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SHORT COMMUNICATION

Phytochemical investigation and antimicrobial activity of an endophytic fungus *Phoma* sp.

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Abstract Phytochemical investigation of the endophytic fungi *Phoma* sp. resulted in the isolation of sclerodin (**1**), 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**), atrovenetinone (**3**), and sclerodione (**4**). Preliminary studies showed that sclerodin (**1**) displayed moderate antialgal activity while 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**), atrovenetinone (**3**), and sclerodione (**4**) displayed moderate antifungal activity. Furthermore 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**) and atrovenetinone (**3**) showed moderate antibacterial activity against *Bacillus megaterium* and additionally atrovenetinone (**3**) showed good antibacterial activity towards *Eurotium repens*. Furthermore atrovenetinone (**3**) and sclerodione (**4**) displayed very strong antifungal activity towards *Ustilago violacea*.

1. Introduction

There is an alarming increase in health related problems which may be directly associated with current day cancers, drug-resistant bacteria, parasitic protozoans and fungi (Hussain et al., 2012b). It has been found that either unusual or rather specialized ecological environments produce some of the most valuable microorganisms for the production of secondary metabolites. Furthermore, intensive studies on these secondary

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metabolites have focused in on the endophytes present, since these have been recognized as having the best possibility for the development of new and unique medicinal agents for addressing the health hazards faced by society today (Hussain et al., 2012b). In continuation of our programme on phytochemical analysis of endophytic fungi (Hussain et al., 2009a,b; Hussain et al., 2011a,b; Hussain et al., 2012a,b), we investigated the endophytic fungus *Phoma* sp., (internal strain No. 7133), which was isolated from *Senecio kleinii* from Gomera and led to the isolation and structural determination of four compounds viz., sclerodin (**1**), 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**), atrovenetinone (**3**), and sclerodione (**4**) (Fig. 1). Compounds **1**–**4** were also isolated from another taxonomical unidentified fungal strain 3004 in our group. Antimicrobial studies showed that sclerodin (**1**) showed moderate antialgal activity while 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**), atrovenetinone (**3**), and sclerodione (**4**) demonstrated moderate antifungal activity. Moreover 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**) and atrovenetinone (**3**) showed moderate antibacterial activity against *B. megaterium* and on the other hand atrovenetinone (**3**) demonstrated good antibacterial activity towards *Eurotium repens*. It is noteworthy that atrovenetinone (**3**) and sclerodione (**4**) displayed very strong antifungal activity towards *Ustilago violacea*.

2. Materials and methods

2.1. General experimental procedure

Ultraviolet (UV) spectra were recorded in methanol on a Hitachi U-3200 spectrophotometer. Infra Red (IR) spectra were measured on Shimadzu-8900 spectrophotometer. EI-MS and HR-EI-MS were carried out using MAT 8200 and Micromass LCT mass spectrometers, in *m/z*. The ^{1}H NMR spectra were recorded on a Bruker AMX-500 spectrometer using TMS as an internal reference. The chemical shifts are reported in ppm (δ) while the coupling constants (J) in Hertz. The ^{13}C NMR spectra were recorded at 125 MHz on the same instrument. Column chromatography (CC) was carried out using silica gel (70–230 and 230–400 mesh; E-Merck, Darmstadt, Germany) and Aluminium sheets precoated with silica gel 60 F 254 (0.2 mm thick; E-Merck) were used for TLC to check the purity of the compounds and were visualized under UV light (254 and 366 nm) followed by ceric sulphate as the spray reagent. Microbiological methods and culture conditions are as described previously (Höller et al., 2000; Schulz et al., 1995).

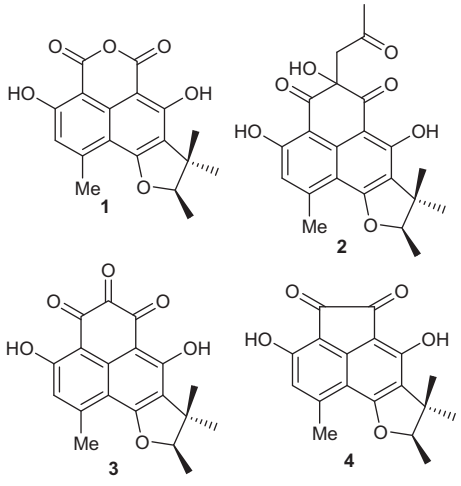

Figure 1 Structure of compounds **1**–**4** isolated from *Phoma* sp.

2.2. Identification, culture, extraction, and isolation

The endophytic fungus *Phoma* sp., (internal strain No. 7133), was isolated from *Senecio kleinii* from Gomera, and was cultivated on biomalt solid agar medium (12 L, 5% w/v) at room temperature for 28 days. The endophytic fungus was identified by Dr. Siegfried Draeger and a voucher specimen (TUB-7133) was deposited in the culture collection of the Institute of Microbiology, Technical University of Braunschweig, Germany. The cultures were extracted with ethyl acetate to afford a residue (4.3 g). The extract was separated into three fractions by column chromatography on silica gel with a gradient of *n*-hexane/ethyl acetate (90:10, 50:50, 0:100) as the eluent. The sub-fraction F_1 was further purified by silica gel column chromatography (CC) and preparative TLC with *n*-hexane/ethyl acetate (10:1 to 5:1) to give pure compounds **1** (4.5 mg), **2** (7.0 mg), **3** (5.3 mg), and **4** (4.3 mg).

2.2.1. Sclerodin (*1*)

Mp: 256 °C; IR (CH_2Cl_2): 3436, 1709, 1623, 1459, 1302, 1035 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.34 (s, 3H, 4′-H), 1.53 (d, 3H, J = 6.6 Hz, 1′-H), 1.59 (s, 3H, 5′-H), 2.83 (s, 3H, 7a-H), 4.75 (q, 1H, J = 6.6 Hz, 2′-H), 6.82 (s, 1H, 8-H), 11.38 (s, 1H, OH), 11.62 (s, 1H, OH); ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 14.9 (C-1′), 21.1 (C-5′), 24.1 (C-7a), 25.8 (C-4′), 43.8 (C-3′), 92.4 (C-2′), 93.7 (s, C-3a), 97.4 (C-9a), 108.8 (C-6a), 117.6 (C-8), 119.5 (s, C-5), 135.6 (s, C-3b), 150.2 (C-7), 164.5 (s, C-4), 165.1 (C-3), 165.7 (C-1), 166.2 (s, C-9), 166.5 (s, C-6).; EIMS (200 °C) *m/z* (%): 328 [M]+ (37), 312 (100), 295 (29), 269 (58), 257 (30); HREIMS: 328.940 (calcd for $C_{18}H_{16}O_6$, 328.947).

2.2.2. 8,9-Dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4H-phenaleno[1,2-b]furan-4,6(5H)-dione (*2*)

Mp: 231 °C; IR (CH_2Cl_2): 3407, 1711, 1645, 1632, 1382 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.30 (s, 3H, 4′-H), 1.33 (s, 3H, 4′-H), 1.49 (d, 3H, J = 6.6 Hz, 1′-H), 1.50 (d, 3H, J = 6.6 Hz, 1′-H), 1.54 (s, 3H, 5′-H), 1.55 (s, 3H, 5′-H), 2.22 (s, 6H, 2c-H), 2.75 (s, 6H, 7a-H), 3.31 (s, 2H, 2a-H), 3.36 (s, 2H, 2a-H), 3.68 (bs, 2H, OH), 4.66 (q, 2H, J = 6.6 Hz, 2′-H), 6.76 (s, 2H, 8-H), 12.84 (d, 2H, OH), 13.36 (d, 2H, OH); ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 14.8 (C-1′), 15.0 (C-1′), 21.0 (C-4′), 24.6 (C-7a), 25.88 (q, C-5′), 26.1 (C-5′), 31.1 (C-2c), 31.2 (C-2c), 43.6 (C-3′), 43.7 (C-3′), 51.9 (C-2a), 52.2 (C-2a), 77.6 (C-2), 91.9 (C-2′), 103.0 (C-3a), 105.9 (C-9a), 110.0 (C-6a), 118.2 (C-8), 118.7 (C-5), 118.8 (C-5), 137.8 (C-3b), 149.4 (C-7), 149.5 (C-7), 165.7 (C-4), 165.8 (C-4), 166.3 (C-9), 166.4 (C-9), 166.5 (C-6), 166.5 (C-6), 197.5 (C-3), 197.5 (C-3), 199.7 (C-1), 206.4 (s, C-2b), 206.7 (C-2b); EIMS (200 °C) *m/z* (%): 398 [M]+ (13), 355 (25), 327 (55), 313 (100), 297 (75), 269 (35); HREIMS: 398.1360 (calcd for $C_{22}H_{22}O_7$, 398.1366).

*2.2.3. Atrovenetinone (**3**)*

Mp: 217 °C; IR (CH_2Cl_2): 3421, 1641, 1610, 1447, 1593 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.31 (s, 3H, 4′-H), 1.32 (s, 3H, 4′-H), 1.48 (d, 3H, J = 6.5 Hz, 1′-H), 1.50 (d, 3H, J = 5.5 Hz, 1′-H), 1.55 (s, 3H, 5′-H), 2.74 (s, 3H, 7a-H), 2.77 (s, 3H, 7a-H), 4.68 (pt, 1H, J = 6.5 Hz, 2′-H), 4.77 (pt, 1H, J = 6.6 Hz, 2′-H), 6.64 (s, 1H, 8-H), 6.71 (s, 1H, 8-H), 12.71 (s, 1H, OH), 12.93 (s, 1H, OH), 1.20 (s, 1H, OH), 13.82 (s, 1H, OH); ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 14.9 (C-1′), 20.9 (C-4′), 24.6 (C-7a), 24.9 (C-7a), 25.6 (C-5′), 25.9 (C-5′), 43.6 (C-3′), 43.6 (C-3′), 86.9 (C-2), 92.2 (C-2′), 92.9 (d, C-2′), 102.2 (C-3a), 105.0 (C-3a), 108.2 (C-9a), 109.8 (C-9a), 110.3 (C-6a), 110.9 (C-6a), 118.4 (C-8), 119.0 (C-8), 119.1 (C-5), 119.5 (C-5), 138.3 (C-3b), 138.6 (C-3b), 150.4 (C-7), 152.1 (C-7), 166.7 (C-4), 167.2 (C-6), 168.2 (C-9), 168.7 (C-9), 177.0 (C-2), 179.2 (C-3), 179.5 (C-1), 193.7 (C-3), 196.0 (C-1); EIMS (200 °C) m/z (%):340 [M]+ (30), 327 (35), 312 (27), 297 (100), 269 (40); HREIMS: 340.0940 (calcd for $C_{19}H_{16}O_6$, 340.0947).

*2.2.4. Sclerodione (**4**)*

Mp: 202 °C; IR (CH_2Cl_2): 3432, 1685, 1634, 1617, 1364 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.31 (s, 3H, 4′-H), 1.50 (d, 3H, J = 6.7 Hz, 1′-H), 1.55 (s, 3H, 5′-H), 2.76 (s, 3H, 7a-H), 4.70 (q, 1H, J = 6.3 Hz, 2′-H), 6.67 (s, 1H, 8-H), 7.63 (s, 1H, OH), 7.89 (s, 1H, OH); ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 14.8 (C-1′), 21.4 (C-4′), 22.4 (C-7a), 26.0 (C-5′), 43.6 (C-3′), 92.3 (C-2′), 106.4 (C-3a), 107.4 (C-9a), 109.5 (C-6a), 117.8 (C-8), 119.9 (C-5), 146.8 (C-7), 151.5 (C-3b), 154.7 (C-9), 155.2 (C-4), 164.7 (C-6), 186.5 (C-3), 190.0 (C-1); EIMS (200 °C) m/z (%):312 [M]+ (28), 297 (75), 285 (20), 269 (100), 241 (20), 213 (32); HREIMS: 312.0990 (calcd for $C_{18}H_{16}O_5$, 312.0998).

2.3. Bioactivity test-agar diffusion test

Tests for antifungal, antibacterial, and antialgal activities were performed as previously described (Schulz et al., 1995). The test organisms for the agar diffusion and screening tests were bacteria *B. megaterium* de Bary (gram positive) and *Escherichia coli* (Migula) Castellani & Chalmers (gram negative), the fungi *U. violacea* (Pers.) Roussel (Ustomycetes), *Mycotypha microspora* Fenner (Zygomycetes), *E. repens* Corda (Ascomycetes) and *Fusarium oxysporum* Schltdl. (Deuteromycetes) and the alga *Chlorella fusca* Shih Krauss (Chlorophyceae), where the inhibition of *C. fusca* is usually correlated with broader antialgal activity (Schulz et al., 1995). Compounds **1**–**4** were dissolved in acetone at a concentration of 1 mg/mL. Fifty microlitres of the solutions (50 µg) was pipetted onto a sterile filter disc (Schleicher & Schuell, 9 mm), which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the test organism (Schulz et al., 1995).

3. Results and discussion

3.1. Structure elucidation

The ethyl acetate extract of endophytic fungus *Phoma* sp. was chromatographed on silica gel to give four compounds **1**–**4**. These four compounds were identified as viz., sclerodin (**1**) (Ayer et al., 1986), 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**) (Ayer et al., 1986), atrovenetinone (**3**) (Ayer et al., 1986), and sclerodione (**4**) (Ayer et al., 1986) (Fig. 1) and their structures were confirmed by a comparison of their spectral data to the literature.

3.2. Antimicrobial activity

Antibacterial, antifungal and antialgal properties of the four pure isolated compounds viz., sclerodin (**1**), 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**), atrovenetinone (**3**) and sclerodione (**4**) are compiled in Table 1. The isolated compounds **1**–**4** were tested in an agar diffusion assay for their antifungal, antibacterial, and antialgal properties towards *Chlorella fusca*, *U. violacea*, *E. repens*, *M. microspora*, *F. oxysporum*, *E. coli*, and *B. megaterium*.

Sclerodin (**1**) which has a pyran-2,6-dione group showed moderate algicidal activity towards *C. fusca* and sclerodione (**4**) which has cyclopent-1,2-dione showed moderate antifungal activity towards *E. repens*. On the other hand 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**) and atrovenetinone (**3**) showed moderate antifungal activities towards *M. microspora*. Moreover compounds **1** and **4** were not active against *M. microspora*. Atrovenetinone (**3**) displayed moderate antifungal activity towards *F. oxysporum* while compounds **1**, **2**, and **4** were not active against *F. oxysporum*. It is noteworthy that compound **3** has a cyclohex-4-ene-1,2,3-trione group while compounds **1**, **2**, and **4** do have said group in their structures. On the other hand compound **2** has a propyl-2-one and hydroxyl group instead of oxygen which is present in sclerodin (**1**). Moreover atrovenetinone (**3**) has a third carbonyl group instead of oxygen which is present in sclerodin (**1**). In addition 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**) and atrovenetinone (**3**) showed moderate antibacterial activity against *B. megaterium*. Furthermore atrovenetinone (**3**) showed good antifungal activity towards *E. repens*. Interestingly atrovenetinone (**3**) and sclerodione (**4**) showed very strong antifungal activity towards *U. violacea* while compounds **1** and **2** were not active against *U. violacea*. It is noteworthy that this compound has a cyclohex-4-ene-1,2,3-trione group while

Table 1 Biological activities of pure metabolites **1**–**4** against microbial test organisms in agar diffusion assay.[a]

Compound	Antialgal	Antifungal				Antibacterial	
	Chl	Ust.	Eur.	Mm	F.o.	Ec	Bm
1	4	1	0	0	0	0	2
2	2	1	0	3	0	0	3
3	2	15	7	3	4	0	4
4	1	10	4	0	0	0	2

[a] 10 mg/mL of compounds **1**–**4** were tested for inhibitions of *Chlorella fusca* (Chl), *Ustilago violacea* (Ust), *Eurotium repens* (Eur), *Mycotypha microspora* (Mm), *Fusarium oxysporum* (F.o), *Escherichia coli* (Ec) and *Bacillus megaterium* (Bm); Radius of zone of inhibition was measured in mm.

compound **4** has cyclopentane-1,2-dione group in their structures. It is important to note that none of these compounds was active against *E. coli*. Compounds **1** and **2** were not active against *E. repens* and *U. violacea*.

4. Conclusion

The overall result of the present study concluded that a phytochemical investigation of the endophytic fungus *Phoma* sp. resulted in the identification of four compounds viz., sclerodin (**1**), 8,9-dihydro-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-4*H*-phenaleno[1,2-*b*]furan-4,6(5*H*)-dione (**2**), atrovenetinone (**3**), and sclerodione (**4**). Preliminary studies showed that atrovenetinone (**3**) displayed good antibacterial activity towards *E. repens*. Furthermore atrovenetinone (**3**) and sclerodione (**4**) showed very strong antifungal activity towards *U. violacea*.

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