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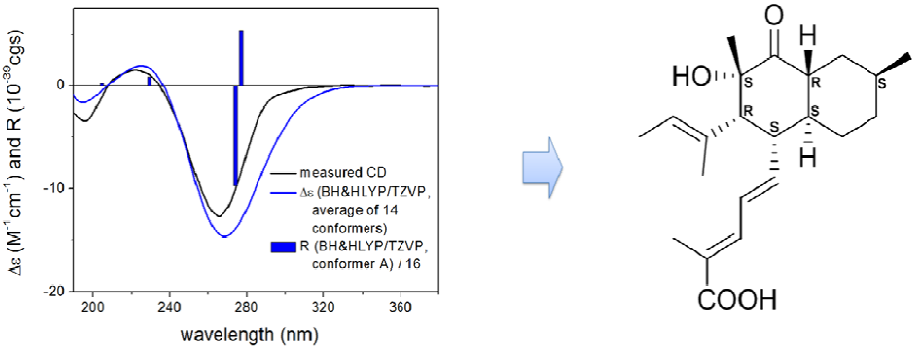
Graphical abstract



A new fusarielin analogue from *Penicillium* sp. isolated from the Mediterranean sponge *Ircinia oros*

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Abstract

The marine-derived fungus *Penicillium* sp. (strain IO1) isolated from the Mediterranean sponge *Ircinia oros* yielded a new fusarielin analogue (**1**) together with the known compounds griseofulvin (**4**) and dechlorogriseofulvin (**5**). The structure of **1** was unambiguously elucidated by comprehensive spectroscopic analysis (1D and 2D NMR, and mass spectrometry) as well as by comparison with the literature, while the absolute configuration of **1** was determined on the basis of TDDFT ECD calculations. A further *Penicillium* sp. (strain IO2) that was isolated from the same sponge *I. oros* yielded the known compounds dehydrocurvularin (**6**), curvularin (**7**), and trichodimerol (**8**). Co-cultivation of both *Penicillium* strains (IO1 and IO2) was found to induce the accumulation of the known norlichexanthone (**2**) and monocerin (**3**) that were not detected in either of the two axenic fungal controls. Compounds **3** and **6** showed pronounced cytotoxicity against the murine lymphoma (L5178Y) cell line with IC_{50} values of 8.4 and 4.7 μM, respectively.

Keywords: *Penicillium* sp.; absolute configuration; co-cultivation; cytotoxicity; sponge-derived fungus

Marine-derived fungi have received considerable attention in recent years due to their capacity to produce structurally unique and bioactive metabolites as potential sources of pharmaceutical leads.[1-3] Examples include neoechinulin B and its analogues, isolated from the marine-derived fungus *Eurotium rubrum*, as potent inhibitors of a panel of influenza viruses including resistant strains,[4] as well as the anthraquinone derivative lunatin from the sponge-derived fungus *Curvularia lunata*, which showed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*.[5] Marine invertebrates, such as sponges (Porifera), continue to be one of the most important sources of fungal isolates when compared to algae, sediment or drift wood.[6] Fungal symbionts of sponges are suggested to play a role in the biosynthesis of bioactive secondary metabolites used as chemical defense by their hosts,[7] which makes them attractive as promising sources of new bioactive metabolites.

In our ongoing studies on bioactive compounds from sponge-associated fungi, we investigated an unknown *Penicillium* sp. (IO1), which was isolated from the Mediterranean sponge *Ircinia oros* collected at Kemer of Antalya in Turkey. Subsequent bioactivity-guided isolation yielded a new fusarielin derivative (**1**) and two known compounds (**4** and **5**). Co-cultivation of this fungus with a further *Penicillium* sp. (IO2) isolated from the same sponge resulted in the accumulation of the known compounds (**2** and **3**) that were not present in either of the fungal axenic controls.

Results and Discussion

From the Mediterranean sponge *I. oros* two *Penicillium* strains (IO1 and IO2) were isolated that differed with regard to their natural products when grown on solid rice medium. The crude EtOAc extract of *Penicillium* sp. strain IO1, was submitted to chromatographic separation using silica gel and Sephadex LH-20 as stationary phases followed by purification with semi-preparative reversed phase HPLC to yield one new compound (**1**) and two known compounds, including griseofulvin (**4**)[8] and dechlorogriseofulvin (**5**)[8] (Figure 1).

Compound **1**[9] was isolated as a pale yellow gel. The molecular formula was determined as

$C_{22}H_{32}O_4$ on the basis of a prominent ion peak at *m/z* 361.2369 $[M + H]^+$ observed in the HRESIMS spectrum, indicating seven degrees of unsaturation. The ^1H NMR spectrum of **1** revealed the presence of three tertiary methyl groups at δ_H 1.94 (3H, d, J = 1.5 Hz, H_3-19), 1.55 (3H, s, H_3-21) and 1.50 (3H, s, H_3-22), two secondary methyl groups at δ_H 1.58 (3H, d, J = 6.0 Hz, H_3-18) and 1.01 (3H, d, J = 7.0 Hz, H_3-20), and four olefinic protons at δ_H 7.13 (1H, dd, J = 11.4, 1.5 Hz, H-3), 6.51 (1H, dd, J = 15.0, 11.4 Hz, H-4), 5.70 (1H, dd, J = 15.0, 9.8 Hz, H-5) and 5.01 (1H, m, H-17) (Table 1). The ^{13}C NMR showed a total of 22 resonances (Table 1) assigned to eight sp^2 carbons [including two carbonyl groups at δ_C 216.5 (C-13) and 171.9 (C-1)], five methyl groups [δ_C 29.1 (C-21), 18.1 (C-20 and C-22), 13.5 (C-18) and 12.7 (C-19)], eight aliphatic sp^3 carbons (including three methylenes and five methines), and one oxygenated quaternary carbon at δ_C 78.5 (C-14), as supported by DEPT and HSQC spectra (Figure S4 and S6).

Detailed analysis of the ^1H-^1H COSY spectrum disclosed the presence of a continuous spin system starting from H-3 and sequentially extending until H-12 with H-10 further correlating with an aliphatic methyl (H_3-20) (Figure 2a). In addition, COSY correlations from H-6 to H-15 and from H-7 to H-12 were observed. The HMBC correlations from H-15 (δ_H 2.76) to C-13, C-14, C-6 (δ_C 49.3), and C-7 (δ_C 45.8); H_3-21 to C-13, C-14 and C-15 (δ_C 66.0); and H-12 (δ_H 2.60) to C-6 and C-13 further extended this substructure from C-12 to C-15 (Figure 2a), thus indicating the presence of a decalone ring similar to that of fusarielins and rapiculin.[10, 11] Further HMBC correlations from H-4 to C-2 (δ_C 127.0), C-3 (δ_C 139.5), and C-6, and from H_3-19 to C-1, C-2 and C-3 corroborated the presence of a 2-methylpenta-2,4-dienoic acid moiety and its connection to the decalone moiety at C-6. Moreover, a but-2-en-2-yl group was attached at C-15, as supported by the COSY correlation between H-17 and H_3-18, as well as by the HMBC correlations from H_3-22 to C-15, C-16 (δ_C 133.8) and C-17 (δ_C 125.6) (Figure 2a), thereby rationalizing the remaining element of unsaturation. Thus, the planar structure of **1** was established as shown in Figure 1.

The geometry of the double bonds in the 2-methylpenta-2,4-dienoic acid side chain was deduced as *E* based on the ROESY correlation between H_3-19 and H-4 and the large coupling constant between H-4 and H-5 ($^3J_{4,5}$ = 15.0 Hz), respectively. Likewise, the ROESY correlation between H_3-22 and H_3-18 indicated the 16*E* configuration of the double bond in

the but-2-en-2-yl group (Figure 2b).

Accordingly, the relative configuration of the stereocenters of the decalone moiety of **1** was deduced by analysis of the coupling constants and the ROESY spectrum (Figure 2b). The axial orientations of H-12, H-7, and H-6 were suggested by the large vicinal coupling constants ($^3J_{6,7}$ = $^3J_{7,12}$ = 11.6 Hz), hence indicating the presence of a trans-decalone ring as observed in fusarielins.[10] This was further corroborated by the absence of a cross-peak between H-7 and H-12 in the ROESY spectrum of **1**. Moreover, the relatively small coupling constant between H-6 and H-15 ($^3J_{6,15}$ = 5.4 Hz) revealed the equatorial orientation for the latter and subsequently the axial orientation for the but-2-en-2-yl group. The ROESY spectrum further showed correlations of H-12 with H_3-20, H_3-21 and H-6, which confirmed the co-facial orientation of these protons and the (6*S**,7*S**,10*S**,12*R**,14*S**,15*R**) relative configuration (Figure 2b).

The conformers differed in the orientation of the C-15 substituent and the carboxyl group (Figure S9). All the conformers reproduced the intense negative CE at 266 nm, and the Boltzmann-weighted TDDFT-ECD spectra computed with three functionals (B3LYP, BH&HLYP, PBE0) and TZVP basis set for (6*S*,7*S*,10*S*,12*R*,14*S*,15*R*)-**1** gave good agreement with the experimental ECD spectrum, although the intensity of the 218 positive CE was underestimated (Figure S10).

In order to improve the agreement, the reoptimization of the MMFF conformers of (6*S*,7*S*,10*S*,12*R*,14*S*,15*R*)-**1** was also carried out at B97D/TZVP level with PCM for acetonitrile, which afforded 14 conformers above 2% population (Figure 3). The Boltzmann-weighted TDDFT-ECD spectra of these conformers also reproduced the positive 218 CE of the experimental spectrum with the BH&HLYP/TZVP method providing the best agreement, which allowed determining the absolute configuration as (6*S*,7*S*,10*S*,12*R*,14*S*,15*R*) (Figure 4).

The ECD calculations also revealed that the negative 266 nm CE is governed by the $\pi\pi^*$ transition of the C-6 and C-15 side-chains (Figure S11). On the basis of the above data, **1** was identified as a new natural product, for which the name fusarielin I is proposed.

The crude EtOAc extract of the second *Penicillium* strain (IO2) analyzed in this study yielded three known compounds namely dehydrocurvularin (**6**),[12] curvularin (**7**),[12] and

trichodimerol (**8**),[13] as evident by comparison of their NMR and mass spectroscopic data with the literature.

Interestingly, co-cultivation of the two *Penicillium* sp. strains (IO1 and IO2) induced the production of two known compounds norlichexanthone (**2**)[14] and monocerin (**3**),[15] which were not detected in either of the axenic fungal controls (Figure S12). Co-cultivation of microorganisms has repeatedly been shown to induce the formation of compounds that are not detected when the respective microorganisms are grown under axenic conditions.[16] This elicitation of natural product accumulation is believed to be caused by competition/antagonism of different microorganisms and has been shown to be due to an activation of biogenetic gene clusters that remain silent under axenic conditions.[17,18]

All compounds analyzed in this study were submitted to a cellular cytotoxicity (MTT) assay employing the L5178Y mouse lymphoma cell line. Compounds **3** and **6** exhibited significant cytotoxicity with IC_{50} values of 8.4 and 4.7 μM, respectively, compared to kahalalide F as a positive control (IC_{50} 4.3 μM). Monocerin (**3**) was previously reported as antifungal, insecticidal, and phytotoxic secondary metabolite from several fungal species.[19,20] The antimicrobial activity of **2** against *Staphylococcus aureus, Sarcina ventriculi, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger* and *Fusarium oxysporum* was also described with MIC values similar to those of ampicillin and nystatin.[21] Therefore, it could be assumed that the production of **2** and **3** during co-cultivation is triggered by one of these fungi as a stress response to suppress the growth of its competitor.

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Supplementary data

Supplementary data (experimental section and compound characterization, as well as

figures of structures and populations of the conformers of **1** obtained by reoptimization of the initial MMFF conformers and Kohn-Sham orbitals of **1** responsible for the 266 nm negative π-π* [HOMO-1 (a)→LUMO (b)] transitions) associated with this article can be found, in the online version.

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9. *Fusarielin I* (**1**): Pale yellow gel; $[\alpha]_D^{20}$ −108 (*c* 0.1, MeOH); UV/Vis (λ_{max}, MeOH) (log ε): 267 (4.1) nm; ECD (MeCN, λ[nm] (Δε), $c = 4.99 \times 10^{-4}$ M): 266 (−12.32), 218 (1.64), 194 (−3.81); ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data in CD_3OD see Table 1; HRESIMS *m/z* 361.2369 $[M + H]^+$ (calcd for $C_{22}H_{33}O_4$ 361.2373).

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Table 1 1D and 2D NMR spectral data of **1** at 600 (^{1}H) and 150 (^{13}C) MHz (in CD_3OD, δ in ppm).

No.	δ_C	δ_H (J in Hz)	HMBC
1	171.9, qC		
2	127.0, qC		
3	139.5, CH	7.13, dd (11.4, 1.5)	1, 2, 5, 19
4	128.5, CH	6.51, dd (15.0, 11.4)	2, 3, 6
5	145.3, CH	5.70, dd (15.0, 9.8)	2, 3, 6, 7
6	49.3, CH	2.88, ddd (11.6, 9.8, 5.4)	4, 5, 7, 8, 15, 16,
7	45.8, CH	1.75, qd (11.6, 2.7)	6, 8, 9, 12
8	28.14, CH_2	1.51, m 1.31, m	
9	32.0, CH_2	1.50, m	
10	28.10, CH	2.12, m	
11	32.5, CH_2	1.80, dd (13.6, 1.8) 1.32, m	9, 10, 12
12	44.9, CH	2.60, td (11.6, 2.9)	6, 7, 8, 11, 13
13	216.5, qC		
14	78.5, qC		
15	66.0, CH	2.76, d (5.4)	5, 6, 7, 13, 14, 16, 17, 21, 22
16	133.8, qC		
17	125.6, CH	5.01, m	
18	13.5, CH_3	1.58, d (6.0)	16, 17
19	12.7, CH_3	1.94, d (1.5)	1, 2, 3
20	18.1, CH_3	1.01, d (7.0)	9, 10, 11
21	29.1, CH_3	1.55, s	13, 14, 15
22	18.1, CH_3	1.50, s	15, 16, 17

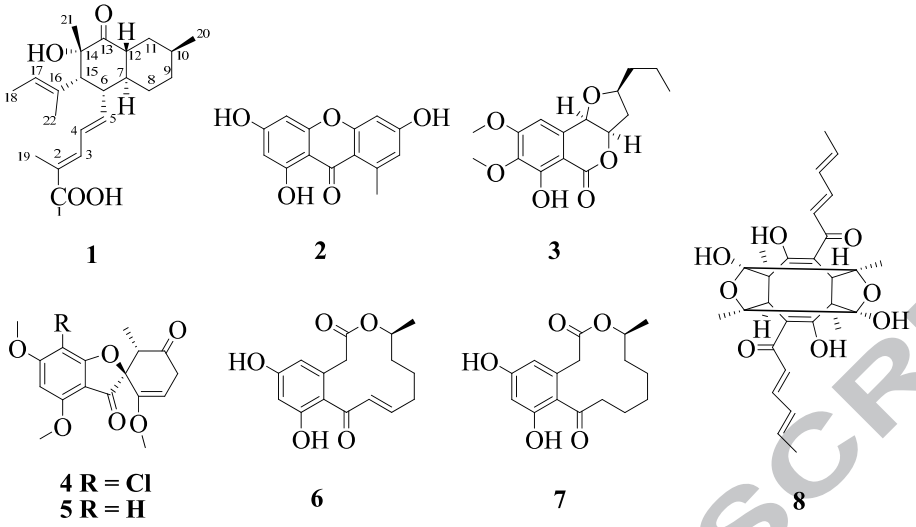
Figure 1 structures of isolated compounds.

Figure 2 (a) ^{1}H-^{1}H COSY (bold lines) and key HMBC (arrows) correlations of **1**.

(b) key ROESY correlations of **1**.

Figure 3 Structures and populations of the conformers of (6*S*,7*S*,10*S*,12*R*,14*S*,15*R*)-**1** obtained by the B97D/TZVP reoptimization of the initial MMFF conformers with PCM for acetonitrile

Figure 4 Comparison of the experimental ECD of **1** (black) with the Boltzmann-weighted BH&HLYP/TZVP ECD spectra of (6*S*,7*S*,10*S*,12*R*,14*S*,15*R*)-**1** (blue) computed for the 14 B97D/TZVP (PCM for acetonitrile) conformers. Bars represent the computed rotational strength of the lowest-energy conformer.



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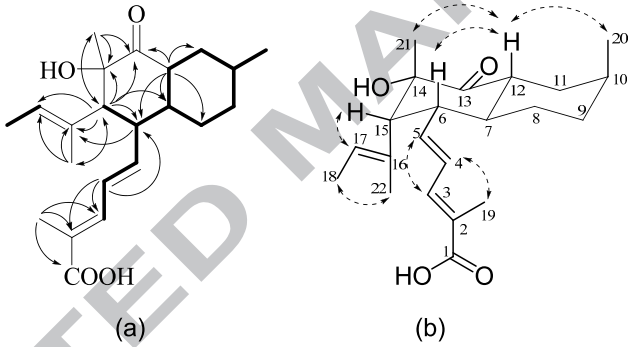

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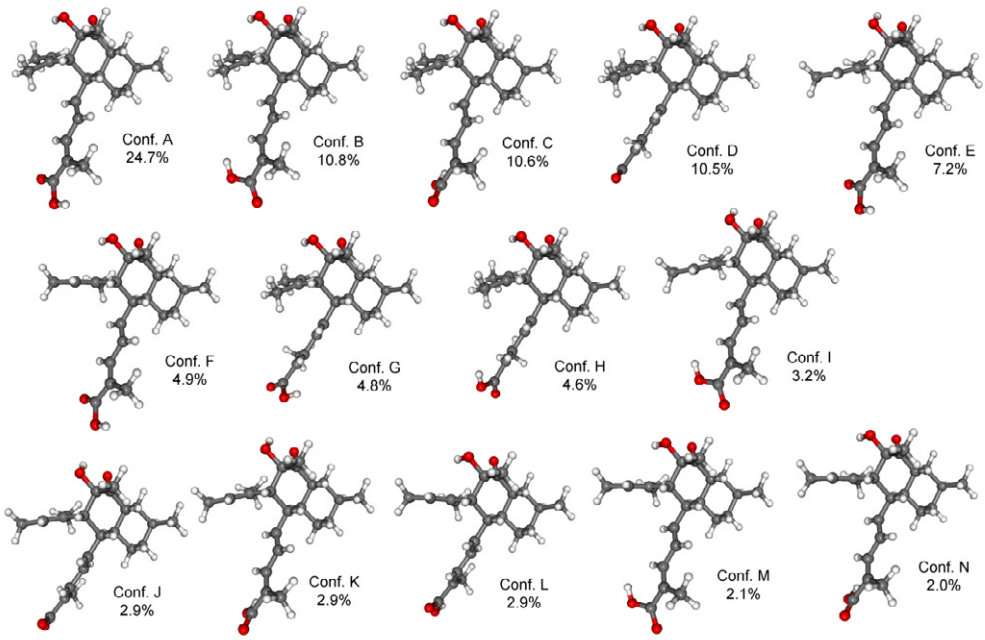

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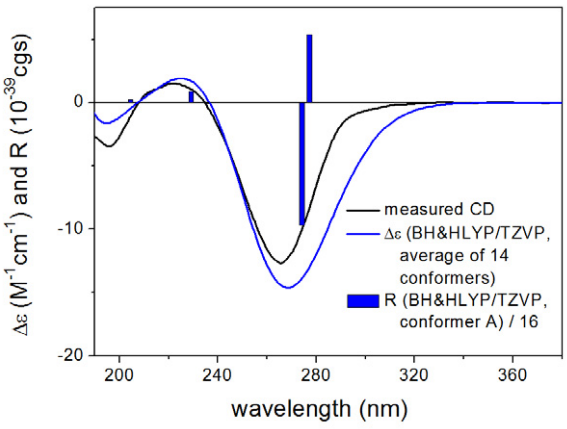

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