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Isolation, Structure Elucidation and Biological Activity of 8-*O*-Methylaverufin and 1,8-*O*-Dimethylaverantin as New Antifungal Agents from *Penicillium chrysogenum*

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In the screening of fungi for bioactive components, 8-*O*-methylaverufin (**1b**) and 1,8-*O*-dimethylaverantin (**2b**) were isolated from the culture broth of *Penicillium chrysogenum*. The structure of these new antibiotics were determined by interpretation of the 1D and 2D NMR spectra and by comparison of the NMR data with those of the structurally related averufin (**1a**) and averantin (**2a**). Both compounds have moderate antifungal activity.

In the course of our screening program for novel bioactive compounds from micro-organisms, the ethyl acetate extract of a *Penicillium chrysogenum* isolate drew our attention due to yellow to orange bands on TLC and a biological activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Mucor miehei*. Their pink colouration with dilute sodium hydroxide solution was typical for *peri*-hydroxy quinones, and the nonpolar properties on TLC indicated the absence of sugar moieties. Working up of the extract resulted in the isolation of 8-*O*-methylaverufin (**1b**) and 1,8-*O*-dimethylaverantin (**2b**) as new antibiotics with anti-fungal activity, together with ergosterol peroxide, and ten known quinones related to sterigmatocystin (**7a**). In this paper we report the isolation, structure elucidation, and biological activity of **1b** and **2b**.

Results and Discussion

Well-grown agar cultures of *Penicillium chrysogenum* served to inoculate 48 of 1 liter-Erlenmeyer flasks each containing 250 ml of M_2 medium. The flasks were incubated at 28°C while rotating with 110 rpm for $4^1/_2$ days and extracted with ethyl acetate using our standard procedure[1]. Repeated chromatography of the crude extract on silica gel with a cyclohexane/ethyl acetate gradient (Figure 1) delivered one colourless and twelve yellow compounds which were sufficiently stable to determine their molecular weight by EIMS measurements. By a search with mass and ^{1}H NMR data in databases[2], averufin (**1a**)[3], 6,8-*O*-dimethylaverufin (**1c**)[4], averantin (**2a**)[5], 6,8-dimethylnidurufin (**3**)[6], norsolorinic acid (**4**)[7], versicolorin C (**5a**)[3,8], (−)-aversin (**5b**)[9], 6,8-*O*-dimethylversicolorin A (**6**)[10], sterigmatocystin (**7a**)[11], 5-methoxysterigmatocystin (**7b**)[12], and ergosterol peroxide[13] were easily identified as known compounds.

Another quinone was obtained as an orange-red solid which showed *quasi*-molecular peaks at *m/z* 787 ($[2M+Na]^{+1}$) and 381 ($[M-H]^{-1}$) in the (+)- and (−)-ESI mass spectra, respectively, implicating a molecular weight of 382. The proton NMR spectrum showed one typical signal of a chelated OH group at δ 13.96. In the sp^2 region, two *meta*-coupled doublets at δ 7.31 and 6.93 and a singlet at δ 7.02 were observed. The aliphatic region delivered a signal at δ 5.28 for a methine group connected to oxygen, a methoxy signal at δ 3.94, a 6-proton multiplet at δ 1.90~1.50 and a methyl singlet at δ 1.50. The ^{13}C NMR spectrum depicted two carbonyl carbon signals of a

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quinone system at δ 183.8 and 182.2, four signals for aromatic carbons connected to oxygen and eight additional sp^2 carbons. In the aliphatic region an acetal carbon signal at δ 99.9, a methine carbon connected to oxygen, a methoxy carbon at δ 55.1, three methylene carbons and a methyl carbon were visible. A search with these NMR data (Table 1) and the molecular formula in AntiBase[2], the Dictionary of Natural Products[14] and the Chemical Abstract was not successful and thus indicating a new structure. The EIHRMS of the molecular signal delivered the molecular formula $C_{21}H_{18}O_7$. As the ^1H and ^{13}C NMR spectra of this compound were very similar to those of averufin (**1a**) and 6,8-*O*-dimethylmethylaverufin (**1c**), a monomethylaverufin was very likely. The structure of 8-*O*-methylaverufin (**1b**) was finally derived by H,H COSY, HMQC and HMBC (Figure 2) experiments.

The atoms C-11 and C-15 of averufin (**1a**) and 6,8-*O*-dimethylaverufin (**1c**) are both known to have *S*-configuration. Since both these compounds were produced

Fig. 1. Isolation scheme for purification of products from the fermentation broth of *Penicillium chrysogenum*.

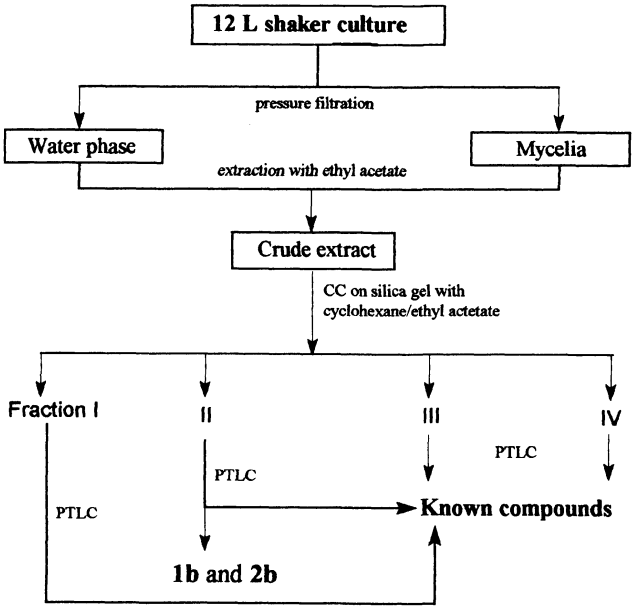


Fig. 2. Structure of 8-*O*-methylaverufin (**1b**) derived by H,H-COSY (↔), HMQC and HMBC (→) couplings.

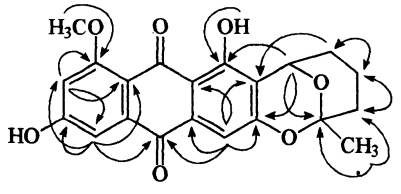


Table 1. ^{13}C (75.5 MHz) and ^1H NMR data (300 MHz) of 8-*O*-methylaverufin (**1b**) and 1,8-*O*-dimethylaverantin (**2b**) in $CDCl_3$ (*J* in [Hz]).

C-No.	Chemical shift (δ)				C-No.	Chemical shift (δ)			
	1b		**2b**			**1b**		**2b**	
1	157.1	-	165.2[a]	-	10	183.8[a]	-	183.5[a]	-
2	115.7	-	116.2	-	10a	135.9	-	138.2	-
3	157.1	-	170.6	-	11	66.1	5.28 (m)	69.6	5.15 (dd, 8.2, 4.8)
4	103.4	7.02	104.6	6.95 (s)	12	26.9	1.90-1.50 (m)	37.8	1.86 (m), 1.77 (m)
4°	132.3	-	132.9	-	13	15.3	1.90-1.50 (m)	32.7	1.58 (m), 1.43 (m)
5	113.7	7.31 (d, 2.5)	111.8	7.27 (d, 2.5)	14	35.3	1.90-1.50 (m)	26.3	1.32 (m)
6	158.6	-	163.2	-	15	99.9	-	23.4	1.32 (m)
7	105.6	6.93 (d, 2.5)	105.1	6.88 (d, 2.5)	16	27.5	1.50 (s)	14.4	0.87 (t, 7.2)
8	164.4	-	162.3[a]	-	1-OMe	-	-	56.7[a]	3.95 (s)
8°	105.6	-	107.9	-	8-OMe	55.1	3.94 (s)	56.3[a]	3.96 (s)
9	182.2	-	185.9	-	1-OH		13.96 (s)	-	-
9a	110.3	-	111.7	-	6-OH		10.04 (s br)	-	-

[a] assignment may be reversed

Table 2. Physico-chemical properties of 8-*O*-methylaverufin (**1b**) and 1,8-*O*-dimethylaverantin (**2b**).

	1b	**2b**
Properties	**orange solid**	**orange solid**
R_f (C_6H_{12}/50 % EtOAc)	0.32	0.42
Molecular formula	$C_{21}H_{18}O_7$	$C_{22}H_{24}O_7$
(+)-ESI-MS	787 ($[2M+Na]^{+1}$)	823 ($[2M+Na]^{+1}$), 423 ($[M+Na]^{+1}$)
(-)-ESI-MS	381 ($[M-H]^{-1}$)	399 ($[M-H]^{-1}$)
IR (KBr) ν cm^{-1}	3427, 2925, 2852, 1619, 1605, 1437, 1382, 1346, 1266, 1244, 1204, 1263, 1126, 1054, 1028, 927, 836, 751	3426, 2927, 2854, 1608, 1594, 1434, 1322, 1262, 1161, 1136, 1057, 1040, 1017, 960, 902, 879, 846, 748
UV/VIS (MeOH): λ_{max} (lg ε)	446 (3.75), 309 (4.12), 290 (4.33), 223 (4.47)	442 (4.55), 306 (sh, 4.91), 287 (5.05), 261 (4.92), 221 (5.20)

in parallel by the fungus investigated here, the 8-*O*-methylaverufin is assumed to have the same configuration as indicated in structure **1b**.

The (−)-ESI and (+)-ESI spectra of the second new compound delivered *quasi*-molecular peaks at *m/z* 399 ($[M-H]^{-1}$) and 423 ($[M+Na]^{+1}$), respectively, which fixed the molecular weight to be 400. High resolution at EI ionisation afforded a molecular formula $C_{22}H_{24}O_7$. The ^1H NMR spectrum was very similar to that of averantin (**2a**). It showed two aromatic doublets as well, each with a *meta*-coupling at δ 7.27 and 6.88 and a sp^2 singlet at δ 6.95, a multiplet for a methine group connected to an oxygen, four methylene signals and a methyl singlet. The main difference was the absence of chelated OH signals, which were substituted by two additional methoxy signals. The second compound was therefore the hitherto unknown 1,8-*O*-dimethyl derivative of averantin (**2a**). Comparison of the ^{13}C NMR data with those of averufin (**1a**), 8-*O*-methylaverufin (**1b**), 6,8-*O*-dimethylaverufin (**1c**), and averantin (**2a**), which were also isolated from the strain, confirmed the structure as 1,8-*O*-dimethylaverantin (**2b**).

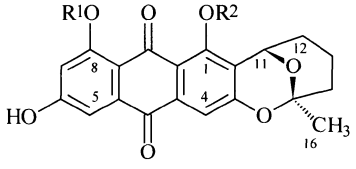
Biological Properties

Antibacterial and antifungal activities were *semi*-quantitatively determined using the agar diffusion method with paper disks (i.d. 9 mm) loaded with 10 μg compound/test plate. The crude extract was inactive against the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*. All compounds isolated from the fungus were inactive against *Streptomyces viridochromogenes* (Tü 57), *Escherichia coli* and *Candida albicans*. The previously reported compound averufin (**1a**) lacks inhibitory activity against the tested bacteria and fungus at the indicated concentration (Table 3), but 8-*O*-methylaverufin (**1b**) showed activity against the fungus *Mucor miehei*. More interestingly, the known compound averantin (**2a**) possesses activity against tested bacteria and lacks activity against the fungus, and 1,8-*O*-dimethylaverantin (**2b**) lacks antibacterial activity but possesses antifungal activity. From such observations, it could be concluded that the methyl substitution at the 8-hydroxyl group of these quinones is likely a key to conferring antifungal activity. However, as exemplified in (−)-aversin (**5b**) and 6,8-*O*-dimethylversicolorin A (**6**), dimethylation at 6- and 8-hydroxyl might cancel such activity. Although the antifungal activities of the subject compounds are moderate, the structure-activity relationship shown in this investigation might be helpful for further studies.

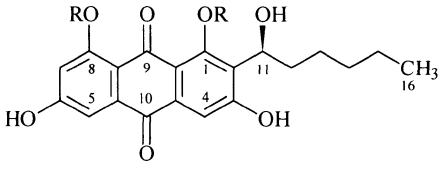
Experimental

Material & methods and antimicrobial tests were used as described earlier[1]. Rf values were measured on Polygram SIL G/UV_{254} with 50% ethyl acetate in cyclohexane. Preparative TLC (PTLC) was performed on 20×40 cm glass plates using 55 g silica gel P/UV_{254} per plate (Macherey-Nagel & Co, Düren, Germany)

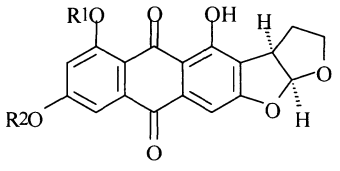
Structures **1**~**7b**



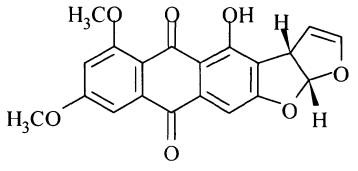
1a: $R^1 = R^2 = H$
1b: $R^1 = Me$, $R^2 = H$
1c: $R^1 = R^2 = Me$



2a: R = H
2b: R = Me



5a: $R^1 = H$, $R^2 = H$
5b: $R^1 = Me$, $R^2 = Me$



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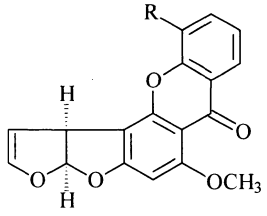

7a: $R^1 = H$
7b: $R^1 = OMe$

Table 3. Antimicrobial activities in the agar diffusion test with 10 μg/test plate i.d. 9 mm (i.d. of inhibition zones [mm]).

Compounds	BS	SA	MM
Averufin (**1a**)	0	0	0
8-*O*-Methylaverufin (**1b**)	0	0	14
6,8-*O*-Dimethylaverufin (**1c**)	0	0	0
Averantin (**2a**)	15	14	0
1,8-*O*-Dimethylaverantin (**2b**)	0	0	14
6,8-Dimethylnidurufin (**3**)	0	0	0
Norsolorinic acid (**4**)	0	0	0
Versicolorin C (**5a**)	17	0	0
(-)-Aversin (**5b**)	0	0	0
6,8-*O*-Dimethylversicolorin A (**6**)	0	0	0
Sterigmatocystin (**7a**)	0	0	18
5-Methoxysterigmatocystin (**7b**)	0	0	14

BS = *Bacillus subtilis*, SA = *Staphylococcus aureus*, MM = *Mucor miehei*

Taxonomy of the Producing Strain

The strain was determined as *Penicillium chrysogenum* Thom (*syn.*=*P. notatum* Westling) (det. W. Helfer) and is deposited in the culture collection of bioLeads company (Heidelberg, Germany). M_2 medium: malt extract (10 g), yeast extract (4 g) and glucose (4 g) were dissolved in artificial sea water (0.5 liter) and tap water (0.5 liter). Before sterilisation, the pH was adjusted to 7.8 by addition of 2 N NaOH

Fermentation of *Penicillium chrysogenum*

The *Penicillium chrysogenum* strain grew very well on agar with M_2 medium in about 72 hours with thick greenish aerial mycelium. 48 of 1 litre Erlenmeyer flasks shaking cultures, each containing 250 ml of M_2 medium, were inoculated with pieces of well grown agar plates and kept for $4^1/_2$ days at 28°C while stirring at 110 rpm. The entire culture broth was mixed with *ca.* 0.5 kg diatom earth, pressed through a pressure filter, and both filtrate and

residue were extracted separately with ethyl acetate. Since both extracts showed the same components on TLC, they were combined and evaporated to dryness to yield 5.01 g of an orange crude extract. This was subjected to silica gel column chromatography (50×3 cm) using a cylohexane-EtOAc gradient (1500 ml *c*-hex/15% EtOAc, 1000 ml *c*-hex/30% EtOAc, 1000 ml *c*-hex/50% EtOAc, 1000 ml *c*-hex/70% EtOAc, 500 ml EtOAc) to give the fractions I (211 mg), II (704 mg), III (676 mg) and IV (651 mg). Separation of fraction I by PTLC (2 plates 20×40 cm, CH_2Cl_2/5% acetone) followed by a further PTLC purification step (2 plates, 20×20 cm, C_6H_{12}/50% EtOAc) afforded ergosterol peroxide (57 mg, Rf=0.67), averufin (**1a**, 28 mg, Rf=0.53), sterigmatocystin (**7a**, 17 mg, Rf=0.46), and norsolorinic acid (**4**, 2 mg, Rf=0.38). Fraction II was similarly purified twice by PTLC (2 plates 20×20 cm, C_6H_{12}/50% EtOAc) to yield averantin (**2a**, 58 mg, Rf=0.48), versicolorin C (**5a**, 10 mg, Rf=0.46), 1,8-*O*-dimethylaverantin (**2b**, 3 mg, Rf=0.42), 6,8-dimethylaverufin (**1c**, 13 mg, Rf=0.40), and 8-*O*-methylaverufin (**1b**, 4 mg, Rf=0.32). Purification of Fraction III and IV (5 plates 20×40 cm, CH_2Cl_2/9% acetone) delivered 6,8-*O*-dimethylversicolorin A (**6**, 3 mg, Rf=0.35) and dimethylnidurufin (**3**, 23 mg, Rf=0.18), and 5-methoxysterigmatocystin (**7b**, 27 mg, Rf=0.37) and aversin (**5b**, 12 mg, Rf=0.30), respectively.

Acknowledgements

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