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# Neobulgarones A~F from Cultures of *Neobulgaria pura*, New Inhibitors of Appressorium Formation of *Magnaporthe grisea*

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Six new dimeric anthraquinone derivatives, neobulgarones A (**3a**), B (**3b**), C (**4a**), D (**4b**), E (**5a**) and F (**5b**), were isolated from the mycelia of the ascomycete *Neobulgaria pura* together with the monomeric carviolin (**1**) and 1-*O*-methylemodin (**2**). All new compounds inhibited the formation of appressoria in germinating conidia of *Magnaporthe grisea* on inductive (hydrophobic) surface. The compounds exhibited moderate cytotoxic, but no antifungal, antibacterial, or phytotoxic activities.

Many plant pathogenic fungi like *Magnaporthe grisea* and *Colletotrichum* species invade their hosts by means of melanized appressoria. In *M. grisea*, these infection structures are needed to generate the turgor pressure necessary for the penetration of leaf epidermal cells[1]. Therefore, appressorium formation could constitute a highly selective target for new fungicides to fight rice blast, the major disease of rice. Differential gene expression has been found to be regulated by a range of signals including contact with a solid surface, the hydrophobicity of the surface, and the presence of plant waxes and cutin monomers *e.g.* 1,16-hexadecandiol or oleyl alcohol[2~4]. Among second messengers, cAMP and 1,2-dioctanoylglycerol are mediators of signals leading to appressorium formation in germinating conidia of *M. grisea*. At least two signal transduction pathways are involved[5,6].

During our search for new selective fungicides, different assays were used to screen for inhibitors of appressorium formation on inductive and non-inductive surfaces[7]. This led to the discovery of glisoprenins and several monounsaturated fatty acids as the first inhibitors of the signal transduction pathway activated by the hydrophobicity of the surface[7~10]. In addition, mycelial extracts from submerged cultures of *Neobulgaria pura*, HA A07-97, were active. Bioactivity-guided isolation yielded six active compounds, and spectroscopic studies suggest that they are diastereomeric pairs of the three new dimeric anthraquinone derivatives shown in Fig. 1. In addition, the inactive anthraquinone monomers carviolin (**1**), known as pigment from cultures of *Penicillium roseo-purpureum*[11], and 1-*O*-methylemodin (**2**)[11b] were isolated. Here we wish to describe the fermentation of *Neobulgaria pura*, HA A07-97, the isolation of the active constituents, the structure determination, and the biological activities of the new compounds.

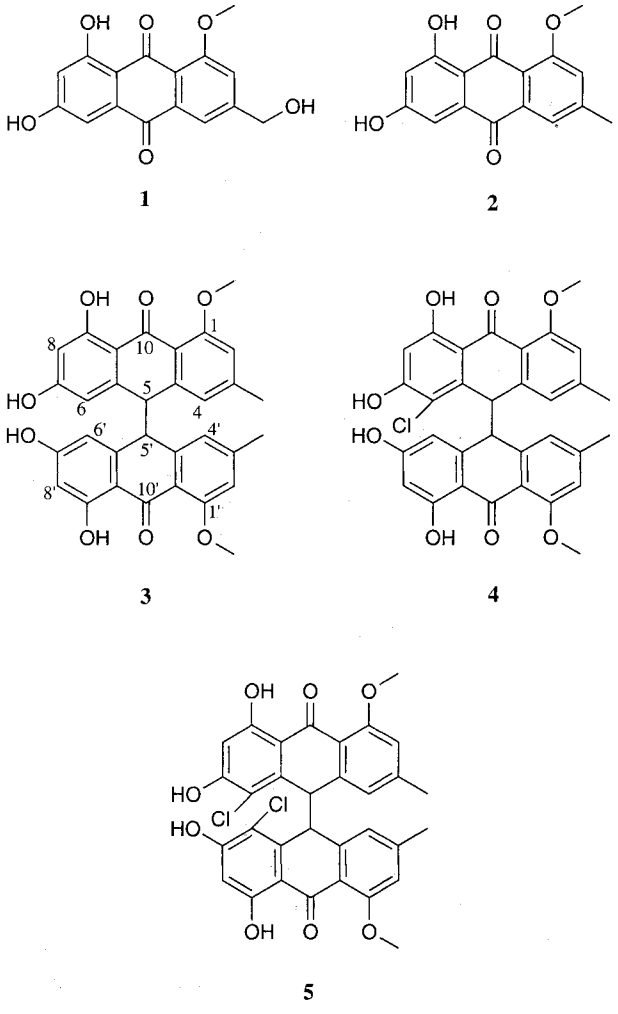
## Materials and Methods

### General

Preparative HPLC was carried out on a JASCO HPLC (PU 980, MD 910), analytical HPLC on a Hewlett-Packard 1090 Type II with LiChrosphere RP18 (10 $\mu$m; 125×4 mm) and a $H_2O$-MeOH gradient. $^1H$ 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 a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals [$\delta_H$ 7.26 and $\delta_C$ 77.0 ppm] were used as reference.

Dedicated to the memory of Sir EDWARD ABRAHAM.

Fig. 1. Structures of the compounds isolated. **a** and **b** designate different diastereomers.




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 optimised for $^{1}J_{CH}=145$ Hz and $^{n}J_{CH}=10$ Hz. The raw data were transformed, and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). MS were recorded on a Hewlett-Packard 1100 LC-MSD (source: APCI; ion mode: positive; Vcap: 4000V; nebulizer: 50 psig; drying gas flow: 6 liters/minute; drying gas temp.: 350°C; corona: 4 $\mu$A; vaporizer: 325°C (mild) 400°C (strong); scan range: 50~800 amu; fragmentor: 80 V (mild) 180 V (strong)) with NUCLEOSIL 100-5 $C_{18}$ (125×2 mm) and a $H_2O$-acetonitrile gradient, while the UV and the IR spectra were recorded with a Perkin-Elmer $\lambda$ 16 and a Bruker IFS 48 spectrometer. The melting points (uncorrected) were determined with a Reichert microscope, and optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22°C.

## Producing Strain

The strain *Neobulgaria pura*, HA A07-97, was obtained from ascospores of fruiting bodies collected in the vicinity of Kaiserslautern. It is deposited in the culture collection of the LB Biotechnology, University of Kaiserslautern. For maintenance on agar slants the fungus was grown on YMG-medium consisting of: yeast extract 0.4%, malt extract 1%, glucose 1% in 1 liter tap water, pH 5.5.

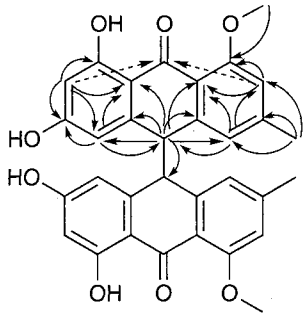
## Biological Assays

Inhibition of appressorium formation in germinating conidia of *M. grisea* strain P1 was measured as described previously[7]. Cytotoxic activities against Jurkat (ATCC TIB 152), HL60 (ATCC CCL 240), HeLa S3 (ATCC CCL 2.2) and B16-F1 (ATCC CRL 6323) were assayed as described in ref. 13. Growth inhibition of germinated seeds of *Setaria italica* and *Lepidium sativum* and antimicrobial activities (plate diffusion assay) were tested as described by ANKE *et al.*[14].

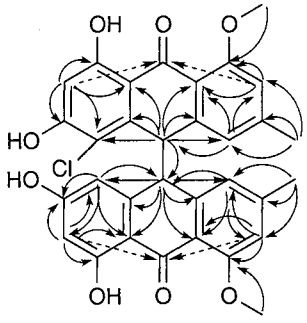
## Fermentation and Isolation

Fermentation was carried out in YMG medium at 24°C in a 20 liter fermentor (Braun Biostat U) with aeration (3.0 liter air/minute) and agitation (120 rpm); 200 ml of a well-grown culture (10 days) in the same medium were used as inoculum. After 256 hours the culture was harvested. The mycelium containing the metabolites was separated from the culture fluid and lyophilised. The compounds were extracted from the lyophilised mycelium (54.7 g) with 5 liters of methanol/acetone 1 : 1. The crude extract (1.7 g) obtained after concentration was fractionated on silica gel (Merck 60, 63~200 $\mu$m) using cyclohexane-EtOAc with increasing EtOAc concentrations. The fractions containing the seven compounds were eluted between 70 and 100% EtOAc. Final purification was achieved by preparative HPLC (LiChrospher RP18, 7 $\mu$m, column size 250×25 mm, flow rate 5 ml/minute) using a $H_2O$-acetonitrile gradient (0~70% acetonitrile in 80 minutes; 70~100% acetonitrile in 10 minutes, retention times and yields: Carviolin (**1**): 58 minutes, 11.7 mg; 1-*O*-methylemodin (**2**): 75 minites, 0.4 mg; neobulgarones A (**3a**): 76 minutes, 2.3 mg; B (**3b**): 78 minutes, 2.1 mg; C (**4a**): 81 minutes, 6.2 mg; D (**4b**): 88 minutes, 7.9 mg; E (**5a**): 84 minutes, 8.1 mg; and F (**5b**), 90 minutes: 8.6 mg). The physico-chemical properties of the neobulgarones are given in Table 1, while the NMR data are given in Table 2 ($^{1}$H) and Table 3 ($^{13}$C).

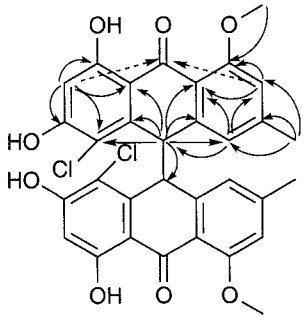
Fig. 2. Significant HMBC correlations observed with the neobulgarones A (**3a**), B (**3b**), C (**4a**), D (**4b**) and E (**5a**). **a** and **b** designate different diastereomers.




**3a and 3b**




**4a and 4b**




**5a**

## Results and Discussion

### Structure Elucidation of the Neobulgarones

The determination of the structures of the new compounds was severely hampered by the fact that no useful mass spectra could be obtained by the conventional ionisation techniques (*i.e.* EI, CI or FAB) suitable for high resolution measurements, only LC-MS experiments (see Materials and Methods for details) yielded useful spectra. Consequently, no high resolution data are available for the neobulgarones. The mass spectra of neobulgarone A (**3a**), neobulgarone C (**4a**) and neobulgarone E (**5a**) are almost identical to those of neobulgarone B (**3b**), neobulgarone D (**4b**) and neobulgarone F (**5b**), respectively, suggesting that the six compounds are three pairs of isomers. The presence of one chlorine in **4a** and **4b** and two chlorines in **5a** and **5b** was indicated by the isotope patterns of the molecular ions observed, and the differences in molecular weight suggest that **4** is monochlorinated and **5** dichlorinated. The 1D NMR spectra of **3a**, **3b**, **5a** and **5b** are relatively simple, which indicated that they actually are dimers, while those of **4a** and **4b** more correspond to a compound with the molecular weight exceeding 500. The resolution obtained in the 1D NMR spectra was relatively poor, and weak couplings were in many cases not resolved. However, the 2D COSY spectra revealed the $^1$H-$^1$H coupling. COSY and especially HMBC spectra (significant HMBC correlations observed are shown in Fig. 2) established the structures of neobulgarones A (**3a**), B (**3b**), C (**4a**), D (**4b**) and E (**5a**) (the structure of neobulgarone F (**5b**) will be further discussed below). The fact that **3a**, **3b** and **5a** are symmetric dimers was demonstrated by the HMBC correlation from 5-H to C-5′ (observed as a correlation to its own carbon), and the C-5/C-5′ connection in **4a** and **4b** was shown by both the corresponding HMBC correlations and the $^1$H-$^1$H coupling between 5-H and 5′-H. In addition, 5-H gives HMBC correlations to C-4, C-4a, C-5a, C-6, C-9a and C-10a (5′-H gives the corresponding HMBC correlations), which together with the correlations between 2-H (and 2′-H) and C-1, C-4 and C-10a, between 3-$CH_3$ (and 3′-$CH_3$) and C-2, C-3 and C-4, between 4-H (and 4′-H) and C-2, C-5 and C-10a, between 8-H (and 8′-H) and C-6, C-7, C-9 and C-9a, and, where appropriate, between 6-H (and 6'-H) and C-5, C-7, C-8 and C-9a establish the structures. A weak HMBC correlation was observed between both 2-H and 8-H (and 2′-H and 8′-H) and the keto carbon, positioning the carbonyl group between the two aromatic rings. The bond between C-5 and C-5′ is readily broken during mass spectrometry, and even mild conditions (acceleration voltage 80 V) in the LC-MS experiment resulted in partial fragmentations. Neobulgarones A (**3a**) and B (**3b**) yielded the fragment 270, in agreement with a dimeric compound composed of two monomers weighing 269, **4a** and **4b** yielded the fragments 270 and 304 (and is consequently composed of two monomers weighing 269 and 303) while **5a** obviously consists of two identical monomers weighing 303. Neobulgarone A (**3a**) must be an isomer of neobulgarone B (**3b**), as neobulgarone C (**4a**) is an isomer of neobulgarone

Table 1. Physico-chemical properties of neobulgarones A (**3a**), B (**3b**), C (**4a**), D (**4b**), E (**5a**) and F (**5b**).

| | **3a** | **3b** | **4a** | **4b** | **5a** | **5b** |
|---|---|---|---|---|---|---|
| Appearance | Yellow crystals | Yellow crystals | Yellow crystals | Yellow crystals | Yellow crystals | Yellow crystals |
| MP (°C) | 218 (decomp.) | 210 (decomp.) | 211 (decomp.) | 222 (decomp.) | 225 (decomp.) | 217 (decomp.) |
| $[\alpha]_D^{22}$ (in methanol) | +150 ° (c 0.5) | +110 ° (c 0.3) | +180 ° (c 0.2) | +130 ° (c 0.2) | +460 ° (c 0.2) | + 220 ° (c 0.2) |
| Molecular formula | $C_{32}H_{26}O_8$ | $C_{32}H_{26}O_8$ | $C_{32}H_{25}O_8Cl$ | $C_{32}H_{25}O_8Cl$ | $C_{32}H_{24}O_8Cl_2$ | $C_{32}H_{24}O_8Cl_2$ |
| LC-MS (*m/z*) | | | | | | |
| Observed M + 1 | 539 | 539 | 573, 575 (30 %) | 573, 575 (30 %) | 607, 609 (70 %) and 611 (10 %) | 607, 609 (70 %) and 611 (10 %) |
| Fragments | 270 | 270 | 270, 304 | 270, 304 | 304 | 304 |
| UV (MeOH) | | | | | | |
| $\lambda_{max}$ nm (ε) | 273 (13,200)<br>348 (12,000) | 262 (17,500)<br>276 (17,700)<br>350 (20,600) | 271 (14,100)<br>356 (12,500) | 262 (18,800)<br>274 (18,200)<br>354 (22,100) | 266 (9,100)<br>363 (7,700) | 267 (20,800)<br>279 (19,200)<br>361 (21,700) |
| IR (KBr) $cm^{-1}$ | 3435, 2935, 1610, 1465, 1385, 1260, 1165, 1140, 1090, 900 and 850 | 3435, 2925, 1610, 1465, 1385, 1260, 1165, 1140, 1090, 900 and 845 | 3430, 2925, 1610, 1465, 1385, 1290, 1255, 1165, 1090, 900 and 845 | 3440, 2925, 1610, 1465, 1385, 1290, 1255, 1140, 1095, 905 and 845 | 3440, 2925, 1610, 1465, 1385, 1290, 1245, 1145, 1090, 905 and 845 | 3440, 2925, 1610, 1465, 1385, 1290, 1245, 1145, 1100, 905 and 845 |

Table 2. $^1H$ (500 MHz) NMR data ($\delta$; multiplicity; *J*) for neobulgarones A (**3a**), B (**3b**), C (**4a**), D (**4b**), E (**5a**) and F (**5b**), in $CDCl_3$ : $CD_3OD$ 19 : 1 with the $CHCl_3$ signal (7.26 ppm) as reference.

| H | **3a** | **3b** | **4a** | **4b** | **5a** | **5b** |
|---|---|---|---|---|---|---|
| 2 | 6.65; s | 6.68; s | 6.58; s | 6.60; s | 6.63; s | 6.72; s |
| 4 | 5.87; s | 6.13; s | 5.46; s | 5.32; s | 5.58; s | - |
| 5 | 4.30; s | 4.21; s | 4.73; d; 2.5 | 4.70; d; 3.2 | 5.04; s | 4.95; s |
| 6 | 6.17; s | 5.77; s | - | - | - | - |
| 8 | 6.29; d; 1.8 | 6.16; s | 6.48; s | 6.46; s | 6.37; s | 6.32; s |
| 1-OMe | 3.81; s | 3.82; q | 3.73; s | 3.77; s | 3.79; s | 3.83; s |
| 3-Me | 2.21; s | 2.25; q | 2.08; s | 2.04; s | 2.05; s | 2.28; s |
| 2' | 6.65; s | 6.68; s | 6.57; s | 6.79; s | 6.63; s | 6.72; s |
| 4' | 5.87; s | 6.13; s | 5.32; s | 7.23; s | 5.58; s | - |
| 5' | 4.30; s | 4.21; s | 4.46; d; 2.5 | 4.45; d; 3.2 | 5.04; s | 4.95; s |
| 6' | 6.17; s | 5.77; s | 6.90; s | 4.95; d; 2.3 | - | - |
| 8' | 6.29; d; 1.8 | 6.16; s | 6.31; s | 6.08; d; 2.3 | 6.37; s | 6.32; s |
| 1'-OMe | 3.81; s | 3.82; q | 3.73; s | 3.88; s | 3.79; s | 3.83; s |
| 3'-Me | 2.21; s | 2.25; q | 2.06; s | 2.49; s | 2.05; s | 2.28; s |

The coupling constants *J* are given in Hz.

Table 3. $^{13}C$ (125 MHz) NMR data ($\delta$; multiplicity) for neobulgarones A (**3a**), B (**3b**), C (**4a**), D (**4b**), E (**5a**) and F (**5b**), in $CDCl_3$ : $CD_3OD$ 19 : 1 with the $CDCl_3$ signal (77.0 ppm) as reference.

| | **3a** | **3b** | **4a** | **4b** | **5a** | **5b** |
|---|---|---|---|---|---|---|
| C | | | | | | |
| 1 | 160.1; s | 160.3; s | 159.9; s | 160.4; s | 159.6; s | 159.8; s |
| 2 | 112.4; d | 112.2; d | 112.5; d | 112.6; d | 113.0; d | 112.4; d |
| 3 | 144.6; s | 144.7; s | 144.7; s | 144.5; s | 144.2; s | 145.1; s |
| 4 | 122.5; d | 122.2; d | 123.2; d | 123.3; d | 123.5; d | 122.7; s |
| 4a | 141.3; s | 142.7; s | 139.0; s | 140.0; s | 139.0; s | 137.6; s |
| 5 | 56.7; d | 56.7; d | 54.2; d | 53.6; d | 49.0; d | 50.8; d |
| 5a | 143.4; s | 142.0; s | 140.5; s | 139.0; s | 140.0; s | 141.6; s |
| 6 | 107.3; d | 107.8; d | 110.1; s | 110.2; s | 110.8; s | 112.9; s |
| 7 | 162.6; s | 162.3; s | 158.5; s | 158.3; s | 158.1; s | 157.8; s |
| 8 | 102.0; d | 101.8; d | 102.6; d | 102.8; d | 102.3; d | 102.7; d |
| 9 | 163.9; s | 163.5; s | 161.5; s | 161.4; s | 161.3; s | 161.6; s |
| 9a | 113.1; s | 112.2; s | 114.0; s | 114.0; s | 114.3; s | 113.5; s |
| 10 | 186.8; s | 186.6; s | 186.5; s | 186.4; s | 185.8; s | 185.6; s |
| 10a | 118.6; s | 119.0; s | 117.6; s | 117.8; s | 119.1; s | 119.3; s |
| 1-OMe | 56.1; q | 56.0; q | 55.9; q | 56.2; q | 56.2; q | 56.0; q |
| 3-Me | 21.8; q | 21.7; q | 21.5; q | 21.5; q | 21.3; q | 21.9; q |
| 1' | 160.1; s | 160.3; s | 160.0; s | 159.8; s | 159.6; s | 159.8; s |
| 2' | 112.4; d | 112.2; d | 112.5; d | 111.9; d | 113.0; d | 112.4; d |
| 3' | 144.6; s | 144.7; s | 144.0; s | 145.3; s | 144.2; s | 145.1; s |
| 4' | 122.5; d | 122.2; d | 123.5; d | 121.2; d | 123.5; d | 122.7; s |
| 4a' | 141.3; s | 142.7; s | 139.4; s | 144.9; s | 139.0; s | 137.6; s |
| 5' | 56.7; d | 56.7; d | 51.5; d | 51.6; d | 49.0; d | 50.8; d |
| 5a' | 143.4; s | 142.0; s | 144.8; s | 139.3; s | 140.0; s | 141.6; s |
| 6' | 107.3; d | 107.8; d | 106.4; d | 109.4; d | 110.8; s | 112.9; s |
| 7' | 162.6; s | 162.3; s | 163.2; s | 161.6; s | 158.1; s | 157.8; s |
| 8' | 102.0; d | 101.8; d | 101.8; d | 101.9; d | 102.3; d | 102.7; d |
| 9' | 163.9; s | 163.5; s | 163.3; s | 163.5; s | 161.3; s | 161.6; s |
| 9a' | 113.1; s | 112.2; s | 113.5; s | 111.9; s | 114.3; s | 113.5; s |
| 10' | 186.8; s | 186.6; s | 186.6; s | 186.0; s | 185.8; s | 185.6; s |
| 10a' | 118.6; s | 119.0; s | 118.5; s | 120.2; s | 119.1; s | 119.3; s |
| 1'-OMe | 56.1; q | 56.0; q | 55.9; q | 55.9; q | 56.2; q | 56.0; q |
| 3'-Me | 21.8; q | 21.7; q | 21.4; q | 22.2; q | 21.3; q | 21.9; q |

D (**4b**), and the only possibility is that they are pairs of diastereomers. No attempt was made to determine the relative stereochemistry of the dimers.

The spectroscopic data of neobulgarone F (**5b**) is very similar to those of neobulgarone E (**5a**), the almost identical mass spectrum (same molecular ion, same isotope pattern, and same fragmentation), IR and UV spectra (see Table 1), as well as $^{13}C$ NMR spectrum (Table 3), strongly suggest that **5b** is the diastrereomer of **5a**. However, as can be seen in Table 2, the signals for 3-H and 3'-H are missing in the $^1H$ NMR spectrum of neobulgarone F (**5b**). At the moment we have no explanation for this inconsistency, and until the matter has been elucidated the structure of neobulgarone F (**5b**) should be regarded as tentative.

The structures of carviolin (**1**)[11] and 1-*O*-methylemodin (**2**)[12] could be established by comparing their spectral data with those reported in the literature.

## Biological Activities

Infection structure formation on hydrophilic surface induced by 1,16-hexadecanediol or the cAMP-analogue chlorophenylthio-cAMP was not affected by the compounds up to a concentration of 20 $\mu$g/ml. At higher concentrations the compounds were no longer soluble. Table 4 shows the effects of the six dimeric anthraquinones, carviolin and 1-*O*-methylemodin on appressorium formation of *M. grisea* strain P1 on the inductive (hydrophobic) side of a GelBond sheet. The inhibition of appressorium formation by neobulgarone D, the most active compound, could not be overcome by the addition of chlorophenylthio-cAMP but by the addition of 1,16-hexadecanediol (data not shown). Therefore, we assume that the signal transduction pathway leading to appressorium formation which is mediated by the second messenger cAMP is not activated by 1,16-hexadecanediol.

In the agar diffusion assay no antimicrobial activity

Table 4. Effects of anthraquinones on appressorium formation in *M. grisea* P1 on the inductive (hydrophobic) surface of a GelBond sheet and cytotoxic activities of the compounds against Jurkat, HL60, HeLa S3 and B16-F1 cells.

| | $AIC_{50/90}$ [$\mu$g/ml] | $IC_{50}$ [$\mu$g/ml] | | | |
|---|---|---|---|---|---|
| Compound | *M. grisea* P1 | Jurkat | HL60 | HeLa S3 | B16-F1 |
| Carviolin | >20/- | >100 | >100 | >100 | >100 |
| 1-*O*-methylemodin | >20/- | n.t. | n.t. | n.t. | n.t. |
| Neobulgarone A | 20/>20 | 10 | 10 | >20 | 20 |
| Neobulgarone B | 10/20 | 5 | 2 | >20 | 10 |
| Neobulgarone C | 20/- | 10 | 5 | >20 | 20 |
| Neobulgarone D | 5/15 | 5 | 2 | >20 | 10 |
| Neobulgarone E | 10/20 | 10 | 10 | >20 | 20 |
| Neobulgarone F | 10/20 | 10 | 5 | >20 | 20 |

$AIC_{50/90}$: Concentration at which appressorium formation was inhibited by 50/90 (±5)%.

- = not tested.

>98% of conidia germinated in all experiments. In the control without inhibitor 97.1 (± 2.2)% of the germinating conidia formed appressoria.

was observed against *Enterobacter dissolvens*, *Bacillus brevis*, *B. subtilis*, *Micrococcus luteus*, *Mucor miehei*, *Nematospora coryli*, *Paecilomyces variotii* or *Penicillium notatum* up to 100 $\mu$g/disc. None of the compounds exhibited phytotoxic activities up to a concentration of 100 $\mu$g/disc in 150 $\mu$l water. The compounds were moderately cytotoxic. The $IC_{50}$ values against different cell lines are also shown in Table 4. Carviolin was not active. 1-*O*-methylemodin was not tested in these assays due to the small amounts available.

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