Antiviral Terpenoid Constituents of *Ganoderma pfeifferi*

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Four sterols and 10 triterpenes were isolated from the fruiting bodies of *Ganoderma pfeifferi*, including the three new triterpenes 3,7,11-trioxo-5α-lanosta-8,24-diene-26-al (lucialdehyde D, **1**), 5α-lanosta-8,24-diene-26-hydroxy-3,7-dione (ganoderone A, **2**), and 5α-lanosta-8-ene-24,25-epoxy-26-hydroxy-3,7-dione (ganoderone C, **3**). The structures of **1**−**3** were determined on the basis of spectroscopic evidence. Antibacterial, antifungal, and antiviral activity were studied for some of the isolated compounds. Ganoderone A (**2**), lucialdehyde B (**4**), and ergosta-7,22-dien-3β-ol (**7**) were found to exhibit potent inhibitory activity against herpes simplex virus.

Ganoderma pfeifferi Bres., a weak parasitic and later saprophytic basidiomycete, is a fungus only found in Europe, living preferentially on *Fagus* spp. and some other deciduous trees such as *Aesculus*, *Acer*, *Fraxinus*, and *Quercus* species. The species can be recognized by its cracked and wrinkled resinous layer on the pileus and its sweet scent in winter. Its dark brown context makes it distinguishable from older specimens of *G. lucidum* and *G. resinaceum*.[1]

In contrast to *G. lucidum* and *G. applanatum*, from which a number of biologically and pharmacologically interesting triterpenes[2−11] and polysaccharides[12−15] have been isolated,[16] *G. pfeifferi* is one of the phytochemically less well examined species of the family Ganodermataceae. Two antibacterial farnesyl hydroquinones were isolated from *G. pfeifferi* in our group,[17] followed by the isolation of antiviral triterpenes.[18] As part of our continuing interest in compounds from *G. pfeifferi,* four sterols and 10 triterpenes were isolated in the present investigation from the fruiting body of this mushroom. In addition to the already known compounds, ergosta-7,22-diene-3-one,[19] ergosta-4,6,8(14),22-tetraene-3-one,[20] 5α,8α-epidioxyergosta-6,22-diene-3β-ol,[22,23] lucialdehyde B (**4**),[21] ganoderol A (**5**),[5] ganoderol B,[5] ganoderal A (**6**),[5] ergosta-7,22-diene-3β-ol (**7**),[19] and applanoxidic acids A,[3] C,[3] and G,[4] we isolated three previously unknown triterpenoid constituents (**1**−**3**). The present paper describes the isolation and structure elucidation of these compounds as well as the biological examination of a number of the isolated terpenes in regard to their antibacterial, antifungal, and antiviral activities.

Results and Discussion

Numerous isolated steroids and triterpenes have been described from fungi in the Ganodermataceae previously. However, it is highly interesting to note that the yields of lucialdehyde B (**4**) and ganoderal A (**5**) in *G. pfeifferi* are much higher than in other *Ganoderma* species. For **4**, we found a more than two 100-fold yield compared with *G. lucidum*, from which it was first isolated.[21]

A molecular formula of $C_{30}H_{42}O_4$ was established for compound **1** from its HRMS (found *m/z* 467.3145, calcd 467.3161 for [M + H]). The ^{1}H NMR spectrum clearly showed an aldehyde moiety (δ_H 9.40) as well as a low-field-shifted alkene proton triplet (δ_H 6.47), suggesting that the aldehyde was conjugated with a double bond. The ^{13}C NMR spectrum showed similarities to the one obtained for lucialdehyde B (**4**).[21] One additional carbonyl group and the typical shifts for an unsaturated diketone (δ_C 149.8 and 151.5) could be observed (Table 1). Correlations in the HSQC and HMBC NMR spectra confirmed the assumption of **1** being the C-11-oxidized derivative of lucialdehyde B. On taking all of the data together, **1** was identified as 3,7,-11-trioxo-5α-lanosta-8,24-dien-26-al and was assigned the trivial name lucialdehyde D.

Compound **2** gave the molecular formula $C_{30}H_{46}O_3$, as determined by HRMS (found *m/z* 455.3530, calcd 455.3525 for [M + H]). The UV absorption at 256 nm and IR bands at 1706 and 1641 cm^{-1} indicated the presence of two keto groups. The ^{13}C NMR data were similar to those of ganoderone B,[24] also known as lucidadiol,[25] except for the absence of the signal for the secondary alcohol at δ_C 77.8 (C-3), which was replaced by a peak at δ_C 214.6 (Table 1). Unambiguous assignments for hydrogens and carbons could be made on the basis of the correlations observed in the HSQC, HMBC, and NOESY NMR spectra. It was not possible to determine the configuration at C-5 by NMR, as no NOESY correlations for H-5 could be observed. However, on biogenetic grounds it is highly probable that H-5 of **2** is α, by analogy with ganoderone B and other similar lanostanoids. On the basis of these data, **2** was identified as 5α-lanosta-8,24-diene-26-hydroxy-3,7-dione. Thus, **2** (ganoderone A) is the corresponding ketone to ganoderone B.

HRFTICRMS of **3** showed a quasi-molecular ion at *m/z* 471.3476, corresponding to the molecular formula $C_{30}H_{46}O_4$ (calcd 471.3474 for [M + H]). The UV and IR spectra were similar to those of **2**, suggesting **3** to be a further ganoderone derivative. In turn, the ^{13}C NMR data showed similarities to those obtained for **2**, including the presence of the ketone signal. However, the signals for the unsaturated carbons C-24 and C-25 at δ_C 126.8 and 134.4,

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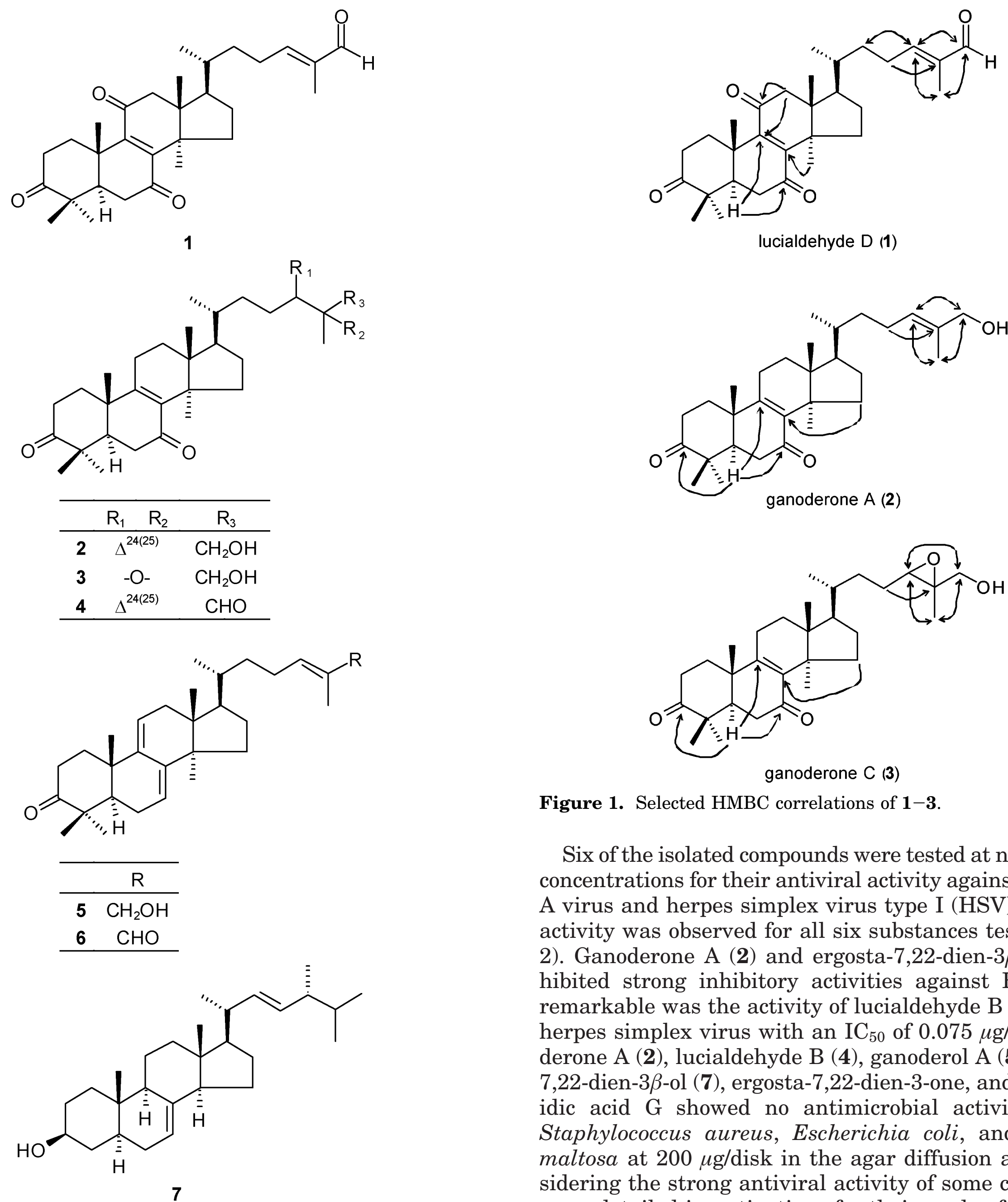

Figure 1. Selected HMBC correlations of **1**−**3**.

respectively, were replaced by signals at δ_C 60.3, 60.5, 60.7, and 61.0 (Table 1). From the HSQC, HMBC, and NOESY NMR spectra, it could be shown that these signals corresponded to an epoxy group at C-24,C-25. Four signals were observed for the two carbons, leading to the assumption that both isomeric forms of the epoxide were present. A close relationship to the ^{13}C NMR data of epoxyganoderiol A was apparent, as well.[26] NOESY NMR correlations showed that, as has been described for epoxyganoderiol A,[26] the bonds of the epoxy oxygen were attached either both α or both *β*. The configuration of C-5 could not be determined, but H-5 can, as described above, be assumed to be attached α on C-5. Thus, **3** was identified as a 1:1 mixture of 5α-lanosta-8-ene-24α,25α-epoxy-26-hydroxy-3,7-dione and 5α-lanosta-8-ene-24*β*,25*β*-epoxy-26-hydroxy-3,7-dione. That **3** was an artifact could be excluded, as it was detectable by GC-MS in a crude cold dichloromethane extract of freshly collected fruiting bodies of *G. pfeifferi*. Compound **3** was named ganoderone C due to the close structural similarities to ganoderones A and B.

Six of the isolated compounds were tested at noncytotoxic concentrations for their antiviral activity against influenza A virus and herpes simplex virus type I (HSV). Antiviral activity was observed for all six substances tested (Table 2). Ganoderone A (**2**) and ergosta-7,22-dien-3*β*-ol (**7**) exhibited strong inhibitory activities against HSV. Most remarkable was the activity of lucialdehyde B (**4**) against herpes simplex virus with an IC_{50} of 0.075 *μ*g/mL. Ganoderone A (**2**), lucialdehyde B (**4**), ganoderol A (**5**), ergosta-7,22-dien-3*β*-ol (**7**), ergosta-7,22-dien-3-one, and applanoxidic acid G showed no antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida maltosa* at 200 *μ*g/disk in the agar diffusion assay. Considering the strong antiviral activity of some compounds, more detailed investigations for their mode of action and for structure−activity relationships are necessary. It is noteworthy that in contrast to ganoderone B (lucidadiol), which has been shown to be inactive against HSV,[18] ganoderone A (**2**) is active against this virus.

Experimental Section

General Experimental Procedures. Melting points were determined using a micro-melting apparatus according to Boetius and are corrected. Optical rotations were measured on a Kruess P3002/RS polarimeter with a cell volume of 1 mL. UV−vis spectra of the new compounds were recorded in MeOH using an AnalytikJena SPECORD 1000 spectrometer fitted with 1 cm quartz cuvettes. IR spectra were recorded with a Perkin-Elmer FTIR1500 FT spectrometer using KBr tablets. NMR spectra were recorded in $CDCl_3$ at 600.13 MHz (^{1}H NMR) and 150.10 MHz (^{13}C NMR and DEPT-135) at ambient temperature using a Bruker Avance-II 600. Spectra were referenced indirectly to tetramethylsilane via the residual signals of the deuterated solvents. Column chromatography was carried out on Sephadex LH-20 (Amersham Biosciences,

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of **1**, **2**, and **3** (J in Hz, recorded in $CDCl_3$)[a]

carbon	1	2	3	proton	1	2	3
1	35.0	35.3	35.4	H (α)	1.75 m	1.77 m	1.79 m
				H (β)	2.97 d (2.5)	2.08 m	2.13 m
2	34.1	34.3	34.3	H (α)	2.48 m	2.46 dd (1.3)	2.46 d (2.1)
				H (β)	2.64 m	2.69 m	2.69 m
3	215.5	214.6	214.6				
4	46.9	47.2	47.2				
5	49.8	50.4	50.4	H (α)	2.26 m	2.09 m	2.14 m
6	37.1	37.1	37.1	H (α)	2.38 m	2.33 m	2.34 d (4.1)
				H (β)	2.54 m	2.53 m	2.54 d (1.5)
7	201.3	198.1	198.0				
8	151.5	139.6	139.5				
9	149.8	162.7	162.6				
10	38.8	39.4	39.4				
11	201.8	23.8	23.8	H_2		2.30 m	2.29 m
12	51.2	30.1	30.1	H (α)	2.75 d (16.9)	1.75 m	1.79 m
				H (β)	2.65 d (16.8)	1.80 m	1.83 m
13	46.7	44.9	44.9				
14	48.7	47.8	47.8				
15	31.9	31.9	31.9	H (α)	1.68 m	1.65 m	1.69 m
				H (β)	2.17 m	2.04 m	2.07 m
16	27.43	28.7	28.7	H (α)	1.34 m	1.38 m	1.37 m
				H (β)	2.04 m	2.00 m	2.00 m
17	49.1	49.0	49.0	H (α)	1.71	1.48 m	1.46 m
18	16.9	15.9	15.9	H_3	0.84 s	0.68 s	0.69 s
19	17.9	17.9	17.9	H_3	1.29 s	1.33 s	1.33 s
20	36.1	36.2	36.2	H (β)	1.46 m	1.40 m	1.43 m
21	18.3	18.7	18.7	H_3	0.96 d (6.5)	0.94 d (5.7)	0.95 m
22	34.5	35.9	32.6	H_2	1.23 m, 1.64 m	1.10 m, 1.53 m	1.16, 1.53
			32.7				
23	25.92	24.5	25.0	H_2	2.23 m, 2.42 m	1.89 m, 2.07 m	1.41 m, 1.49 m
			25.2				1.58 m, 1.66 m
24	154.6	126.8	60.3	H	6.47 t (7.0)	5.39 t (5.4)	3.00 m
			60.5				
25	139.4	134.4	60.7				
			61.0				
26	195.2	69.0	65.5	H/H_2	9.40 s	4.00 s	3.57 d (12.1),
							3.67 d (12.1)
27	9.2	13.6	14.2	H_3	1.75 s	1.67 s	1.28 s
			14.3				
28	27.44	25.4	25.4	H_3	1.13 s	1.09 s	1.09 s
29	20.3	21.4	21.4	H_3	1.11 s	1.11 s	1.11 s
30	25.87	24.9	24.9	H_3	1.23 s	0.93 s	0.94 s

[a] The number of protons directly attached to each carbon was verified by DEPT experiments. Signal assignments by HSQC and HMBC techniques.

Table 2. Antiviral Activity of Compounds **2**−**7** (IC_{50} in μg/mL)

compound	influenza A (MDCK cells)	HSV (Vero cells)
2	no activity	0.3
3	2.6	no activity
4	3.0	0.075
5	no activity	0.75
6	no activity	0.03
7	0.78	0.03
amantadine sulfate	15.0	n.d.[a]
aciclovir	n.d.	0.1

[a] Not determined

Uppsala, Sweden) and silica gel (0.06−0.2 mm, 4 nm pore diameter, Acros Organics, Geel, Belgium). Fractions were monitored by TLC (silica gel 60 254 nm) using toluol−ethyl acetate−acetic acid (7:3:0.1) as mobile phase. LC-MS investigation was done using electrospray ionization under atmospheric conditions (APIES) or chemical ionization using isobutane (APCI), using an Agilent Technologies 1100 series HPLC system and an Agilent Technologies G1946C quadrupole mass spectrometer. HRMS was performed by FTICR mass spectrometry using a Bruker Daltonics APEX III FT-ICR MS equipped with a 7.0 T shielded superconducting magnet. Ions were generated from an external electrospray ionization source. Mass spectra were acquired in both positive and negative mode. All experimental sequences, including scan accumulation and data processing, were performed with XMASS 6.1.2. GC-MS data were obtained using an Agilent 6890N Network GC system including a 5973 mass selective detector and a 7683 injector system. GC and MS data were recorded using version SWG1701CA C.00.00 21-Dec-1999 of the Chemstation software by Agilent Technologies. Analytical HPLC was performed on a Dionex HPLC system using an ASI-100 autosampler and the UVD-340S diode array detector (200−595 nm). The system was runing on a Gynkotek HP480 pump. An end-capped RP_{18} column (125 × 4.6 mm, 5 μm) was used at a flow rate of 1.5 mL/min. A solvent system consisting of methanol (eluent A) and 0.1% (m/m) phosphoric acid (eluent B), starting from an initial ratio of 10% A and 90% B and reaching 100% A within 13.0 min, was used. Semipreparative HPLC was perfomed on a YMC-Pack ODS-AQ column (250 × 20 mm, 10 μm) using a Dionex HPLC system including a ASI-100 autosampler, a Dionex P580 pump, and a Gynkotek UVD-160 variable-wavelength detector. The gradients of the solvents MeOH and water are summarized in the Supporting Information (SI). All HPLC runs were recorded using the Chromeleon HPLC data system version 6.30 SP1 built 587. All chemicals were used as received, and solvents were distilled prior to use.

Fungal Material. Mature fruiting bodies of *G. pfeifferi* were collected in the vicinity of Greifswald (Germany) in September 2002. They were taken from the same stump of *Fagus* sp. from which the fruiting bodies were collected in 1995.[17] They were again kindly identified by Prof. Dr. H. Kreisel, Department of Biology, Ernst-Moritz-Arndt-Universität, Greifswald, Germany. A voucher specimen (herbarium accession number PI-

28) is deposited at the Department of Pharmaceutical Biology, Ernst-Moritz-Arndt-Universität.

Extraction and Isolation. Air-dried and powdered fruiting bodies of *G. pfeifferi* (288 g) were extracted with CH_2Cl_2 in a Soxhlet apparatus for 18 h to give a crude extract (8.5 g). The crude extract was rinsed with 450 mL of *n*-hexanes−CH_2Cl_2 (2:7) on a Sephadex LH-20 column (open column, 3 × 35 cm, water cooled, flow 24 mL/h; F1-1). The fraction not eluting was washed off with MeOH (F1-2). This fraction was not examined further. The first fraction, F1-1, was subjected to separation on Sephadex LH-20 (open column, 2 × 35 cm, flow 20 mL/h) using acetone as eluent to give three fractions (F1-1-1 to F1-1-3). F1-1-1 was not examined further. Chromatography of F1-1-2 on silica gel (open column, 2 × 35 cm, 30 mL/h), using *n*-hexanes−ethyl acetate (3:1; 300 mL), (2:1; 300 mL), (1:1; 300 mL) and acetone (200 mL), gave nine fractions (F1-1-2-1 to F1-1-2-9). F1-1-2-1 was recrystallized from acetone to give ergosta-7,22-dien-3-one (yield 37.3 mg). Further separation by semipreparative HPLC (HPLC 1; see SI) gave ganoderal A (**6**, 29.9 mg). HPLC (HPLC 2) of F1-1-2-2 gave ergosta-4,6,8(14),-22-tetraen-3-one (4.7 mg). F1-1-2-4 was recrystallized from acetone to give ergosta-7,22-dien-3β-ol (**7**, 18.8 mg). HPLC (HPLC 3) of F1-1-2-5 gave lucialdehyde D (**1**, 6.5 mg), lucialdehyde B (**4**, 52.4 mg), and ganoderol A (**5**, 27.4 mg). Fraction F1-1-2-6 was recrystallized from MeOH to give 5α,8α-epidioxyergosta-6,22-dien-3β-ol (74.8 mg). Further separation of its mother liquid by HPLC (HPLC 4) gave ganoderol B (8.0 mg). HPLC (HPLC 5) of F1-1-2-7 gave applanoxidic acid C (38.4 mg). Fraction F1-1-2-8 was recrystallized from MeOH to give ganoderone A (**2**, 23.8 mg). HPLC (HPLC 6) of F1-1-2-9 gave ganoderone C (**3**, 13.7 mg). Fraction F1-1-3 was chromatographed on silica gel (open column, 2 × 35 cm, 30 mL/h) using ethyl acetate−acetone (4:1; 400 mL), (3:1; 200 mL), (1:1; 100 mL) and acetone (200 mL), giving four fractions (F1-1-3-1 to F1-1-3-4). HPLC of F1-1-3-2 (HPLC 7) and F1-1-3-3 (HPLC 8) gave applanoxidic acid G (97.5 mg) and applanoxidic acid A (47.5 mg), respectively.

Lucialdehyde D (3,7,11-trioxo-5α-lanosta-8,24-dien-26-al, 1): colorless oil; $[\alpha]^{20}_D$ +46.8 (*c* 0.100, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 234 (4.06), 267 (3.64) nm; IR (KBr) ν_{max} 2967, 2931, 2886, 2874, 1710, 1686, 1465, 1380, 1229, 1174 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 466 (100), 448 (25), 381 (17), 286 (22), 259 (28); HRFTICRMS *m/z* $[M + H]^+$ 467.3145 (calcd for $C_{30}H_{43}O_4$, 467.3161).

Ganoderone A (5α-Lanosta-8,24-diene-26-hydroxy-3,7-dione, 2): white needles (MeOH); mp 136−137 °C; $[\alpha]^{21}_D$ +2.4 (*c* 0.110, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 204 (4.26), 256 (4.02) nm; IR (KBr) ν_{max} 2977, 2969, 2922, 2875, 2860, 1706, 1641, 1577, 1457, 1379, 1372 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 454 (27), 421 (43), 353 (23), 325 (28), 121 (46), 109 (58), 55 (57), 43 (100); HRFTICRMS *m/z* $[M + H]^+$ 455.3530 (calcd for $C_{30}H_{47}O_3$, 455.3525).

Ganoderone C (5α-Lanosta-8-ene-24,25-epoxy-26-hydroxy-3,7-dione, 3): pale white to yellow amorphous solid; $[\alpha]^{20}_D$ −29.7 (*c* 0.120, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 254 (3.92) nm; IR (KBr) ν_{max} 2967, 2931, 2880, 1709, 1662, 1467, 1457, 1381, 1372 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 470 (54), 455 (29), 379 (63), 221 (87), 55 (86), 43 (100); HRFTICRMS *m/z* $[M + H]^+$ 471.3476 (calcd for $C_{30}H_{47}O_4$, 471.3474).

Antibacterial and Antifungal Testing. An agar diffusion method according to the European Pharmacopoeia was used to determine antibacterial and antifungal activity.[27] Sterile Oxoid CM 432 antibiotic medium No. 1 (Oxoid Ltd, Basingstoke, England) was inoculated with bacterial cells (200 μL of bacterial cell suspension in 20 mL of medium) and poured into sterile Petri disks. Sterile paper disks impregnated with 200 μg of substance were applied on the cooled agar. Plates were kept for 2 h in a refrigerator to enable prediffusion of the substances into the agar and were then incubated for 18 h at 37 °C (*C. maltosa* at 27 °C). Inhibition zone diameters around each of the disks were measured and recorded at the end of the incubation time. Penicillin G was used as positive control. Bacterial strains used: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11229, and *Candida maltosa* SBUG 700.

Antiviral Testing. Compounds were tested for antiviral activity using a test system described before.[28] For the preparation of the stock solution, each 1.0 mg of test compound was solubilized in 100 μL of methanol prior to dissolving in MEM and stocked at a concentration of 1 mg/mL.

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Supporting Information Available: Additional information on general methods; isolation scheme; ^1H, ^{13}C, and DEPT-135 NMR spectra, EIMS, and UV spectra of all isolated compounds; IR spectra and HSQC, HMBC, and NOESY NMR spectra of new compounds; additional references concerning all isolated compounds and compounds structurally similar to the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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