militarinones A−D (**1**−**4**), the shift differences between C-20 and C-19 signals were 2.1−2.3 ppm, and we thus propose their *syn*-configuration. The stereochemistries at C-5 and C-6 in the tetramic acids **2** and **3** could not yet be clarified due to scarcity of material.

Given that the mycelial extract of *P. militaris* RCEF 0095 and **4** had shown pronounced neuritogenic properties in PC-12 cells,[13,15] compounds **1**−**3** were also tested in this assay. At the highest concentration tested (100 μM), marginal spike induction was observed for **2** and **3**, whereas **1** was inactive. Cytotoxicity in PC-12 cells was assessed with a lactate dehydrogenase assay. At concentrations of 100 and 33 μM, compound **1** showed significant cytotoxicity (74.0% and 30.7%, respectively). Militarinone B (**2**) was weakly cytotoxic at 100 μM (16.8%), whereas militarinone C (**3**) exhibited no cytotoxcity.

Militarinones A (**4**)[15] and D (**1**) belong to the pyridone alkaloids, a comparably small class of natural products which, however, includes well-known metabolites such as bassianin, tenellin,[6] and ilicicolin H,[30] along with minor representatives, such as fischerin,[31] funiculosin,[32] apiosporamide,[33] leporin A,[34] pyridoxatin,[35] fusaricide,[36] sambutoxin,[37] harzianopyridone,[38] and oxysporidinone.[39] Despite numerous biosynthetic studies on tenellin and ilicicolin H,[40−45] the biosynthesis of pyridone alkaloids remains a matter of controversy. Our first report of co-occurrence of pyridone alkaloids and related tetramic acids provides new insights on the crucial steps in the formation of the pyridone ring.

It is generally accepted that the biosynthesis involves the aromatic amino acids phenylalanine (or tyrosine) and an activated polyketo acid,[40] but the condensation of these precursors and the subsequent steps leading to the pyridone moiety remained uncertain. Early studies on tenellin biosynthesis[40,45] led to the hypothesis that phenylalanine would undergo a transformation to 3-amino-2-phenylpropionic acid prior to its condensation with an activated polyketo acid to the pyridone alkaloid (Figure 1, pathway A). This pathway could not be confirmed in subsequent feeding experiments with [3-^{13}C]-labeled 3-amino-2-phenylpropionic acid.[42] An alternative pathway was proposed in which the condensation of phenylalanin and the polyketo acid would provide an acyltetramic acid (Figure 1, pathway B). Upon *p*-hydroxylation of the aromatic ring, a reactive quinone methide intermediate would lead to ring expansion to the corresponding pyridone. However, feeding experiments with the hypothetical tetramic acid precursor for tenellin produced no incorporation into the target molecule.[43] Recently, a radical-induced ring expansion of tetramic acids has been proposed, but no data have been published so far in support of this mechanism.[46]

The identification of militarinones A−D supports pyridone alkaloid biosynthesis via an acyl tetramic acid intermediate. The proposed biogenetic relationship between these compounds is depicted in Figure 2. Tyrosine (**5**) and the polyketo acid (**6**) would react to acyltetramic acid **3**, which in turn would be oxidized to the corresponding 6-hydroxy derivative **2**. The fact that tetramic acids **2** and **3** bear already a *p*-OH group at C-4′ is in disaccord with the hypothesis of a *p*-hydroxylation being the initial step for the ring expansion of the pyrrolidin-2,4-dione. Dehydration at C-5 and C-6 and tautomerization could lead to a quinone methide intermediate that would react to the corresponding pyridone **1**. This compound could undergo further reactions such as N-oxidation, and several oxidative and reductive steps in the aromatic ring would lead to the *cis*-(1,4-dihydroxycyclohexyl) moiety of **4**. Compound **4** is the major pigment in *P. militaris*, and this fact supports its putative role as the end product of the pyridone alkaloid pathway in *P. militaris*. The extracts of *P. militaris* RCEF 0095 contain additional minor pigments awaiting structural characterization. It can be anticipated that these metabolites will help to elucidate the biosynthesis of acyl tetramic acids and pyridone alkaloids in more detail.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured with a Polartronic E polarimeter (Schmidt and Haensch, Berlin) equipped with a microcell (3 × 50 mm i.d.). UV spectra were measured on a Beckman DU640 spectrophotometer. NMR spectra of **2** and **3** were recorded in d_6-DMSO (300 K) with Bruker DPX 300 (300.13 MHz for ^{1}H; 75.48 MHz for ^{13}C) (1D spectra) and with Bruker

DRX-500 (500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C) (2D spectra) spectrometers equipped with 5 mm probeheads. NMR spectra of **1** were measured in $CDCl_3$ (300 K) in 2.5 mm microprobe tubes on a Bruker DRX-500 (500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C). ESI LC-MS spectra were recorded in positive and negative ion modes on a PE Biosystems API 165 single quadrupole instrument. HRESIMS spectra were recorded on a Thermo Finnigan MAT 95 XL mass spectrometer. TLC was carried out on precoated silica gel 60 F_{254} Al sheets (Merck, Darmstadt) with $CHCl_3$−MeOH−H_2O (80:18:2) and on precoated RP-18 F_{254s} Al sheets (Merck, Darmstadt) with MeOH−0.02 M CH_3COONH_4 (pH 5) (9:1). Detection was at UV 254 and 365 nm, followed by spraying with Godin's reagent.[47] The equipment for gel chromatography consisted of a Pharmacia LKB Pump P-1, a Knauer K-2501 variable wavelength detector, a Pharmacia LKB REC 2 chart recorder, and a Pharmacia LKB SuperFrac fraction collector. Pyridone **1** was isolated by analytical HPLC with a Hewlett-Packard 1100 HPLC series system consisting of a binary pump, autosampler, column oven, DAD, and a HP workstation. Preparative LPLC was performed with a Knauer Wellchrom HPLC PUMP K-1001, a 6-way valve with a 2 mL sample loop, a Knauer K-2500 UV detector, and a Pharmacia RediFrac fraction collector. Solvents and chemicals were of analytical or HPLC grade.

Fungal Strain and Preservation. The fungus was isolated from a Lepidopteran pupa collected in Anhui province, China, and identified by one of us (Z.L.) as a *P. militaris*. It has been catalogued as strain RCEF 0095 in the culture collection of the Entomogenous Research Centre, Anhui Agricultural University. Long-term preservation of mycelial pieces is 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. Mycelium preserved on agar slants with a medium containing 40 g/L sucrose, 10 g/L peptone, 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 20 °C with the same medium. For fermentation, mycelial pieces were transferred to 56 Erlenmeyer flasks (500 mL) containing 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 fitration. The mycelium was freeze-dried to afford a solid residue (44.2 g), which was extracted 2× with MeOH (2 × 900 mL) for 24 h at room temperature under shaking. Given the light sensitivity of the *Paecilomyces* pigments, all extraction and chromatographic steps were carried out under light protection. The combined extracts were filtered, and the solvent was removed in vacuo. The freeze-dried crude extract (25.9 g) was treated with H_2O (500 mL) to remove H_2O-soluble substances. After ultrasonication and centrifugation at 10 °C and 800 U/min, the aqueous supernatant was decanted. The insoluble and viscous dark yellow gum was freeze-dried (5.13 g). The residue was extracted with MeOH (2 × 10 mL) and centrifugated as described. The combined MeOH-soluble fraction (3.42 g) was divided into four portions, which were each submitted to gel permeation chromatography on a Sephadex LH-20 column (25−100 μm; 90 × 5.5 cm i.d.) with MeOH as the mobile phase. Fractions were collected every 10 min (flow 1.2 mL/min). On the basis of UV detection (400 nm) and TLC pattern, nine fractions (1−9) were collected. Fraction 9 (2472−2736 mL, 5.9 mg) was dissolved in MeOH and submitted to HPLC on a LiChrospher 100 RP-18 column (5 μm; 125 × 4 mm i.d.; Merck) with MeOH−H_2O (86:14) as eluent. The flow rate was 1 mL/min, and detection was at 400 nm. Multiple injections (120 μg in 20 μL) afforded **1** (1.1 mg). Fraction 7 (1740−2016 mL, 162 mg) was a mixture of two yellow pigments. An aliquot (120 mg) was dissolved in CH_3CN and purified by solid phase extraction on a LiChroprep RP-18 cartridge (25−40 μm) and successive elution with CH_3CN and CH_3CN−0.1% TFA (75:25). The effluent was dried (66 mg), redissolved in CH_3CN−0.1% TFA (75:25), and separated by RP LPLC on a Merck LiChroprep RP-18 column (40−63 μm, 310 × 25 mm i.d.) with CH_3CN−0.1% TFA (75:25) as mobile phase. The flow rate was 6 mL/min, and detection at 400 and 254 nm. Five fractions (a−e) were collected. Fractions b (t_R 57 min, 330−354 mL, 22.9 mg) and e (t_R 94 min, 552−582 mL, 13.8 mg) were passed over a Sephadex LH-20 column (MeOH) leading to compounds **2** (15.1 mg) and **3** (7.9 mg).

Militarinone D (1): yellow amorphous powder; mp 132−138 °C; R_f 0.70, silica gel 60 F_{254}, $CHCl_3$−MeOH−H_2O (80:20:2); R_f 0.15, RP-18 F_{254s}, MeOH−0.02 M CH_3COONH_4 (pH 5) (9:1); $[\alpha]^{25}_D$ −74.4° (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ϵ) 253 (4.43), 384 (4.55) nm; UV (MeOH + HCl, pH 2) 253 (4.44), 388 (4.57) nm; UV (MeOH + NaOH, pH 12) 256 (4.42) 335 (4.46) nm; ^{1}H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; ESI MS (pos. ion mode) *m/z* 444.2 $[M + Na]^+$, 422.3 $[M + H]^+$; ESI MS (neg. ion mode) *m/z* 420.3 $[M - H]^-$; HRESIMS *m/z* 444.21526 $[M + Na]^+$ (calcd for $C_{26}H_{31}NaNO_4$ 444.21508).

Militarinone B (2): yellow amorphous powder; mp 135−139 °C; R_f 0.45, silica gel 60 F_{254}, $CHCl_3$−MeOH−H_2O (80:20:2); R_f 0.26, RP-18 F_{254s}, MeOH−0.02 M CH_3COONH_4 (pH 5) (9:1); $[\alpha]^{25}_D$ −553.2° (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ϵ) 259 (4.14), 358 (4.53) nm; UV (MeOH + HCl, pH 2) 261.5 (4.04), 397.5 (4.71) nm; UV (MeOH + NaOH, pH 12) 243 (4.43) 351 (4.61) nm; ^{1}H NMR (d_6-DMSO, 300 MHz), see Table 1; ^{13}C NMR (d_6-DMSO, 75 MHz), see Table 1; ESI MS (pos. ion mode) *m/z* 462.2 $[M + Na]^+$, 440.4 $[M + H]^+$, 422.3 $[M + H - H_2O]^+$, ESI MS (neg. ion mode) *m/z* 438.0 $[M - H]^-$, 422.6 $[M - H - O]^-$; HRESIMS *m/z* 462.22080 $[M + Na]^+$ (calcd for $C_{26}H_{33}NaNO_5$ 462.22128).

Militarinone C (3): yellow amorphous powder; mp 86−92 °C; R_f 0.60, silica gel 60 F_{254}, $CHCl_3$−MeOH−H_2O (80:20:2), R_f 0.19, RP-18 F_{254s}, MeOH−0.02 M CH_3COONH_4 (pH 5) (9:1); $[\alpha]^{25}_D$ −430.2° (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ϵ) 258.5 (4.14), 359 (4.57) nm; UV (MeOH + HCl, pH 2) 262 (3.98), 400.5 (4.75) nm; UV (MeOH + NaOH, pH 12) 239 (4.36) 350.5 (4.63) nm; ^{1}H NMR (d_6-DMSO, 300 MHz), see Table 1; ^{13}C NMR (d_6-DMSO, 75 MHz), see Table 1; ESI MS (pos. ion mode) *m/z* 446.2 $[M + Na]^+$, 424.2 $[M + H]^+$, ESI MS (neg. ion mode) *m/z* 422.5 $[M - H]^-$; HRESIMS *m/z* 446.22830 $[M + Na]^+$ (calcd for $C_{26}H_{33}NaNO_4$ 446.22854).

Assay for Neuritogenic Activity. PC-12 cells, obtained from DSMZ (Braunschweig, Germany), were grown in DMEM (1% HS, 200 U/mL penicillin, 200 μg/mL streptomycin) and 50 ng/mL NGF at 37 °C (5% CO_2). After 5 days, the medium was replaced by DMEM (10% HS, 5% FKS, penicillin 200 U/mL, and streptomycin 200 μg/mL). Cell aggregates were separated by passage through a 21-gauge needle, and aliquots of cell suspension (1 mL, 10^5 cells/mL) were transferred to each well of collagen-coated 24-well plates. Cultures were preincubated for 4 h, then methanolic solutions of samples were added. Final concentrations of test compounds in the assay were 100, 33, 10, and 3.3 μM, and the MeOH concentration was 1%. Wells containing NGF (50 and 25 ng/mL in PBS, PBS 1%), MeOH (1%), and PBS (1%) were used as positive and negative controls, respectively. All assays were carried out in duplicate. After 16 h of incubation, cells were fixed with 0.5% glutardialdehyde and stained with hematoxylin/eosin for visualization of cell bodies and spikes. Cells in three different areas of each well were analyzed (200−300 cells in total) and spikes scored as follows: cells with no neurite outgrowth were considered "−", cells with spike length equal to cell diameter scored 1, cells with spike length 2−3 × cell diameter scored 2, and cells with spikes ≥3 cell diameter or extended networks scored 3.

Assay for Cytotoxicity. Cytotoxicity of test extracts was assessed by the amount of lactate dehydrogenase (LDH) released by the cells. Preparation of PC-12 cells and concentrations for extracts and NGF were as in the assay for neuritogenic activity. PC-12 cells were exposed to test extracts for 24 h at 37 °C (5% CO_2). Untreated cells were analyzed for determination of spontaneous LDH release (negative control, 0% value), whereas Triton X-100 (1%)-lyzed cells were taken

as a measure for maximum LDH release (100% value). The LDH test was carried out with a commercial LDH kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Spectrophotometric measurement was carried out at 492 nm using a microplate reader. Cytotoxicity of extracts (%) was calculated relative to control values. Assays were carried out in quadruplicate.

Acknowledgment. We are indebted to Dr. F. Gollmick, Hans-Knöll-Institute for Natural Product Research, Jena, Germany, and to Dr. W. Günther, Institute of Organic and Macromolecular Chemistry, Friedrich-Schiller-University, Jena, for recording NMR spectra. Thanks are due to Mrs. S. Schönau, Institute of Inorganic and Analytical Chemistry, Friedrich-Schiller-University, Jena, and Mrs. A. Perner, Hans-Knöll-Institute for Natural Product Research, Jena, for measurement of HRESIMS. The skillful assistance of Dr. B. Schubert for the fermentation is kindly acknowledged.

Supporting Information Available: Selective COSY, HMBC, and NOESY correlations of compounds **1**, **2**, and **3** (Figures 1−4) are available free of charge via the Internet at http://pubs.acs.org.

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NP020430Y