Trichopyrone and Other Constituents from the Marine Sponge-Derived Fungus *Trichoderma* sp.

Ahmed Abdel-Lateff[a,b], Katja Fisch[a,c], and Anthony D. Wright[d,*]

[a] Institute for Pharmaceutical Biology, University of Bonn, Nussallee 6, D-53115 Bonn, Germany
[b] Current address: Pharmacognosy Department, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt
[c] Current address: Kekulé Institute of Organic Chemistry and Biochemistry, University of Bonn, Gerhard-Domagk-Straße 1, D-53121 Bonn, Germany
[d] College of Pharmacy, University of Hawaii at Hilo, 34 Rainbow Drive, Hilo 96720, Hawaii, USA. Fax: +1 80 89 33 29 74. E-mail: adwright@hawaii.edu

* Author for correspondence and reprint requests

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The fungus *Trichoderma viride* was isolated from the Caribbean sponge *Agelas dispar*, which was collected from waters around the island of Dominica. Its EtOAc extract, exhibiting mild radical scavenging properties, was mass cultivated and found to produce a new pyranone derivative, trichopyrone (**1**), and ten compounds, namely four sorbicillinoid polyketide derivatives, trichodermanone A–D (**2**–**5**), two hexaketide derivatives, rezishanone (**6**) and vertinolide (**7**), three known dodecaketides, trichodimerol (**8**), bislongiquinolide (trichotetronine, **9**), and bisvertinol (**10**), as well as 2-furancarboxylic acid (**11**). The structures of all compounds were determined by interpretation of their spectroscopic data (1D and 2D NMR, MS, UV and IR). The biological activities of all isolates were evaluated in a series of bioassays (radical scavenging, antioxidant, antimicrobial, inhibition of HIV-1 RT). The majority had very weak or no effects in the applied test systems.

Key words: Marine Fungi, Pyranone, Trichopyranone

Introduction

Marine-derived fungi are a known source of structurally diverse, biologically active natural compounds. In this context, fungi associated with sponges have been found to yield a variety of structurally diverse natural products, *e. g*. microsphaeropsin and ulocladol (König and Wright, 1996, 1999; König *et al*., 2006; Höller *et al*., 2000). Fungi of the genus *Trichoderma* are widespread in both terrestrial and marine environments (Andrade *et al*., 1992), *e. g*. soil, and in association with higher plants and sponges (Sperry *et al*., 1998). *Trichoderma* sp. are noted for their diverse secondary metabolite chemistry that is not characterized by any clear pattern concerning the structural types so far discovered from fungi belonging to this genus. To date, some 500 compounds have been reported from fungi of the genus *Trichoderma*, including the bicyclic dodecanone koninginin G (Cutler *et al*., 1999), the carotane derivatives trichocaranes A–D (Macías *et al*., 2000), and the cyclopentenones pentenocins A and B (Matsumoto *et al*., 1999).

Pyranone derivatives are widespread in nature, commonly occurring in a number of higher plant families and fungi, such as arzanol (Rosa *et al*., 2007), ampelopyrone (Aly *et al*., 2008), and citreo-γ-pyrone (Takashi *et al*., 1999).

The current secondary metabolite investigation was undertaken with the fungus *Trichoderma viride* (Hypocreaceae, Ascomycota) (Persoon, 1974), isolated from the Caribbean sponge *Agelas dispar* J. (order: Agelasida, family: Agelasidae), collected from the waters around the island of Dominica. The fungus was cultivated on a solid glucose biomalt medium with added artificial sea water. Successive fractionation of the EtOAc extract, produced by extraction of the cultivation media and associated fungal mycelia, by vacuum liquid chromatography (VLC) over reversed phase (RP-18) silica followed by reversed phase (RP-18) HPLC yielded a new pyranone derivative, trichopyrone (**1**), and ten known compounds, namely four sorbicillinoid polyketide derivatives, trichodermanone A–D (**2**–**5**) (Neumann *et al*., 2007), two hexaketide derivatives, rezishanone (**6**) (Maskey

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et al., 2005) and vertinolide (**7**) (Trifonov *et al.,* 1982), three dodecaketides, trichodimerol (**8**) (Abe *et al.*, 1998a), bislongiquinolide (trichotetronine, **9**) (Abe *et al.,* 1999), and bisvertinol (**10**) (Trifonov *et al.,* 1986), as well as 2-furancarboxylic acid (**11**) (Corey *et al.*, 1958) (Fig. 1).

Results and Discussion

The molecular formula of **1** was determined as $C_{12}H_{16}O_4$ from positive ion mode ESIMS, HREIMS and NMR measurements. In the ^{13}C NMR spectrum of **1**, 12 resonances were observed and assigned to two methyl groups, a methylene group, three sp^2-hybridized methine groups, an aliphatic methine group, a methoxy group and four sp^2-hybridized quaternary carbon atoms; see Table I. It was evident from these data that only four of five degrees of unsaturation within **1** are due to multiple bonds, three carbon-carbon double bonds and a carbonyl group. The molecule is monocyclic. The ^1H and ^{13}C NMR spectral data of **1** could explain all but one hydrogen atom; the remaining hydrogen must be present as part of a hydroxy function, a deduction supported by the IR data (λ_{max} 3384 cm^{-1}). This latter deduction meant that the two remaining oxygen atoms had to be present in the form of ethers or esters. Also evident from the ^1H and ^{13}C NMR data of **1** were resonances consistent with the presence of a tri-substituted pyran-2-one ring, a deduction supported by the UV maximum at 278 nm (Cutignano *et al*., 2007). After association of all ^1H and ^{13}C NMR resonances, associated with C–H one-bond interactions, from cross-peaks seen in the ^1H-^{13}C 2D NMR shift-correlated (HMQC) spectrum of **1**, it was possible to deduce the planar structure of the molecule by interpretation of its ^1H-^1H COSY and ^1H-^{13}C HMBC spectral data. From the ^1H-^1H COSY spectrum of **1**, a ^1H-^1H spin system from H_3-10 to H-6 was evident, giving rise to this part of the molecule. Support for this molecular fragment also came from diagnostic cross-peaks in the HMBC spectrum observed between H_3-10 and both C-9 and C-8, and between H_2-8 and C-7. The *Z*-geometry was assigned to $\Delta^{6,7}$ on the basis of $J_{H\text{-}6,\,H\text{-}7}$ being 15.7 Hz. Because of characteristic long-range correlations observed between H-4 and C-2, C-3, C-5 and C-6, it was evident that the C-6 to C-10 side-chain was attached to the pyrone ring through a bond between C-5 and C-6. A characteristic long-range correlation, this time observed between H_3-12 and C-3, meant that the methoxy group is attached to C-3. Further, long-range ^1H-^{13}C HMBC correlations observed from the resonance of H_3-11 to those of C-1, C-2, C-3 and C-4, enabled the remaining methyl group to be placed at C-2, leaving the hydroxy group to be positioned at C-9 (δ 68.2, d). For **1**, a new pyrone derivative, the trivial name trichopyrone is

Table I. ^1H [$(CD_3)_2CO$, 300 MHz] and ^{13}C NMR [$(CD_3)_2CO$, 75.5 MHz] spectral data of **1**[a], and ^{13}C NMR [$(CD_3)_2CO$, 75.5 MHz] spectral data of **12**.

Position	δ_C **1**	δ_C **12**	δ_H **1**	HMBC
1	165.1 s	165.1 s		
2	103.3 s	102.0 s		
3	168.6 s	165.9 s		
4	96.9 d	91.6 d	6.44 (1H, s)	C-2, C-3, C-5, C-6
5	158.7 s	160.2 s		
6	126.1 d	126.1 s	6.20 (1H, d, 15.7)	
7	137.0 d	135.3 d	6.65 (1H, d, 15.7)	
8	44.5 t	30.7 t	2.35 (2H, dd, 7.7, 2.9)	
9	68.2 d	22.2 t	3.91 (1H, dq, 5.9, 2.9)	
10	25.2 q	13.9 q	1.16 (3H, d, 5.9)	C-8, C-9
11	10.8 q	8.6 q	1.84 (3H, s)	C-1, C-2, C-3, C-4
12	57.8 q	56.0 q	3.95 (3H, s)	C-3
13		12.4 q		
OH-9			3.98 (1H, m)	

[a] All assignments are based on 1D and 2D measurements (HMBC, HMQC, COSY). Implied multiplicities were determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q). *J* in Hz.

proposed (Fig. 1). Prior to the isolation of **1**, the 1-methyl-9-dehydroxy derivative **12** was reported (Schueffler *et al.*, 2007); its NMR spectroscopic data are in good agreement with those reported here for **1** at corresponding centres.

Together with the new compound **1** the previously reported compounds **2**–**11** were identified by comparison of their spectroscopic data with published values. The EtOAc extract and all compounds, except for **11**, were tested in ELISA-based assays for their HIV-1 reverse transcriptase inhibitory activity (Eberle and Seibel, 1992), compound **6** being the only one to show any activity (63.8% at 200 μg/mL). Antimicrobial activities were measured for all compounds, and the EtOAc extract, using various test organisms in agar diffusion assays (Höller *et al.,* 2000); no significant activity was found in these assays. All compounds and the EtOAc extract were tested for their estrogenic effects (Routledge and Sumpter, 1996) with no activity being observed. The antioxidative properties of the total extract and all compounds except for **10** and **11** were assessed using DPPH radical (Amarowicz *et al.*, 2000) and TBARS (thiobarbituric acid reactive substances) (Wallin *et al.*, 1993) assays. The results of these assays are shown in Tables II and III, and are in a good agreement with published values (Abe *et al.*, 1998a, b). The antioxidant activity of the known compound **6** is published for the first time. The total extract and all compounds, except for **11**, were tested for cytotoxicity against ten human cancer cell lines, with no significant activity being observed, except for some very weak activity of the total extract.

Experimental

General procedure

HPLC was carried out using a Merck-Hitachi system equipped with an L-6200A intelligent pump, an L-4500 photodiode array detector, a

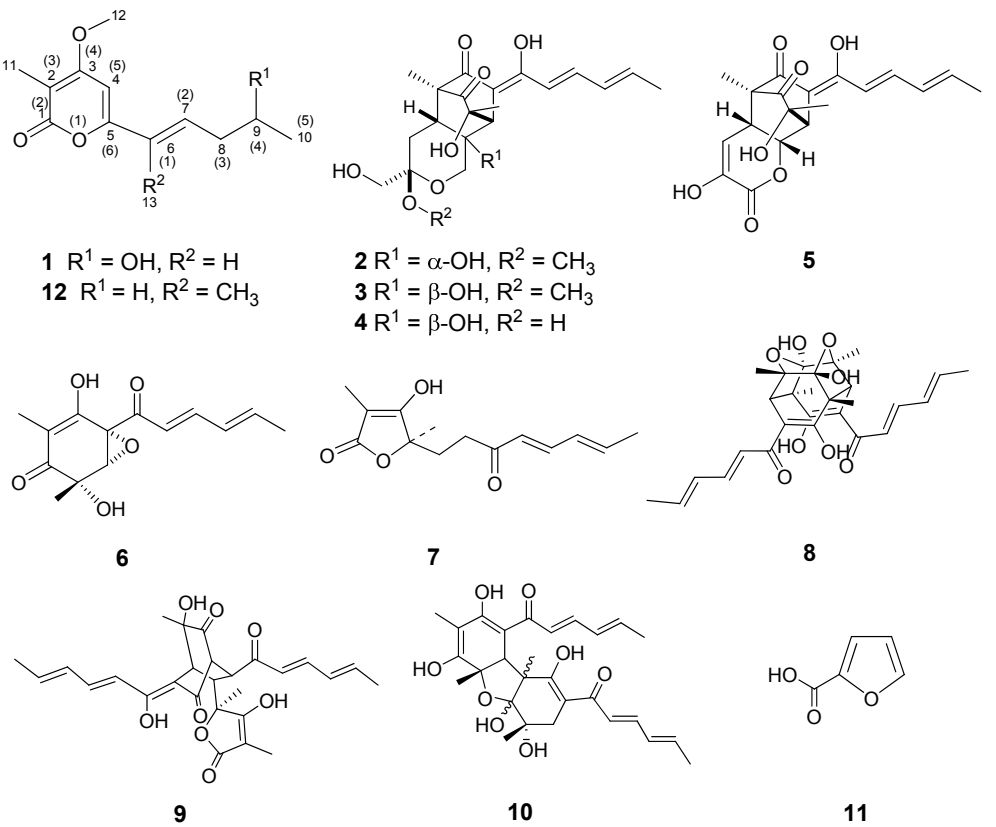

Fig. 1. Chemical structures of isolates **1**–**11**. The numbers in brackets are used for systematic naming.

Table II. DPPH radical scavenging effects of the EtOAc extract of *Trichoderma* sp. and **1**–**9**.

Material	Scavenging (%)[a]			
	6[b]	23	115	230
EtOAc extract	–2.3	–0.6	7.3	12.5
1	–0.9	–0.9	1.0	3.7
2	–0.9	8.5	11.2	8.6
3	–1.1	0.0	1.0	1.3
4	–0.8	0.7	3.1	3.4
5	–0.6	–1.4	2.1	4.7
6	1.8	8.6	30.2	43.8
7	–1.5	1.9	10.1	15.5
8	–0.1	4.8	23.0	37.2
9	0.4	4.0	16.7	23.8
BHT[c]	1.0	4.0	17.3	32.8
Vit. E	10.9	38.2	94.3	94.0

[a] Scavenging (%) = 100 – [A(sample) · 100/A(control)], where A is the absorbance of solutions measured at both 532 and 600 nm.
[b] Concentrations in μmol/L.
[c] BHT, butylated hydroxytoluene.

D-6000 interface with D-7000 HSM software, and a Rheodyne 7725i injection system. UV and IR spectra were obtained using Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. Optical rotations were recorded on a Jasco DIP 140 polarimeter. HREIMS was measured on a Kratos MS 50 spectrometer. LC-ESIMS was performed using an API 2000 LC MS/MS system from Applied Biosystems/MDS Sciex. All NMR spectra were recorded on Bruker Avance 300 DPX and 500 DRX spectrometers in $(CD_3)_2CO$. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 2.04/29.8 [$(CD_3)_2CO$].

Isolation and taxonomy of the fungal strain

The sponge *Agelas dispar* J. was collected in December 1993 by divers, using a self-contained underwater breathing apparatus (SCUBA), from the waters around the Caribbean island of Dominica. The sponge was identified by Dr. R. Desqueroux-Faundez, Musee d'Histoire Naturelle, Geneva, Switzerland. The fungus was isolated by inoculating small pieces of the sponge's inner tissue onto a medium containing cellulose (10 g/L), yeast extract (1 g/L), benzylpenicillin (250 mg/L), streptomycin sulfate (250 mg/L), agar (15 g/L) and artificial sea water (ASW) (800 mL/L). ASW contained the following compounds (g/L): KBr (0.1), NaCl (23.48), $MgCl_2 \cdot 6H_2O$ (10.61), $CaCl_2 \cdot 6H_2O$ (1.47), KCl (0.66), $SrCl_2 \cdot 6H_2O$ (0.04), Na_2SO_4 (3.92), $NaHCO_3$ (0.19), H_3BO_3 (0.03). The fungal strain was identified as belonging to the genus *Trichoderma* by Dr. S. Draeger, Institute for Microbiology, Technical University of Braunschweig, Braunschweig, Germany.

Table III. Antioxidative effects (TBARS assay) of the EtOAc extract of *Trichoderma* sp. and **1**–**9**.

Material	Inhibition (%)[a]		
	8[b]	33	164
EtOAc extract	6.4	1.5	–5.3
1	1.9	3.2	1.6
2	2.4	–3.0	–7.2
3	0.7	7.6	6.0
4	7.1	6.3	5.5
5	6.2	6.0	6.2
6	14.4	11.7	17.8
7	3.2	4.3	5.2
8	9.1	9.7	19.9
9	6.8	0.9	–0.1
BHT[c]	20.3	50.8	69.2
Vit. E	12.7	67.4	73.6

[a] Inhibition (%) = 100 – [A(sample) – A(sample blank)] · 100/[A(control) – A(blank)], where A is the absorbance of solutions measured at both 532 and 600 nm.
[b] Concentrations in μmol/L.
[c] BHT, butylated hydroxytoluene.

Cultivation

The fungus was cultivated at room temperature for two months in 5.75 L (23 Fernbach flasks) of solid medium containing 50 g/L biomalt (Villa Natura Gesundheitsprodukte GmbH, Kirn, Germany), 10 g/L glucose (J. T. Baker), and 15 g/L agar (Fluka Chemie AG) in ASW.

Extraction and isolation

Mycelia and medium were homogenized using an Ika Ultra-Turrax instrument at 8000 rpm. The resulting mixture was exhaustively extracted with EtOAc (3 × 6 L) and filtered. The filtrate was evaporated to yield 2.5 g of a yellowish brown extract. This extract was fractionated by reversed phase vacuum liquid chromatography (RP-VLC) (2.5–20 cm, RP C-18 material, 70 g), employing gradient elution from H_2O/MeOH 10:90 to MeOH, to yield 9 fractions. According to the differences in the composition of the fractions as detected by ^{1}H NMR spectroscopy, especially with respect to several low-field resonances in the δ 6–8 region, fractions 3, 4, 5 and 6 seemed promising for further investigation. These fractions were combined and fractionated by normal phase (NP) VLC (2–15 cm, silica gel 60, 30 g, Merck 7739), using gradient elution from petroleum ether to EtOAc, followed by MeOH, to yield 15 fractions. Fraction 3.6 was purified by RP-18 HPLC (Eurospher-100, 5 μm, 250 × 8 mm ID, Knauer), employing gradient elution from H_2O/MeOH 6:4 to MeOH in 60 min, 2 mL/min, to yield five peaks (fraction 3.6.3, t_R = 15 min, 2.5 mg; fraction 3.6.4, t_R = 25 min, 20 mg; fraction 3.6.5, t_R = 26 min, 18 mg; fraction 3.6.6, t_R = 29 min, 16 mg; fraction 3.6.7, t_R = 35 min, 3 mg). The material isolated that corresponded to fraction 3.6.3 was identified as **1**.

The remaining three fractions (obtained from the first VLC) were fractionated by RP- and NPSi-VLC, using different eluents of different polarities, followed by RP-HPLC, employing a variety of solvent systems. This led to the isolation of compounds **2**–**11**. The structures of all compounds were determined/confirmed by interpretation of their spectroscopic data (1D and 2D NMR, CD, MS, UV and IR) (Neumann *et al.*, 2007).

Trichopyrone [6-(4-hydroxy-1-pentenyl)-4-methoxy-3-methyl-2*H*-pyran-2-one] (**1**): Yellowish viscous oil (2.5 mg, 0.001% of extract, 0.044% of media). – $[\alpha]_D^{22}$ –10.3° (*c* 0.1, MeOH). – UV (MeOH): λ_{max} (log ε) = 334 (3.5), 278 (3.5), 227 (4.0) nm. – IR (film): λ_{max} = 3384, 2930, 2360, 1676, 1549, 1458, 1258, 1144 cm^{-1}. – ^{1}H and ^{13}C NMR: see Table I. – ESIMS (+ve): *m/z* = 225 $[M+H]^+$. – EIMS: *m/z* (% rel. int.) = 224 $[M^+]$ (25), 180 (100), 152 (25), 139 (20), 125 (30), 111 (60), 83 (45), 71 (55), 57 (65). – HREIMS: *m/z* = 224.1046; calcd. for $C_{12}H_{16}O_4$ *m/z* = 224.1049.

Antioxidative activity assay

The thiobarbituric acid reactive substances (TBARS) assay was used to test the EtOAc extract of *Trichoderma* sp. and compounds **1**–**9**; it was adapted from Wallin *et al.* (1993) and modified and performed as previously described (Abdel-Lateff *et al.*, 2002): Briefly, linolenic acid methyl ester was oxidized in 50 mM phosphate buffer (pH 7.2), under $FeSO_4$ catalysis at 50 °C. Butylated hydroxytoluene (BHT) in ethanol was added to prevent further oxidation. TBARS were determined using trichloroacetic acid and thiobarbituric acid at 60 °C for 30 min. The absorbance was read at 532 nm less the background absorbance at 600 nm.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effects

The assays were performed in flat-bottom polystyrene 96-well microtiter plates. The DPPH radical scavenging effects of the EtOAc extract of *Trichoderma* sp. and compounds **1**–**9** were determined using a modified, previously established methodology (Amarowicz *et al.*, 2000). To 100 μL of each sample (1 mg/mL) in EtOH 25 μL of DPPH (1 mM) in EtOH and 75 μL of EtOH were added to give a final volume of 200 μL. The resultant mixture was briefly shaken and maintained at room temperature in the dark for 30 min. At the end of this period the absorbance of the mixture was measured at 517 nm, using a SLT Spectral Rainbow microtiter plate reader (SLT Labinstruments, Crailsheim, Germany).

The method for calculation of values for both antioxidant assays was as reported previously (Fisch *et al.*, 2003).

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