Antimicrobial phenalenone derivatives from the marine-derived fungus *Coniothyrium cereale*†

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Received 25th August 2010, Accepted 11th October 2010
DOI: 10.1039/c0ob00625d

The marine-derived fungus *Coniothyrium cereale* was isolated from the green alga *Enteromorpha* sp. and found to produce the new phenalenone derivatives **1–7** as well as the known compounds lactone **8**, (–) sclerodin (**9**), lamellicolic anhydride (**10**), (–) scleroderolide (**11**), and (–) sclerodione (**12**). The structures of these closely related compounds were established from extensive spectroscopic investigations on the basis of one and two dimensional NMR spectroscopic studies (^{1}H, ^{13}C, COSY, NOESY, HSQC and HMBC) as well as mass spectrometric analysis (LC/MS, HREIMS and HRESIMS), UV and IR spectra. Compounds **5** and **11** showed the same antimicrobial activity toward *Staphylococcus aureus* SG 511 with an MIC value of 24 μM. The presence of a diketo-lactone ring as in compounds **5** and **11** was found to be essential for this activity. In agar diffusion assays with *Mycobacterium phlei* considerable inhibition zones were observed for compounds **2**, **4** and **7**. Compounds **1**, **5** and **9** showed potent inhibition of human leukocyte elastase (HLE) with IC_{50} values of 7.2, 13.3 and 10.9 μM, respectively.

Introduction

Marine fungi have been established as a promising resource for novel and biologically active molecules. The potential of fungi associated with marine macroorganisms like sponges and algae, however has only been explored to a very limited extent.[1] In particular, fungi living in the inner tissue of marine algae (endophytes) were found capable of producing a structurally and biologically most intriguing array of natural products.[2] Associations between micro- and macro-organisms are a prominent feature of marine ecosystems, and in some cases the microbial part of this association was found to be responsible for secondary metabolite production.[3]

Biological and chemical screening of fungal strains isolated from the inner tissue of algae directed our attention to the fungus *Coniothyrium cereale*. As part of our program for novel and biologically active natural products of marine-fungal origin,[4] this paper reports on the isolation and structure elucidation of new phenalenone derivatives **1–7** and the known compounds lactone **8**, (–) sclerodin (**9**), lamellicolic anhydride (**10**), (–) scleroderolide (**11**) and (–) sclerodione (**12**, Fig. 1). Antimicrobial and cytotoxic activities as well as inhibitory activity toward a panel of pharmacologically important proteases and esterases for the isolated compounds were investigated.

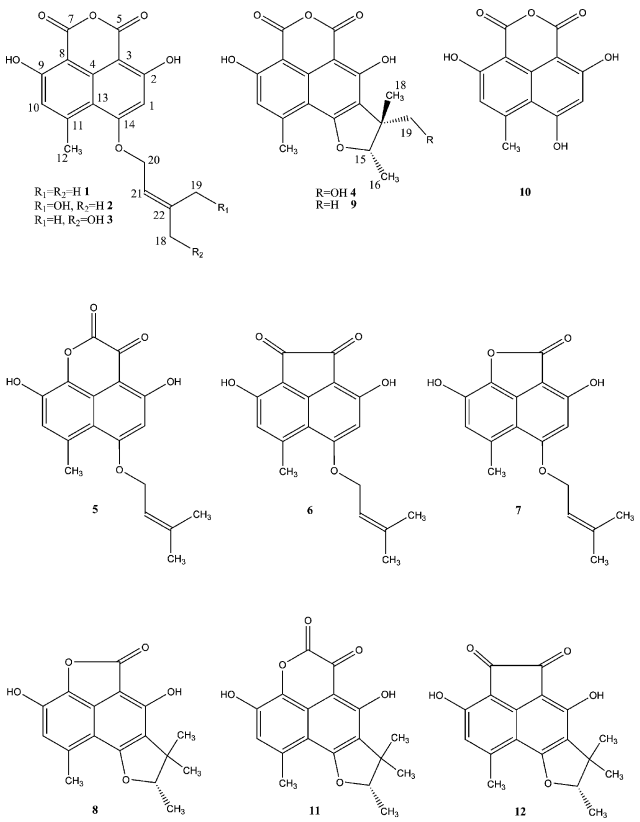

Fig. 1 Chemical structures of phenalenone derivatives (**1–12**).

Results and discussion

The molecular formula of compound **1** was deduced from accurate mass measurements to be $C_{18}H_{16}O_6$ (351.0878, $[M + Na]^+$) which

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† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra and other relevant information are included for compounds **1–12**. See DOI: 10.1039/c0ob00625d

Table 1 ^{1}H NMR spectroscopic data for compounds **1–7**

	1[a]	**2**[b]	**3**[b]	**4**[a]	**5**[b]	**6**[b]	**7**[b]
Position	δ_H (mult, *J* in Hz)	δ_H (mult, *J* in Hz)	δ_H (mult, *J* in Hz)	δ_H (mult, *J* in Hz)	δ_H (mult, *J* in Hz)	δ_H (mult, *J* in Hz)	δ_H (mult, *J* in Hz)
1	6.46, s	6.67, s	6.70, s		6.56, s	6.46, s	6.59, s
10	6.84, s	6.93, s	6.94, s	6.82, s	6.93, s	6.72, s	6.73, s
12	2.81, s	2.84, s	2.86, s	2.80, s	2.70, s	2.73, s	2.62, s
15				4.77, q (6.6)			
16				1.65, d (6.6)			
18	1.85, s	1.87, s	4.05, s	1.49, s	1.83, s	1.80, s	1.79, s
19	1.80, s	4.26, s	1.81, s	a: 3.96, d (11.7) b: 3.86, d (11.7)	1.83, s	1.80, s	1.79, s
20	4.72, d (6.6)	5.00, d (6.6)	4.99, d (6.3)		4.91, d (6.6)	4.77, d (6.3)	4.74, d (6.6)
21	5.55, brt (6.6)	5.71, brt (6.6)	5.94, brt (6.3)		5.66, brt (6.6)	5.61, brt (6.3)	5.59, brt (6.6)
OH-2	11.57, s			11.72, s			
OH-9	11.33, s			11.37, s			

[a] In $CDCl_3$. [b] In acetone-d_6.

Table 2 ^{13}C NMR spectroscopic data for compounds **1–7**

	1[c]	**2**[d]	**3**[d]	**4**[d]	**5**[d]	**6**[d]	**7**[d]
Position	δ_C, mult.[a]	δ_C, mult.[a]	δ_C, mult.[a]	δ_C, mult.[a]	δ_C, mult.[a]	δ_C, mult.[a]	δ_C, mult.[a]
1	97.0, CH	98.1, CH	98.1, CH	114.7, C	97.5, CH	98.2, CH	99.3, CH
2	168.0, C	168.7, C	168.8, C	164.0, C	171.6, C[e]	157.8, C	161.0, C
3	92.6, C	93.7, C	93.9, C	93.3, C	106.5, C	105.7, C	95.1, C
4	135.1, C	136.1, C	136.1, C	135.6, C	122.9, C	152.5, C	134.9, C
5	164.7, C[e]	165.7, C[e]	165.6, C	164.8, C[e]	172.6, C[e]	185.4, C[e]	165.8, C
6	—	—	—	—	155.8, C	—	—
7	165.2, C[e]	166.1, C[e]	n.d.[b]	165.2, C[e]	—	187.7, C[e]	—
8	97.2, C	98.4, C	98.6, C	97.2, C	131.3, C	108.8, C	130.3, C
9	165.3, C	165.8, C	165.9, C	166.1, C	146.1, C	155.2, C	138.3, C
10	118.3, CH	118.8, CH	118.8, CH	117.5, CH	119.6, CH	119.2, CH	119.4, CH
11	150.7, C	151.3, C	151.3, C	150.1, C	137.3, C	147.2, C	132.9, C
12	26.0, CH_3	26.0, CH_3	26.1, CH_3	23.8, CH_3	24.7, CH_3	24.1, CH_3	21.9, CH_3
13	112.4, C	113.2, C	113.4, C	108.5, C	113.1, C	113.0, C	111.5, C
14	166.7, C	167.5, C	167.7, C	167.4, C	170.2, C[e]	165.3, C	165.6, C
15	—	—	—	91.9, CH	—	—	—
16	—	—	—	14.5, CH_3	—	—	—
17	—	—	—	48.8, CH	—	—	—
18	25.8, CH_3	21.4, CH_3	66.8, CH_2	20.8, CH_3	25.8, CH_3	25.8, CH_3	25.8, CH_3
19	18.4, CH_3	61.6, CH_2	14.3, CH_3	64.5, CH_2	18.3, CH_3	18.3, CH_3	18.3, CH_3
20	66.6, CH_2	67.1, CH_2	67.5, CH_2	—	67.9, CH_2	66.9, CH_2	66.9, CH_2
21	117.5, CH	120.3, CH	117.2, CH	—	118.9, CH	119.5, CH	119.7, CH
22	140.2, C	143.5, C	143.8, C	—	140.5, C	139.7, C	139.4, C

[a] Implied multiplicities determined by DEPT. [b] Not detected. [c] In $CDCl_3$. [d] In acetone-d_6. [e] Interchangeable.

requires 11 degrees of unsaturation. An UV maximum at 353 nm clearly evidenced that compound **1** has an extended aromatic system, whereas the IR spectrum showed an OH stretching vibration at 3325 cm^{-1} which corresponds to hydroxy groups in the molecule.

The ^{1}H-NMR spectrum of compound **1** (Table 1) was characterised by resonances due to three tertiary methyl groups (δ_H 2.81 for H_3-12, δ_H 1.85 for H_3-18 and δ_H 1.80 for H_3-19), one of them bound to an aromatic moiety (H_3-12) and two of them located at a double bond (H_3-18, H_3-19) as deduced from their downfield chemical shifts. A further ^{1}H-NMR resonance was due to an oxygen-substituted methylene moiety (δ_H 4.72 for H_2-20), whereas two ^{1}H-NMR singlet resonance signals arose from aryl protons (δ_H 6.46 for H-1 and δ_H 6.84 for H-10). These aryl protons (H-1 and H-10), each had a distinctive set of correlations in the ^{1}H-^{13}C HMBC spectrum suggesting that each of these protons is attached to a different benzene ring. Resonances for an olefinic proton (δ_H 5.55 for H-21), and two downfield shifted ^{1}H-NMR signals due to chelated hydroxy groups (δ_H 11.57 for OH-2 and δ_H 11.33 for OH-9) were also detected.

The ^{13}C-NMR spectrum (Table 2) disclosed 18 resonances, 11 of them for quaternary carbons. Carbons C-3, C-4, C-8, C-11 and C-13 are sp^2 quaternary aromatic carbon atoms and not attached to electronegative substituents, whereas carbons C-2, C-9 and C-14 are sp^2 quaternary aromatic carbon atoms attached to oxygen as judged from their chemical shifts (Table 2). The remaining quaternary carbons were accounted for as two carbonyl carbons, C-5 and C-7, which are probably part of an anhydride moiety, and C-22 being part of an olefinic double bond. In the ^{1}H-^{13}C HMBC spectrum, H-1 showed cross peak correlations with C-2, C-3, C-13 and C-14, whereas H-10 had correlations with C-8, C-9, C-12 and C-13. CH_3-12 had heteronuclear couplings

to C-10, C-11 and C-13. OH-2 showed cross peak correlations with C-1, C-2 and C-3; and OH-9 with C-8, C-9 and C-10. This pattern of heteronuclear correlations, together with the UV and ^{1}H-NMR data indicated two connected penta-substituted benzene rings, *i.e.* a naphthalene-type compound substituted at C-2 and C-9 with hydroxy groups and at C-11 with a methyl group. The quaternary aromatic carbon C-14 had to be further connected to an oxygen containing substituent according to its ^{13}C-NMR chemical shift. The presence of the l,l-dimethyl-1-propenyl group in **1** was proven as follows: the ^{1}H-NMR spectrum contained two singlet resonances at δ_H 1.85 and 1.80 due to a geminal dimethyl group attached to an allylic carbon. This was corroborated by the ^{1}H-^{13}C HMBC cross peak correlations between H_3-18 and H_3-19 and C-22. The ^{1}H-^{1}H COSY spectrum showed cross peak correlations for a ^{1}H-^{1}H-spin system ranging from both terminal methyl protons *via* H-21 to H_2-20. The isoprenyl unit is attached to C-14 through oxygen due to a cross peak correlation in the ^{1}H-^{13}C HMBC spectrum between H_2-20 and C-14.

Comparison of our spectroscopic data with those of known compounds in the literature indicated that compound **1** is a phenalenone derivative closely similar to sclerodin (**9**).[5] Since ^{13}C-NMR shifts of all carbons of the polyketide nucleus (C-2 to C-14) are close to identical to those found for the respective carbon atoms of sclerodin, the carbonyl groups C-5 and C-7 had to be located at C-3 and C-8. The degree of unsaturation required the presence of three rings in compound **1**. In sclerodin (**9**) carbonyl C-7 and C-5 are linked *via* oxygen to form a cyclic anhydride, which is also the case for compound **1**. Compound **1** is thus a prenylated phenalenone-type compound for which the trivial name coniosclerodin is suggested. Compound **1** was described before as a synthetic compound by etherification of lamellicolic anhydride (**10**) with dimethylallyl bromide.[6] The ^{1}H NMR spectroscopic data of compound **1** are in good agreement with literature data.

Spectroscopic data proved that compound **2** is closely similar in structure to compound **1**, the only difference being the presence of the methylene group CH_2-19 instead of the methyl group CH_3-19 in compound **1**. This methylene group is attached to oxygen due to its chemical shifts in both ^{1}H- and ^{13}C- NMR spectra ($\delta_{H/C}$ 4.26/61.6), and connected to C-22 due to the cross peak correlation in the ^{1}H-^{13}C HMBC spectrum of H_2-19 and the quaternary C-22.

HREIMS, UV, IR, and NMR spectroscopic data (Tables 1 and 2) indicated that compound **3** has the same planar structure as compound **2**, the only difference being the configuration of the double bond $\Delta^{21,22}$ which is *E* in the case of compound **3** and *Z* in compound **2**, *i.e* **2** and **3** are *E*-*Z* configurational isomers. The deduction of the double bond configuration in both compounds was determined from the ^{13}C-NMR chemical shift of the respective methyl group. CH_3-19 of compound **3** resonates at δ_C 14.3 which necessitates that it is on the same side of the double bond as CH_2-20. CH_3-18 of compound **2** is more downfield shifted (δ_C 21.4) and hence the *Z* configuration for $\Delta^{21,22}$ was deduced. Compounds **2** and **3** are thus 19-hydroxylated derivatives of compound **1** and hence the trivial names (*Z*)-coniosclerodinol and (*E*)-coniosclerodinol are suggested for compounds **2** and **3**, respectively.

^{1}H- and ^{13}C-NMR spectra of compound **4** are similar to those of compound **1**, especially concerning the C-1 to C-14 part of the molecule. This evidences that both molecules share the same polycyclic aromatic nucleus. A ^{1}H NMR resonance for H-1 is, however missing in compound **4**. Mass spectrometric analysis of compound **4** revealed an accurate mass of 367.0781 ($C_{18}H_{16}NaO_7$, $[M + Na]^+$), requiring 11 ring double bond equivalents. Seven ring double bond equivalents have been accounted for the aromaticity within the carbon skeleton and for two carbonyl groups suggesting that the molecule is tetracyclic. The fourth ring includes a hydroxylated methylene moiety (δ_H 3.96 for H-19a and δ_H 3.86 for H-19b), two methyl groups (14.5 for C-16 and 20.8 for C-18), of which CH_3-16 is connected to the oxygenated methine CH-15 due to the observed ^{1}H-^{1}H coupling. HMBC correlations allowed to connect the C-15 to C-19 part of the molecule, which is an oxygenated hemiterpene unit. C-17 of this partial structure was attached to C-1 of the aromatic moiety due to heteronuclear long range couplings of CH_3-18 and CH_2-19 to C-1. Ring closure to a dihydrofuran ring occurred *via* the oxygen atom at C-15 to C-14 of the aromatic nucleus. Further proof for this structure came from comparison of the spectroscopic data with those of the known compound **9**.[5] The negative specific optical rotation ($[\alpha]^D$ –140.0) which this compound has in common with the known metabolite **9** and further natural products such as trypethelon having the same structural features,[7] suggested the *S*-configuration at C-15. NOESY correlation between H-15 and CH_3-18 indicated that these substituents are on the same side of the molecule and suggesting the 17*S* configuration. We propose the trivial name sclerodinol for compound **4**.

The molecular formula of compound **5** was deduced by accurate mass measurement to be $C_{18}H_{16}O_6$ (351.0837, $[M + Na]^+$) which requires 11 degrees of unsaturation. The ^{1}H- and ^{13}C-NMR spectra of compound **5** (Tables 1 and 2) are similar to those of compound **1**. Differences in the spectra arose from the arrangement of the carbonyl groups. In compound **5**, an α-keto-lactone group is present, instead of the cyclic anhydride as in compound **1**. This is confirmed by the more downfield shifted ^{13}C-NMR resonance of C-8 (δ_C 131.3 in **5**, δ_C 97.2 for **1**) due to its direct connection to oxygen. Furthermore, comparison of spectroscopic data of **5** with those of compound scleroderolide (**11**) confirmed the α-keto-lactone moiety.[8] Therefore, compounds **5** and **1** are positional isomers. We propose the name coniosclerodero lide for compound **5**.

The ^{1}H- and ^{13}C-NMR spectroscopic data of compound **6** (Tables 1 and 2) are similar to those of compounds **5** and **12**. The ^{13}C-NMR chemical shift of C-8 in comparison to **5** was shifted upfield to δ_C 108.8, indicating that this carbon is not oxygenated. The third ring within this metabolite connects C-8 and C-3 *via* a diketo-bridge which is confirmed by comparing its spectroscopic data with those of sclerodione (**12**).[5,8] The trivial name coniosclerodione is suggested for compound **6**.

In case of compound **7**, NMR resonances for the terpenoid moiety were identical to those of compounds **1**, **5** and **6**. The ^{13}C NMR spectrum of compound **7** (Table 2), however had only one signal for a carbonyl group (C-5, δ_C 165.8). As in compound **5**, the ^{13}C NMR resonance of C-8 is downfield shifted to 130.3 ppm, a value characteristic for an oxygenated aromatic carbon. Clear evidence for the lactone oxygen to be placed at C-8 and not at the close to equivalent position C-3, came from the ^{1}H-^{13}C HMBC correlation between H-10 and C-8. Mass spectrometric analysis suggested the presence of a tricyclic molecule with a lactone

Table 3 MIC values (in μM) toward *Staphylococcus aureus* SG 511 and *Saccharomyces cerevisiae* ATCC 9763 and inhibition zones in agar diffusion assays using *Micrococcus luteus* and *Mycobacterium phlei* (diameter in mm at a concentration of 20 μg/disk)

Compound no.	*Staphylococcus aureus*	*Saccharomyces cerevisiae*	*Micrococcus luteus*	*Mycobacterium phlei*
1	n.d.[a]	n.d.	n.a.[b]	n.a.
2	>300	n.d.	n.a.	16
3	>300	n.d.	n.d.	n.d.
4	n.d.	n.d.	n.a.	20
5	23.8	n.d.	n.a.	10
6	65.7	n.d.	10	12
7	n.d.	n.d.	n.a.	22
8	52.0	>300	n.a.	12
9	n.d.	n.d.	n.a.	n.a.
10	n.d.	n.d.	n.d.	n.d.
11	23.8	95.4	12	14
12	200.0	n.d.	n.a.	10

[a] n.d. = not determined. [b] n.a. = not active. All compounds were not active against *Klebsiella pneumonia* and *Pseudomonas aeruginosa* from HZI as well as *Klebsiella pneumonia* I-10910 and *Pseudomonas aeruginosa* 4991 in agar diffusion assays; all compounds were not active toward *Mycobacterium smegmatis* ATCC 70084 in serial dilution assays.

ring between C-3 and C-8. We give the name coniolactone to compound **7**.

Extensive spectroscopic analyses including 1D- and 2D-NMR, LC/MS and specific optical rotations of compounds **8**, **9**, **10**, **11** and **12** proved that they are lactone **8**,[5] (–) sclerodin (**9**),[5] lamellicolic anhydride (**10**),[6,9] (–) scleroderolide (**11**),[10] and (–) sclerodione (**12**),[5] respectively. ^{13}C NMR spectroscopic data for lactone **8** are described for the first time.

Biological activity

All compounds were evaluated for their antimicrobial and cytotoxic activity. Additionally, inhibitory activity toward the enzymes human leukocyte elastase (HLE), bovine chymotrypsin, bovine trypsin, human thrombin, papain from *Carica papaya*, porcine cholesterol esterase, and acetylcholinesterase from *Electrophorus electricus* was determined. Compounds **5**, **6**, **8**, and **11** showed antimicrobial activity toward *Staphylococcus aureus* SG 511 with MIC values of 24, 66, 52, and 24 μM, respectively (Table 3). Antibacterial activity seems to correlate with the presence of a diketo-lactone ring as found in compounds **5** and **11**, whereas cyclisation of the hemiterpene unit does not influence the activity. Furthermore, the aromatic nucleus alone does not convey activity, since compound **10** has no antibacterial properties. In agar diffusion assays with *Mycobacterium phlei* considerable inhibition zones (>15 mm) were observed for compounds **2**, **4** and **7**, however serial dilution assays toward *M. smegmatis* ATCC 70084 did not reveal any activity (Table 3). Compound **11** had marginal activity against *Saccharomyces cerevisiae* ATCC 9763 with a MIC value of 95 μM (Table 3). Compounds **5** and **11** showed very weak *in vitro* cytotoxicity toward an epithelial bladder carcinoma cell line with IC_{50} values of 27 and 41 μM, respectively. Cytotoxicity was also determined using an MTT assay with mouse fibroblast cells. In these assays only compound **12** had significant activity with an IC_{50} value of 6.4 μM. Compounds **1**, **5** and **9** showed potent inhibition of HLE with IC_{50} values of 7.2, 13.3 and 10.9 μM, respectively (Table 4). HLE belongs to the chymotrypsin family of serine proteinases. Excessive activity of this enzyme can result in diseases such as pulmonary emphysema, rheumatoid arthritis, and cystic fibrosis.[11] All compounds were inactive against chymotrypsin, trypsin, thrombin, papain, cholesterol esterase and acetylcholinesterase.

Table 4 Inhibitory activities toward Human Leukocyte Elastase (HLE)

Compound no.	IC_{50}/μM
1	7.16 ± 1.4
2	n.d.[a]
3	n.d.[a]
4	>20
5	13.3 ± 1.7
6	>20
7	>20
8	>20
9	10.9 ± 2.4
11	>20
12	>20

[a] n.d. = not determined.

Compounds with a phenalenone skeleton occur in higher plants and fungi. Phenylphenalenones originating from plants are phenylpropanoid-derived and suggested to function as phytoalexins with activity towards plant fungal pathogens.[12,13] In contrast, phenalenones produced by fungi lack the phenyl ring and are biosynthesized merely from acetate building blocks. Based on biosynthetic investigations of the known compounds sclerodione, scleroderolide and sclerodin, compounds **1–12** are suggested to arise from a heptaketide intermediate, which after the formation of an intact phenalenone skeleton is oxidatively degraded.[14]

Conclusion

In summary, this paper describes the isolation and structure elucidation of seven new as well as five known phenalenone derivatives from the marine-derived fungus *Coniothyrium cereale*, some of which have potent biological activities. Three of the compounds (**2**, **4**, **7**) showed activity against *Mycobacterium phlei* and two compounds (**5**, **11**) against *Staphylococcus aureus* SG 511. Compounds **1**, **5** and **9** showed potent inhibition of human leukocyte elastase (HLE).

Experimental

General procedures

Melting points were measured on a Büchi 535 apparatus. Optical rotations were measured on a Jasco DIP 140 polarimeter. UV and IR spectra were obtained employing Perkin–Elmer Lambda 40 and Perkin–Elmer Spectrum BX instruments, respectively. All NMR spectra were recorded in $CDCl_3$ employing a Bruker Avance 300 DPX or 500 DRX spectrometer. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 7.26/77.0 for $CDCl_3$, $\delta_{H/C}$ 2.50/39.5 for DMSO-d_6 and $\delta_{H/C}$ 2.04/29.8 for acetone-d_6. HRESIMS were recorded on a Finnigan MAT 95 spectrometer. ESI-MS measurements were recorded employing an API 2000, Applied Biosystems/MDS Sciex. VLC grade material (Macherey–Nagel, Polygoprep 60—50, C_{18}) was used for vacuum liquid chromatography. All organic solvents were distilled prior

to use. HPLC was carried out using a Waters system, controlled by Waters Millenium software, consisting of a 600E pump, a 996 PDA, and a 717 plus autosampler.

Origin of the algal sample, isolation and taxonomy of the fungus

An algal sample *Enteromorpha* sp. was collected from Fehmarn, Baltic Sea. The isolation of the fungus was carried out using an indirect isolation method. Algal samples were rinsed three times with sterile H_2O. After surface sterilization with 70% EtOH for 15 s the alga was rinsed in sterile artificial seawater (ASW).[15] Subsequently, the algae were aseptically cut into small pieces and placed on agar plates containing isolation medium: agar 15 g L^{-1}, ASW 800 mL L^{-1}, glucose 1 g L^{-1}, peptone from soymeal 0.5 g L^{-1}, yeast extract 0.1 g L^{-1}, benzyl penicillin 250 mg L^{-1}, and streptomycin sulfate 250 mg L^{-1}. The fungus growing out of the algal tissue was separated on biomalt medium (biomalt 20 g L^{-1}, agar 10 g L^{-1}, ASW 800 mL L^{-1}) until the culture was pure. The fungus (strain number 401 of the culture collection of the Institute for Pharmaceutical Biology) was identified as *Coniothyrium cereale*. Identification of the fungus was done by Dr C. Decock, BCCM/MUCL, Belgium.

Cultivation

The fungal strain *Coniothyrium cereale* was cultivated for 40 days on 10 L solid BMS medium (Gesundheitsprodukte GmbH, Kirn, Germany) with agar (15 g L^{-1}) at room temperature in 40 Fernbach flasks.

Extraction and isolation

Fungal biomass and media were homogenized using an Ultra-Turrax apparatus and extracted with 8 L EtOAc to yield 10.0 g of crude extract. This material was fractionated by RP VLC using a stepwise gradient solvent system of increasing polarity starting from 50% methanol and 50% water to 100% methanol which yielded 12 fractions. RP-HPLC separation of the subfraction 6 (column: Waters Atlantis C_{18}, 250 × 4.6 mm, 5 µm; acetonitrile–H_2O (50 : 50), 2 mL min^{-1}) afforded compounds **1** and **4–9**, and **11–12**. RP-HPLC separation of the subfraction 12 (column: Waters Atlantis C_{18}, 250 × 4.6 mm, 5 µm; acetonitrile–H_2O (60 : 40), 2 mL min^{-1}) afforded compounds **2** and **3**. RP-HPLC separation of the subfraction 9 (column: Waters Atlantis C_{18}, 250 × 4.6 mm, 5 µm; acetonitrile–H_2O (42 : 58), 2 mL min^{-1}) afforded compound **10**.

Coniosclerodin (1). Yellowish white crystals (100 mg; 10 mg L^{-1}); mp 190–193 °C; UV λ_{max} MeOH/nm (log ε) 250 (4.1), 353 (3.9); IR ν_{max}/cm^{-1} (ATR) 3325, 2922, 1715, 1663, 1599, 1455, 1386, 1300, 1182, 1036; ^1H NMR and ^{13}C NMR data (see Tables 1 and 2); (+)-HRESIMS *m/z* 351.0878 ($C_{18}H_{16}NaO_6$ [M + Na]$^+$ requires 351.0839).

(*Z*)-Coniosclerodinol (2). White crystals (10 mg; 1.0 mg L^{-1}); mp 187–189 °C; UV λ_{max} MeOH/nm (log ε) 260 (4.5), 350 (3.9); IR ν_{max}/cm^{-1} (ATR) 3335, 2919, 1718, 1663, 1597, 1458, 1300, 1181, 1036, 808, 755; ^1H NMR and ^{13}C NMR data (see Tables 1 and 2); (+)-HRESIMS *m/z* 367.0801 ($C_{18}H_{16}NaO_7$ [M + Na]$^+$ requires 367.0788).

(*E*)-Coniosclerodinol (3). White crystals (3 mg; 0.3 mg L^{-1}); UV λ_{max} MeOH/nm (log ε) 274 (4.3), 354 (4.0); IR ν_{max}/cm^{-1} (ATR) 3330, 2920, 1718, 1662, 1597, 1458, 1300, 1181, 1036, 808, 755; ^1H NMR and ^{13}C NMR data (see Tables 1 and 2); HREIMS *m/z* 344.0896 ($C_{18}H_{16}O_7$ [M]$^+$ requires 344.0891).

(15*S*, 17*S*)-(–)-Sclerodinol (4). Yellow amorphous powder (7 mg; 0.7 mg L^{-1}); mp 252–256 °C; $[\alpha]_D^{24}$ – 140 (*c* 0.33 in $CHCl_3$); UV λ_{max} MeOH/nm (log ε) 213 (4.2), 274 (4.0); IR ν_{max}/cm^{-1} (ATR) 3355, 2922, 1741, 1624, 1597, 1364, 1309, 1186, 1032, 855; ^1H NMR and ^{13}C NMR data (see Tables 1 and 2); (+)-HRESIMS *m/z* 367.0781 ($C_{18}H_{16}NaO_7$ [M + Na]$^+$ requires 367.0788).

Coniosclerodinol (5). Yellow crystals (10 mg; 1 mg L^{-1}); mp 201–202 °C; UV λ_{max} MeOH/nm (log ε) 245 (4.6), 428 (4.0); IR ν_{max}/cm^{-1} (ATR) 3335, 2925, 1736, 1669, 1583, 1465, 1361, 1308, 1180, 1031; ^1H NMR and ^{13}C NMR data (see Tables 1 and 2); (+)-HRESIMS *m/z* 351.0837 ($C_{18}H_{16}NaO_6$ [M + Na]$^+$ requires 351.0839).

Coniosclerodione (6). Red crystals (8 mg; 0.8 mg L^{-1}); UV λ_{max} MeOH/nm (log ε) 251 (4.5), 354 (3.9); IR ν_{max}/cm^{-1} (ATR) 3330, 2924, 1708, 1606, 1441, 1379, 1299, 1187, 1035; ^1H NMR and ^{13}C NMR data (see Tables 1 and 2); (+)-HRESIMS *m/z* 335.0902 ($C_{18}H_{16}NaO_5$ [M + Na]$^+$ requires 335.0890).

Coniolactone (7). Reddish white crystals (15 mg; 1.5 mg L^{-1}); mp 216–217 °C; UV λ_{max} MeOH/nm (log ε) 260 (4.3), 340 (3.8); IR ν_{max}/cm^{-1} (ATR) 3330, 2923, 1715, 1675, 1381, 1182, 1017; ^1H NMR and ^{13}C NMR data (see Tables 1 and 2); (+)-HRESIMS *m/z* 323.0896 ($C_{17}H_{16}NaO_5$ [M + Na]$^+$ requires 323.0890).

(–)-7,8-Dihydro-3,6-dihydroxy-1,7,7,8-tetramethyl-5*H*-furo-[2′,3′:5,6]naphtho[1,8-bc]furan-5-one (8). Reddish crystals (12 mg; 1.2 mg L^{-1}); mp 230–232 °C; $[\alpha]_D^{24}$ – 55 (*c* 0.6 in $CHCl_3$); UV λ_{max} MeOH/nm (log ε) 261 (4.3), 358 (3.7); IR ν_{max}/cm^{-1} (ATR) 3295, 2923, 1704, 1643, 1625, 1529, 1481, 1378, 1140; ^1H NMR (300 MHz; $CDCl_3$) δ 6.63 (1H, brs, H-10), 4.62 (1H, q, *J* = 6.6 Hz, H-15), 2.57 (3H, s, H_3-12), 1.49 (3H, s, H_3-18), 1.44 (1H, d, *J* = 6.6 Hz, H-16), 1.24 (3H, s, H_3-19); ^{13}C NMR (75 MHz; $CDCl_3$) δ 167.4 (C, C-5), 164.9 (C, C-14), 156.3 (C, C-2), 137.1 (C, C-9), 133.8 (C, C-4), 132.5 (C, C-11), 128.9 (C, C-8), 120.9 (C, C-1), 118.3 (CH, C-10), 107.3 (C, C-13), 94.9 (C, C-3), 92.0 (CH, C-15), 43.3 (C, C-17), 25.5 (CH_3, C-18), 20.9 (CH_3, C-12), 20.0 (CH_3, C-19), 14.4 (CH_3, C-16); (+)-ESIMS *m/z* 301.0 [M + H]$^+$.

(–)-Sclerodin A (9). White crystals (100 mg; 10 mg L^{-1}); $[\alpha]_D^{24}$ – 73 (*c* 1.0 in $CHCl_3$), (+)-ESIMS *m/z* 329.4 [M+H]$^+$. All spectroscopic data were identical with the previously reported data.[5]

Lamellicolic anhydride (10). Pale yellow crystals (5 mg; 0.5 mg L^{-1}); ^1H NMR (300 MHz; DMSO-d_6) δ 6.86 (1H, brs, H-10), 6.36 (1H, s, H-1), 2.81 (3H, s, H_3-12), ^{13}C NMR (75 MHz; DMSO-d_6) δ 166.4 (C, C-2), 164.2 (C, C-9), 150.4 (C, C-11), 116.7 (CH, C-10), 112.3 (C, C-13), 99.9 (CH, C-1), 97.7 (C, C-8), 90.9 (C, C-3) 24.8 (CH_3, C-12); not detected (C-4, C-5, C-7, C-14); (+)-ESIMS *m/z* 261.3 [M + H]$^+$. All spectroscopic data were identical with the previously reported data.[9]

(–) Scleroderolide (11). Yellow crystals (15 mg; 1.5 mg L^{-1}); $[\alpha]_D^{24}$ – 119 (*c* 0.2 in $CHCl_3$), (+)-ESIMS *m/z* 329.4 [M + H]$^+$. All spectroscopic data were identical with the previously reported data.[10]

(–) Sclerodione (12). Red crystals (11 mg; 1.1 mg L^{-1}); $[\alpha]_D^{24}$ – 118 (*c* 0.3 in $CHCl_3$), (+)-ESIMS *m/z* 313.3 $[M+H]^+$. All spectroscopic data were identical with the previously reported data.[5]

HLE inhibition assay

HLE was assayed spectrophotometrically at 405 nm at 25 °C.[16] Assay buffer was 50 mM sodium phosphate buffer (500 mM NaCl, pH 7.8). A stock solution of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-*para*-nitroanilide was prepared in DMSO and diluted with assay buffer. Inhibitor stock solutions (10 mM) were prepared in DMSO. Final concentration of DMSO was 1.5%, the final concentration of the substrate was 100 μM. Assays were performed with a final HLE concentration of 50 ng mL^{-1}. An inhibitor solution (10 μL) and the substrate solution (50 μL) were added to a cuvette that contained the assay buffer (890 μL), and the solution was thoroughly mixed. The reaction was initiated by adding the HLE solution (50 μL) and was followed over 10 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate. HLE inhibition by active compounds was determined in duplicate experiments with five different inhibitor concentrations. Inactive compounds ($IC_{50} > 20$ μM) were evaluated in duplicate measurements at a concentration of 25 μM.

Enzyme assays

The other enzyme assays were performed as described.[17,18] Compounds were evaluated in duplicate measurements at a single concentration of 25 μM (chymotrypsin, trypsin, papain, cholesterol esterase, acetylcholinesterase) or 100 μM (thrombin). IC_{50} values were calculated ($IC_{50} = [I]/(v_0/v - 1)$), and limits $IC_{50} > 500$ μM (trypsin), $IC_{50} > 100$ μM (chymotrypsin, papain), $IC_{50} > 75$ μM (acetylcholinesterase), $IC_{50} > 50$ μM (thrombin, cholesterol esterase) were considered for inactive compounds.

Cytotoxicity test

Human urinary bladder carcinoma cells 5637 [ATCC HTB-9] were grown as a monolayer in RPMI (Lonza, Verviers, BE) supplemented with 10% fetal bovine serum (Sigma, Deisenhofen, Germany) and 1% penicillin-streptomycin solution (penicillin 10 000 IE/mL; streptomycin 10 000 μg mL^{-1}, Biochrom AG, Berlin, Germany). Cells were grown at 37 °C in 95% air humidity, and 5% CO_2, and sub-cultured twice weekly using trypsine/EDTA (0.05%/0.02%, Lonza).

For assays, 5600 cells from a suspension of 2.5×10^4 cells mL^{-1} were seeded into each well of 96-well plates (TPP, Trasadingen, CH) and incubated for 24 h. After washing with HBSS (PAA, Cölbe, Germany), fresh medium was added. Test substances were diluted in assay medium using a stock solution (40 mM in vehicle DMSO). Final vehicle concentration did not exceed 0.05%. Plates were left undisturbed for 70 h at 37 °C. Finally, plates were washed twice with HBSS and incubated with freshly prepared neutral red in RPMI (3.3 μg mL^{-1}) in the incubator for 3 h. After removing the supernatant and extensive washing, neutral red was dissolved in acidic ethanol and O.D. at 540 nm was measured. Etoposide was used as toxic control (IC_{50} 0.32 μM), cell culture medium as non-toxic control. Cell viability was calculated as percentage of vehicle control after background reduction. All experiments were carried out twice or thrice with 6 replicates for each concentration tested. Where applicable, IC_{50} values were calculated by linear regression.

MTT assays with mouse cell line L-929

An MTT assay was used to measure the influence of compounds on the propagation and viability of L-929 mouse fibroblasts (DSMZ ACC2) in 96-well plates. Cells are able to reduce MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] (Sigma) to a violet formazan product. The resulting purple colour gives a measure of the metabolic activity in each well. Cells were kept in DME medium supplemented with 10% FBS.

60 μL of serial dilutions of the test compounds were added to 120 μL aliquots of a cell suspension (50.000/mL) in each well. Blank and solvent controls were incubated under identical conditions. After 5 days 20 μL MTT in phosphate buffered saline (PBS) were added to a final concentration of 0.5 mg mL^{-1}. After 2 h the precipitate of formazan crystals was centrifuged, and the supernatant discarded. The precipitate was washed with 100 μL PBS and dissolved in 100 μL isopropanol containing 0.4% hydrochloric acid. The microplates were gently shaken for 20 min to ensure a complete dissolution of the formazan and finally measured at 590 nm using a plate reader. All experiments were carried out in two parallel experiments, the percentage of viable cells was calculated as the mean with respect to the controls set to 100%. An IC_{50} value was determined from the resulting dose-response curves.

Agar diffusion assays

a) Agar plates containing 15 mL of medium were inoculated with bacterial or yeast suspensions in liquid broth to give a final O.D. of 0.01 (bacteria) or 0.1 (yeasts). In the case of molds, spores were collected from well-grown Petri dishes that were rinsed with 10 mL sterile aqua dest. 1 mL of the spore suspension was added to 100 mL of molten agar medium. 20 μL of test samples in methanol (1 mg mL^{-1}) were applied onto 6 mm cellulose discs. The methanol was allowed to evaporate and the discs were placed upon the inoculated agar plate. The diameters in mm of the resulting growth zones were determined after 24 h of incubation at 30 °C (test organisms: *Micrococcus luteus*; *Mycobacterium phlei*; *Klebsiella pneumonia*; *Pseudomonas aeruginosa*; all from HZI).

b) Agar plates (5% sheep blood, Columbia agar) were overlaid with 3 mL tryptic soy soft agar, incoculated with 10^4 colony forming units of the strains to be tested. All compounds were diluted to a concentration of 1 mg mL^{-1} with DMSO and 5 μL of this dilution were placed on the surface of the agar; the diameter of the inhibition zone was measured after 24 h incubation at 37 °C (test organisms: *Klebsiella pneumonia* I-10910; *Pseudomonas aeruginosa* 4991).

MIC determinations

MIC determinations were carried out in microtiter plates according to NCCLS standards with twofold serial dilutions of the compounds. Strains were grown in half-concentrated Mueller–Hinton broth (Oxoid). Bacteria were added at an inoculum of 10^5 CFU/mL in a final volume of 0.2 mL. After incubation for 24 h

at 37 °C the MIC was read as the lowest compound concentration causing inhibition of visible growth.

Acknowledgements

Financial support came from the Egyptian Government for M. E. We are grateful for financial support from the Deutsche Forschungsgemeinschaft (FOR 845). The authors wish to thank Stephanie Hautmann, Wera Collisi and Qian Lu for technical assistance. This paper is dedicated to Professors: Hany M. Helal, Hassan Elrady A. Saad and Tharwat Abd-Elhameed for their support.

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