Notes

New Variotin Analogues from *Aspergillus viridi-nutans*[||]

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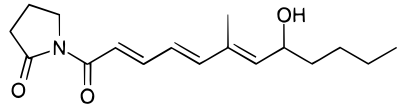
Besides the antifungal agents variotin (**1**), wasabidienone B_0 (**4**), and phomaligin A (**5**), two new but inactive metabolites, viriditin (**2**) and *O*-methylviriditin (**3**), were isolated from extracts of the culture filtrate of liquid cultures of a strain of *Aspergillus viridi-nutans*. In addition, wasabidienone B_1 (**6**) was isolated and characterized by spectroscopy.

Soil-inhabiting microorganisms are rich sources of potentially useful secondary metabolites exhibiting remarkable diversity in both chemical structures and biological activities.[1,2] In a screening of extracts of soil-borne fungi for antimicrobial activities, the culture filtrate extracts of liquid cultures of a strain of *Aspergillus viridi-nutans* was found to contain antifungal metabolites. Bioassay-guided fractionation of the extracts yielded variotin (**1**), an antibiotic previously isolated from *Paecilomyces variotii*[3] but reported here for the first time from an *Aspergillus* species, together with two new compounds assigned the trivial names viriditin (**2**) and *O*-methylviriditin (**3**). In addition, wasabidienone B_0 (**4**), a metabolite of *Phoma wasabiae*,[4] and phomaligin A (**5**), recently reported from mutants of the blackleg fungus *P. lingam*,[5] were obtained in smaller amounts, together with the structurally related wasabidienone B_1 (**6**). Although the structure of **6** has been reported,[6,7] this is the first report of its spectroscopic data. This paper reports on the production and isolation of the six compounds, the structure determination by spectroscopic techniques of the new compounds, and some biological activities.



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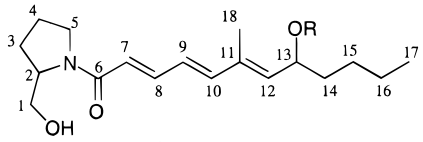

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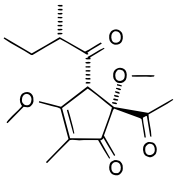
3 R=CH_3



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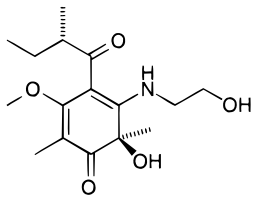

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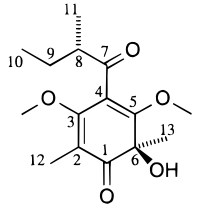

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The isolation of the metabolites is described in the Experimental Section. The structures of viriditin (**2**) and *O*-methylviriditin (**3**) were determined by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. The molecular ions in the EIMS appear at *m*/*z* 307 and 321, suggesting the difference between the two compounds is that **3** contains an additional methylene group. High-resolution mass spectrometry experiments were consistent for the molecular composition of **2** and **3** as $C_{18}H_{29}NO_3$ and $C_{19}H_{31}NO_3$, respectively. ^{1}H and ^{13}C NMR spectroscopy suggest the presence of three carbon−carbon double bonds and one carbonyl group in both **2** and **3**, thus the fifth degree of unsaturation is one ring in each compound. The NMR data (See Tables 1 and 2) of the two compounds are quite similar, except for the presence of signals for a methoxy group in the spectra of **3**, indicating that **3** is an *O*-methylated derivative of **2**. Both compounds were subjected to extensive 2D NMR spectroscopy, and correlations observed in COSY, NOESY, HMQC, and HMBC spectra (for pertinent HMBC correlations in **3**, see Figure 1) established the structures unambiguously. HMBC correlations between 18-H_3 and C-10, between 18-H_3 and C-11, and between 18-H_3 and C-12, together with COSY correlations established the C-7/C-17 chain, and HMBC correlations between 7-H and C-6 and between 8-H and C-6 showed that this chain is attached to the carbonyl group. The presence of a 2-hydroxymethylpyrrolidine moiety could also be demonstrated by COSY and HMBC correlations, and the place-

[||] Dedicated to Prof. Dr. Axel Zeeck on the occasion of his 60th birthday.

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Table 1. ^{1}H (500 MHz) NMR Data for Viriditin (**2**), 13-*O*-Methylviriditin (**3**), and Wasabidienone B_1 (**6**) in $CDCl_3$[a]

	δ_H, mult. (*J*)		
H	**2**	**3**	**6**
1a	3.67, dd (2.5, 11.1)	3.69, dd (2.5, 11.1)	
1b	3.62, m	3.63, m	
2	4.30, m	4.32, m	
3a	2.04, m	2.05, m	
3b	1.63, m	1.62, m	
4a	1.97, m	1.97, m	
4b	1.89, m	1.90, m	
5	3.60, m	3.60, m	
7	6.21, d (14.8)	6.22, d (14.7)	
8	7.37, dd (11.2, 14.6)	7.39, dd (11.1, 14.7)	2.64, m
9a	6.36, dd (11.2, 15.3)	6.36, dd (11.2, 15.2)	1.78, m
9b			1.36, m
10	6.55, d (15.3)	6.59, d (15.3)	0.92, t (7.5)
11			1.10, d (7.0)
12	5.63, d (8.6)	5.53, d (8.8)	1.92, s
13	4.48, dt (8.5, 6.6)	4.00, dt (8.9, 6.5)	1.53, s
14a	1.63, m	1.65, m	
14b	1.47, m	1.44, m	
15	1.32, m	1.30, m	
16	1.32, m	1.30, m	
17	0.89, t (6.9)	0.88, t (6.8)	
18	1.83, s	1.84, s	
3-OCH_3			3.81, s
5-OCH_3			3.97, s
13-OCH_3		3.24, s	

[a] The chemical shifts are given in parts per million relative to the solvent signal (7.26 ppm), and the coupling constants (*J*) are in hertz.

ment of the amide bond between the carbonyl group and the pyrrolidine nitrogen is the only remaining possibility. This was also supported by a weak HMBC correlation between 5-H_2 and C-6, as well by the strong M-100 fragment in the EIMS of **2** and **3**. The carbon−carbon double bonds were all shown to be *E* by the magnitude of the ^{1}H−^{1}H couplings and by NOESY correlations. The structure of wasabidienone B_1 (**6**) was determined in the same way, based on the HMBC correlations summarized in Figure 1. The structures of variotin (**1**), wasabidienone B_0 (**4**), and phomaligin A (**5**) were determined by comparing their spectral data with those published.[3−5]

Variotin (**1**) is a well-known antifungal compound produced by *Paecilomyces variotii* Bainer var. *antibioticus*[3] (Deuteromycota), although its isolation from an *Aspergillus* sp. is unprecedented. The production of variotin by *A. viridi-nutans* increased intensively in the trophophase but dropped rapidly when the production of viriditin (**2**) and *O*-methylviriditin (**3**) started. This could indicate that **2** and **3** are formed by the same biosynthetic pathway via variotin (**1**), for which the pyrrolidone moiety has been shown to originate from glutamic acid and the 8-hydroxy-6-methyldodeca-2,4,6-trienoyl moiety from a methylated hexaketide.[8,9] However, this would require extensive reduction of both the carboxylic acid (to a primary alcohol) and the lactam carbonyl group (to a methylene group) of the glutamic lactam moiety, compared to a relatively facile decarboxylation to yield variotin (**1**), and this remains to be clarified. The carbonyl group in the pyrrolidone moiety of variotin (**1**) is obviously important for antifungal activity, as viriditin (**2**) and *O*-methylviriditin (**3**) possess no antifungal activity (up to concentrations of 100 μg/mL, see Experimental Section), while variotin (**1**) is active (MIC, 1 μg/mL) against most of the fungi tested (except *Fusarium oxysporum*). Of the six compounds isolated, only viriditin (**2**) possesses any cytotoxicity toward Hela S3, L1210, and HL60 cells, with an IC_{90} value of 25 μg/mL.

Table 2. ^{13}C (125 MHz) NMR Data for Viriditin (**2**), 13-*O*-Methylviriditin (**3**), and Wasabidienone B_1 (**6**) in $CDCl_3$[a]

	δ_C (mult.)		
C	**2**	**3**	**6**
1	67.3 (t)	67.6 (t)	202.6 (s)
2	61.4 (d)	61.5 (d)	111.0 (s)
3	28.2 (t)	28.3 (t)	168.6 (s)
4	24.4 (t)	24.4 (t)	117.4 (s)
5	48.0 (t)	48.0 (t)	162.4 (s)
6	167.5 (s)	167.6 (s)	76.6 (s)
7	120.8 (d)	120.9 (d)	206.5 (s)
8	143.4 (d)	143.4 (d)	49.1 (d)
9	126.0 (d)	125.7 (d)	25.1 (t)
10	144.5 (d)	144.4 (d)	11.6 (q)
11	134.6 (s)	136.0 (s)	14.8 (q)
12	139.1 (d)	138.0 (d)	9.0 (q)
13	68.5 (d)	67.6 (d)	29.6 (q)
14	37.1 (t)	35.1 (t)	
15	27.4 (t)	27.4 (t)	
16	22.6 (t)	22.7 (t)	
17	14.0 (q)	14.0 (q)	
18	12.7 (q)	12.9 (q)	
3-OCH_3			61.2 (q)
5-OCH_3			60.7 (q)
13-OCH3		56.2 (q)	

[a] The chemical shifts are given in parts per million relative to the solvent signal (77.0 ppm).

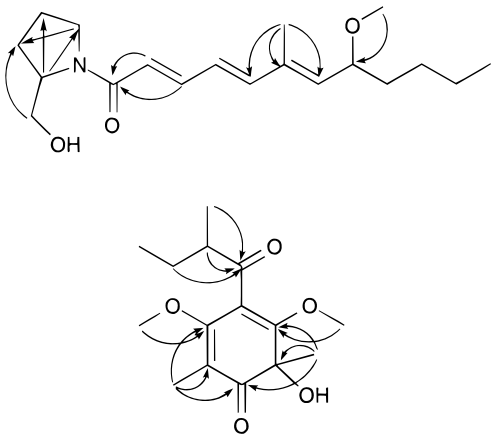

Figure 1. Significant HMBC correlations observed with 13-*O*-methylviriditin (**3**) (top) and wasabidienone B_1 (**6**) (bottom).

Experimental Section

General Experimental Procedures. Preparative HPLC was carried out using a Jasco MD910 equipped with a diode array detector for compounds **1**−**3** and a Gilson model 302 with a variable UV detector for compounds **4**−**6**. UV and IR spectra were recorded with a Perkin-Elmer λ_{16} and a Bruker IFS48 spectrometer, respectively. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5-mm probehead equipped with shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (δ_H 7.26 and δ_C 77.0) were used as reference. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy, the refocusing delays were optimized for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed, and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a JEOL SX102 spectrometer, while the optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Fermentation. *A. viridi-nutans* was isolated from a soil sample collected at the shore of Lake Victoria in Kenya and is preserved in the culture collection of the Department of

Biotechnology, University of Kaiserslautern (accession number JO297). The strain was cultured in a medium consisting of yeast extract (0.4%), malt extract (1.0%), and glucose (1.0%) (YMG), in a 20-L fermentor [type C6 apparatus (Biolafitte, Paris)] with agitation (120 rpm) and aeration (3 L air/min). During fermentation, 100-mL aliquots were regularly drawn from the main culture broth in order to follow the production of bioactive metabolites, mycelial weight, pH, and glucose and maltose contents. The fermentation was stopped when the antifungal activity had reached its peak (after 7 days) and the carbon sources were depleted.

Isolation of Metabolites. The culture filtrate (16 L) was separated from mycelium by filtration, and the filtrate was passed through a Mitsubishi HP 21 resin column (4 × 40 cm), previously packed and equilibrated with distilled water. The column was immediately washed with 2 L of distilled water, then eluted with 1:1 acetone/water mixture and, finally, with pure acetone. The eluted solutions were mixed and the acetone removed under reduced pressure to give an aqueous phase, which was extracted three times with 1 L of EtOAc. The organic extracts were combined, dried using anhydrous Na_2SO_4, and filtered, and the solvent removed under reduced pressure, yielding 2 g of a crude residue. This residue was fractionated on a Si gel column with mixtures of cyclohexane and EtOAc as eluents stepwise (starting with 25% EtOAc, over 50% and 75%, and finally with pure EtOAc. Compounds **1**−**3** were purified by semipreparative HPLC on a RP18 column (25 × 1 cm) with a water/methanol linear gradient as eluent (starting with 30% water for 10 min and then to pure methanol in 20 min, 2 mL/min). Elution times were as follows: **2**, 17 min; **1**, 19 min; and **3**, 23 min. Compounds **4**−**6** were purified on a Diol column (25 × 2 cm) with a linear gradient of cyclohexane and *tert*-butyl methyl ether as eluent (from pure cyclohexane to pure *tert*-butyl methyl ether in 30 min, 2 mL/min). Elution times were as follows: **4**, 20 min; **6**, 24 min; and **5**, 26 min. Approximately 10 mg of **1**, 8 mg of **2**, 3 mg of **3**, 1 mg of **4**, 2 mg of **5**, and 10 mg of **6** were obtained per liter of fermentation broth.

Viriditin (2): yellowish oil, $[\alpha]_D$ −65° (*c* 1.2, $CHCl_3$); UV (MeOH), λ_{max} (log ϵ) 303 (4.53) nm; IR (KBr) ν_{max} 3405, 2930, 1635, 1580, 1435, 1190, 1160, 1125, 1050, 1000, 860 cm^{-1}; NMR data, see Tables 1 and 2; EIMS *m*/*z* 307 $[M]^+$ (36), 276 (21), 250 (10), 222 (44), 207 (67), 189 (12), 180 (18), 123 (35), 121 (53), 102 (22), 85 (100); HREIMS *m*/*z* 307.2157 (calcd for $C_{18}H_{29}NO_3$, 307.2147).

13-*O*-Methylviriditin (3): yellowish oil, $[\alpha]_D$ −67° (*c* 0.4 $CHCl_3$); UV (MeOH), λ_{max} (log ϵ) 302 (4.62) nm; IR (KBr) ν_{max} 3415, 2930, 1635, 1585, 1435, 1190, 1160, 1120, 1090, 1000, 860 cm^{-1}; NMR data, see Tables 1 and 2; EIMS *m*/*z* 321 $[M^+]$ (68), 305 (15), 290 (34), 264 (41), 221 (77), 189 (100), 163 (62), 161 (47), 133 (35), 105 (33), 102 (31); HREIMS *m*/*z* 321.2300 (calcd for $C_{19}H_{31}NO_3$, 321.2304).

Wasabidienone B$_1$ (6): yellowish oil, $[\alpha]_D$ +118° (*c* 0.9 $CHCl_3$); UV (MeOH), λ_{max} (log ϵ) 333 (3.20) nm; IR (KBr) ν_{max} 3430, 2970, 1700, 1645, 1565, 1460, 1375, 1325, 1220, 1145, 1050, 1000 cm^{-1}; NMR data, see Tables 1 and 2; EIMS *m*/*z* 282 $[M^+]$ (12), 239 (100), 225 (21), 221 (26), 207 (10), 198 (13), 194 (24), 183 (12), 179 (11), 165 (14), 155 (35); HREIMS *m*/*z* 282.1460 (calcd for $C_{15}H_{22}O_5$, 282.1467).

Bioassays. Antifungal activity against *Nadsonia fulvescens* (ATCC 24236), *Nematospora coryli* (ATCC 10647), *F. oxysporum* (CBS 149.25), *P. variotii* (ETH 114646), and *Rhodotorula glutinis* (ATCC 26085) was assayed in micro-titer plates as previously described.[10] The size of inoculum was 1 × 10^5 spores/mL, and activity was evaluated after 24 h of incubation at 27 °C. All the compounds isolated in this work were tested for cytotoxicity toward Hela S3 (ATCC CCL 2.2), L1210 (ATCC CCL 219), and HL60 (ATCC CCL 240) cell suspensions, as previously described.[11,12]

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