Two New Xanthone Derivatives from the Algicolous Marine Fungus *Wardomyces anomalus*

Ahmed Abdel-Lateff, Christine Klemke, Gabriele M. König,* and Anthony D. Wright

Institute for Pharmaceutical Biology, University of Bonn, Nussallee 6, 53115 Bonn, Germany

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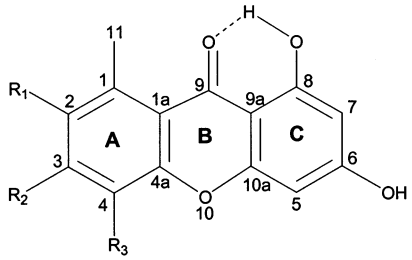
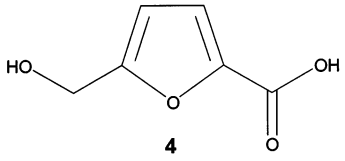
A marine fungal isolate, identified as *Wardomyces anomalus*, was cultivated and found to produce two new xanthone derivatives, 2,3,6,8-tetrahydroxy-1-methylxanthone (**1**) and 2,3,4,6,8-pentahydroxy-1-methylxanthone (**2**), in addition to the known xanthone derivative 3,6,8-trihydroxy-1-methylxanthone (**3**) and the known fungal metabolite 5-(hydroxymethyl)-2-furanocarboxylic acid (**4**). The structures of all compounds were determined on the basis of extensive spectroscopic measurements (1D and 2D NMR, MS, UV, and IR). Compounds **1** and **4** showed significant antioxidant activities. The total extract and **1**, **3**, and **4** were shown to be inhibitors of p56^{lck} tyrosine kinase.

Marine-derived fungi are a source of structurally diverse, biologically active natural compounds.[1−7] Xanthone derivatives are widespread in nature, commonly occurring in a number of higher plant families and fungi.[8,9] Some fungal species are well known as sources of xanthone derivatives, e.g., *Penicillium raistrickii*,[10] *Phomopsis* sp.,[11] *Actinoplanes* sp.,[12] *Ascodesmis sphaerospora*,[13] and *Humicola* sp.[14] Xanthones are known to have a variety of biological activities, e.g., antimicrobial, antitubercular, antitumor, and antiviral.[8,9] In continuation of our projects aimed at finding new natural products with biological activity and/or novel chemical structures from marine-derived fungi,[15] *Wardomyces anomalus* Brooks & Hansford *(*Microascaceae, Ascomycetes), isolated from the green alga *Enteromorpha* sp. (Ulvaceae) collected around Fehmarn island in the Baltic Sea,[16] was investigated. The crude extract showed antimicrobial effects toward *Microbotryum violaceum* and *Eurotium repens*, inhibition of HIV-1 reverse transcriptase (HIV-1-RT) (65.4% at 66 μg/mL), and inhibition of p56^{lck} tyrosine kinase (TK) (98% at 200 μg/mL). Radical scavenging activity was observed using α,α-diphenyl-β-picrylhydrazyl (DPPH) as indicator (71.9% at 500 μg/mL). An extensive computer survey indicated there to be no prior investigation of *W. anomalus*. On this basis, the current secondary metabolite investigation was undertaken, which afforded the two new xanthone derivatives **1** and **2**, in addition to the known xanthone derivative **3**[17] and the known furan derivative **4**.[18] The fungus was cultivated on a solid biomalt medium with added artificial sea salt. Successive fractionation of the EtOAc extract over a reversed-phase Sep Pack column, followed by reversed-phase HPLC, yielded compounds **1**−**4**.



1 $R_1 = R_2 = OH, R_3 = H$
2 $R_1 = R_2 = R_3 = OH$
3 $R_1 = R_3 = H, R_2 = OH$
5 $R_1 = H, R_2 = R_3 = OH$



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Compound **1** has the molecular formula $C_{14}H_{10}O_6$, as established by high-resolution mass measurement. The ^{13}C NMR spectrum of **1** contained 14 carbon resonances attributable to 10 × C, 3 × CH, and 1 × CH_3 groups (Table 1). It was also evident from these data that seven of the 10 elements of unsaturation within **1** were present as C=C double bonds and a carbonyl group (this deduction being supported by an IR absorption at ν_{max} 1646 cm^{-1}); the molecule was thus tricyclic. As the ^{13}C and ^1H NMR spectral data enabled all but four of the hydrogen atoms within **1** to be accounted for, it was evident that the remaining four must be present as OH groups, a deduction that was supported by IR data (ν_{max} 3275 cm^{-1}). UV maxima at 234, 258, 311, 358 nm suggested a xanthone nucleus.[9] The ^1H NMR spectral data revealed the presence of three aromatic protons, two of them (H-5 and H-7) with a meta coupling, the third (H-4) one a singlet, an aryl methyl group (11-CH_3), and a hydrogen-bonded phenolic OH (8-OH). After association of all protons with directly bonded carbons via a 2D NMR (HMQC) spectral measurement, it was possible to deduce the substitution pattern of the xanthone nucleus from HMBC correlations. Thus, HMBC correlations were seen between H-5 and C-6, C-7, C-9 (weak), C-9a, and C-10a, between H-7 and C-5, C-6, C-9 (weak), and C-9a, between H-4 and C-1 (weak), C-1a, C-2, C-3, C-4a, and C-9 (weak), and also between H_3-11 and C-1, C-1a, C-2, C-4a (weak), and C-9 (weak). The HMBC correlations associated with CH_3-11 clearly showed it to reside at C-1. As the 8-OH proton formed a strong hydrogen bond with oxygen, it was likely that this occurred with the oxygen of the C-9 carbonyl group. From HMBC correlations as described previously, it was evident that the C-6 resonated at δ 165.1 and thus was hydroxylated. The ^{13}C NMR chemical shifts of all of the A-ring carbons were then found to be most consistent with the remaining

* To whom correspondence should be addressed. Tel: +49-228-733747. Fax: +49-228-733250. E-mail: g.koenig@uni-bonn.de. Internet: http://www.uni-bonn.de/pharmbio/queen/GAWK.html.

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Table 1. ^{1}H and ^{13}C NMR Spectral Data in Acetone-d_6 at 300/75.5 MHz for Compounds **1**−**3**[a]

	1			**2**		**3**	
no.	δC[b]	δH[c]	HMBC[d]	δC[f]	δH[c]	δC[b]	δH[c]
1	125.6 C			111.9		144.4 C	
1a	112.5 C			118.0		113.0 C	
2	141.8 C			142.8		116.9 CH	6.70 br s
3	152.7 C			n[g]		158.1 C	
4	100.9 CH	6.78 s	C-1 (w),[h] C-1a, C-2, C-3, C-4a, C-9 (w)	n[g]		101.5 CH	6.71 br s
4a	153.5 C			n[g]		160.3 C	
5	93.6 CH	6.26 d (2.2)	C-6, C-7, C-9 (w), C-9a, C-10a	93.9	6.31 d (2.1)	94.0 CH	6.31 d (1.8)
6	165.1 C			165.5		164.9 C[e]	
7	98.4 CH	6.15 d (2.2)	C-5, C-6, C-9 (w), C-9a	98.6	6.16 d (2.1)	98.7 CH	6.17 d (1.8)
8	164.8 C			165.6		163.6 C[e]	
9	183.5 C			183.0		183.0 C	
9a	103.8 C			104.9		103.8 C	
10a	158.0 C			158.4		165.5 C[e]	
11	13.9 CH_3	2.76 s	C-1, C-1a, C-2, C-3 (w), C-4 (w), C-4a (w), C-9 (w)	13.3	2.70 s	23.4 CH_3	2.77 s
2-OH		8.97 br s			7.75 s[e]		
3-OH		8.97 br s			8.80 s[e]		9.79 br s[e]
4-OH					9.20 s[e]		
6-OH		8.97 br s			9.54 s[e]		9.71 br s[e]
8-OH		13.58 s	C-8		13.62 s		13.44 s

[a] All assignments are based on 1D and 2D measurements (HMBC, HMQC). [b] Multiplicities were determined by DEPT. [c] δ/ppm, *J* in Hz. [d] Correlations were reported from proton resonances to carbon resonance. [e] Tentative assignment. [f] Data were obtained indirectly from a HMBC spectrum. [g] Not observed in HMBC spectrum. [h] w = weak signal.

Table 2. Antioxidative Effects (TBARS Assay)[a] and DPPH[b] Radical Scavenging Effects of the Total Extract and Compounds **1**, **3**, and **4**

	% of inhibition[c]		% scavenging effect of DPPH[f]			
compd	7.4[h]	37.0	25	50	100	500
extract	−29.2	−50.2	7.8	11.3	20.2	71.9
1	17.0	37.0	94.7	94.8	95.2	95.4
3	−7.4	−11.1	6.2	12.9	25.3	90.6
4	2.6	7.4	30.7	53.4	87.7	97.0
BHT[d]	42.8	48.5	73.4	83.1	89.1	87.8

[a] TBARS = thiobarbituric acid reactive substances. [b] DPPH = α,α-diphenyl-β-picrylhydrazyl. [c] % inhibition = 100 − (*A* sample[e] − *A* sample blank[e]) × 100/(*A* control − *A* blank). [d] BHT = butylated hydroxytoluene. [e] *A* = absorbance of test blank, and control solutions are measured at both 532 and 600 nm. [f] Scavenging % = 100 − (*A* sample[g] × 100/*A* control[g]). [g] Absorbance of sample and control measured at 517 nm. [h] Concentration in μg/mL.

OH groups to be positioned at C-2 and C-3. Further analysis of the ^{1}H and ^{13}C NMR spectral data showed resonances at δ 6.78 and 100.9 which supported the location of the remaining aromatic proton at C-4. Compound **1**, for which the trivial name anomalin A is suggested, is a new xanthone derivative that is a structural isomer of **5**, a compound from *Penicillium raistrickii*.[10]

The molecular formula of compound **2** was shown to be $C_{14}H_{10}O_7$ on the basis of HRMS. Comparison of ^{1}H NMR and MS data with those of **1** revealed **2** to contain a phenolic group more than **1**. This hydroxyl group replaced the aromatic proton H-4, a deduction supported by ^{1}H NMR spectral data, which showed the absence of the signal at 6.78 ppm, s (H-4 in **1**). Compound **2**, for which the trivial name anomalin B is suggested, is a new xanthone derivative.

3,6,8-Trihydroxy-1-methylxanthone (**3**)[17] and 5-(hydroxymethyl)-2-furanocarboxylic acid (**4**) were identified by comparison of the spectroscopic data with published values.[18]

The antioxidative properties of all compounds, except **2**, were assessed using DPPH radical and TBARS assays.[15] The results of these assays are shown in Table 2. Compounds **1** and **4** have significant DPPH radical scavenging effects (94.7 and 30.7%, respectively, at 25.0 μg/mL) and are also able to inhibit peroxidation of linolenic acid (17.0 and 2.6%, respectively, at 7.4 μg/mL). ELISA-based bioassays with HIV-1-RT and TK p56^{lck} allowed determination of enzyme inhibitory activities. The total extract and **1**, **3**, and **4** had significant TK p56^{lck} enzyme inhibitor activity (100% enzyme inhibition at 200 μg/mL) (Table 3). Finally, only minor antimicrobial activity was observed for **1**−**4** in agar diffusion assays.

Experimental Section

General Experimental Procedures. UV and IR spectra were obtained using Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. All NMR

Table 3. Activity of the Extract and Compounds Toward HIV-1 Reverse Transcriptase and Tyrosine Kinase p56^{lck} and Antimicrobial Activity

assay	extract	**1**	**3**	**4**
tyrosine kinase[a,c,d]	2	0	0	0
HIV-1-RT[a,b,d]	35.6	82.2	82.9	98.3
bacteria[e]	not active	not active	1 mm for *B.m*	not active
fungi[f]	3 mm[g] for *M.v* 2 mm for *E.r*	1 mm for *M.v*	1 mm for *E.r*	not active
Chlorella fusca	1 mm	not active	not active	not active

[a] Percentage of enzyme activity observed relative to negative control (100% HIV-1-RT or TK p56^{lck}). [b] Reduction of enzyme activity to 80% or less was regarded as a significant inhibition. Foscarnet (10 μM, rest activity of HIV-1-RT 13%) was used as a positive control. [c] Reduction of enzyme activity to 40% or less was regarded as a significant inhibition. Piceatannol (3 mM, rest activity of 2%) was used as a positive control. [d] Sample concentration for HIV-1-RT test 66 μg/mL, and for TK p56^{lck} test 200 μg/mL. [e] Tested against *Escherichia coli* (*E.c*) and *Bacillus megaterium* (*B.m*).[h] [f] Tested against *Microbotryum violaceum* (*M.v*), *Eurotium repens* (*E.r*), *Mycotypha microspora* (*M.m*).[h] [g] The radii of the resultant zone of inhibition was measured from the edge of the filter disk. [h] The concentration of the extract and pure compound is 250 and 50 μg/disk, respectively.

spectra were recorded on Bruker Avance 300 DPX and 500 DRX spectrometers. Spectra were referenced to residual solvent signals [$\delta_{H/C}$ 2.04/29.8, $(CD_3)_2CO$]. Mass spectra were measured on a Kratos MS 50 spectrometer. HPLC was carried out using a Merck-Hitachi system consisting of a L-6200A pump, an L-4500 photodiode array detector, and a D-6000 interface.

Isolation and Taxonomy of the Fungal Strain. Algal material (*Enteromorpha* sp., Ulvaceae) was collected by divers around Fehmarn Island in the Baltic Sea. After sterilization with 70% ethanol, algal samples were rinsed with sterile water and pressed onto agar plates to detect any residual fungal spores on the surface of algae. Sterilized algae were then cut into pieces and placed on agar plates containing isolation medium: 15 g/L agar and 1 L sea water from the sample collecting site, plus the antibiotics benzyl penicillin and streptomycin sulfate (250 mg/L). Fungal colonies growing out of the algal tissue were transferred to medium for sporulation (1.0 g of glucose, 0.1 g of yeast extract, 0.5 g of peptone from meat, enzymatic digest, 15 g of agar, and 1 L of artificial sea water, pH 8) in order to enable taxonomy of the isolates. Fungal strain OS4T3-2-1 was identified by Dr. S. Draeger (Institute for Microbiology, Technical University of Braunschweig).

Cultivation. *W. anomalus* was cultivated for 2 months on 100 mL of media, composed of biomalt (5 g/L) [Biomalt, Villa Natura Gesundheitsprodukte GmbH, Germany], agar (15 g/L) [Fluka Chemie AG], and artificial sea water containing the following salts (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).[19]

Extraction and Isolation. Cultivated *W. anomalus* mycelia and medium were diluted with water (100 mL/L) and homogenized using a Waring blender. The resulting mixture was exhaustively extracted with EtOAc (3 × 0.5 L) to yield 66 mg of highly viscous brownish black oil. The EtOAc extract was fractionated over a Bakerbond SPE column (octadecyl C-18, 40 μm, APD, 60 Å, J. T. Baker), gradient elution from H_2O to MeOH, and three fractions were collected, 20 mL of each. Fraction 1 (16.4 mg, 160.4 mg/L) was identified as **4**. Fraction 2 (20 mg) was subjected to HPLC (Eurospher-100 C-18, 5 μm, 250 × 8 mm i.d., Knauer), eluted with 8:2 H_2O/MeOH, and yielded **2** (1 mg, 10 mg/L). Fraction 3 (26.6 mg) was subjected to reversed (RP-18) phase HPLC, eluted with 4:6 H_2O/MeOH, and yielded **1** (6 mg, 60 mg/L) and **3** (2 mg, 20 mg/L).

Biological Activities. The antimicrobial effects were determined as described by Schulz et al.[20] HIV-1 reverse transcriptase and tyrosine kinase $p56^{lck}$ inhibitory activities were measured as reported by Kirsch et al.[21] Finally, the antioxidant assays were performed according to Abdel-Lateff et al.[15]

2,3,6,8-Tetrahydroxy-1-methylxanthone (anomalin A, 1): (6 mg) yellowish brown amorphous powder; UV λ_{max} (MeOH) (log ϵ) 234 (4.86), 258 (4.86), 311 (4.72), 358 (4.50) nm; IR (film) 3275, 2838, 1646, 1456, 1292 cm^{-1}; ^1H and ^{13}C NMR spectral data, Table 1; EIMS *m*/z (% rel int) 274 [M^+] (100), 245 (28), 217 (7), 137 (7), 69 (5); accurate mass *m*/z 274.0465, calcd for $C_{14}H_{10}O_6$ 274.04774.

2,3,4,6,8-Pentahydroxy-1-methylxanthone (anomalin B, 2): (1 mg) yellowish brown amorphous powder; UV λ_{max} (MeOH) (log ϵ) 258 (4.70), 327 (4.48), 370 (sh), nm; IR (film) 3260, 2925, 1647, 1507, 1275 cm^{-1}; ^1H NMR spectral data, Table 1; EIMS *m*/z (% rel int) 290 [M^+] (30), 248 (25), 190 (100), 161 (35); accurate mass *m*/z 290.0422, calcd for $C_{14}H_{10}O_7$ 290.0426.

3,6,8-Trihydroxy-1-methylxanthone (3):[17] (2 mg) yellowish brown amorphous powder; UV λ_{max} (MeOH) (log ϵ) 238 (4.72), 310 (4.60) nm; IR (film) 3260, 2925, 1647, 1507, 1275 cm^{-1}; ^1H and ^{13}C NMR spectral data, Table 1; EIMS *m*/z (% rel int) 258 [M^+] (100), 229 (10), 69 (5); accurate mass *m*/z 258.0524, calcd for $C_{14}H_{10}O_5$ 258.0528.

5-(Hydroxymethyl)-2-furanocarboxylic acid (4): (16.4 mg) identified by comparison of the ^1H and ^{13}C NMR data with those previously published.[18]

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