Hydronaphthalenones and a Dihydroramulosin from the Endophytic Fungus PSU-N24

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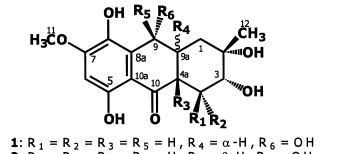
Three new hydronaphthalenone derivatives (1—3) and one new dihydroramulosin derivative (4), were isolated from the endophytic fungus PSU-N24 together with eight known compounds. Their structures were elucidated by spectroscopic methods. Griseofulvin (9) displayed strong antifungal activity against *Microsporum gypseum* SH-MU-4 with a minimum inhibitory concentration (MIC) value of 2 μg/ml while all metabolites exhibited very weak antibacterial activity (MIC value$\geq$128 μg/ml) against *Staphylococcus aureus*, both standard and methicillin-resistant strains. 3-(2-Hydroxypropyl)benzene-1,2-diol (10) showed moderate antimalarial activity against *Plasmodiun falciparum* with an IC_{50} value of 6.68 μg/ml. For antimycobacterial activity against *Mycobacterium tuberculosis*, compound 3 gave the best activity with the MIC value of 12.50 μg/ml.

Key words endophytic fungus; dihydronaphthalenone derivative; dihydroramulosin derivative; antifungal activity; antimalarial activity; antimycobacterial activity

Endophytic fungi grow in the intercellular spaces of higher plants. Many endophytic fungi have the ability to produce substances with antimicrobial,[1)] antimalarial,[2)] antimycobacterial,[2)] antifungal,[3)] and herbicidal[4)] activities. In the course of our ongoing search for bioactive metabolites from the endophytic fungi, the crude extract of the unidentified fungus PSU-N24, isolated from *Garcinia nigrolineata*, showed interesting antimicrobial,[1)] antimalarial[2)] and antimycobacterial[2)] activities. Further investigation of this extract led to the isolation and structural elucidation of three new hydronaphthalenone derivatives (**1**—**3**) and one new dihydroramulosin derivative (**4**) together with eight known compounds; (*R*)-mellein (**5**),[5)] *cis*-4-hydroxymellein (**6**),[6)] 8-dihydroramulosin (**7**),[7)] 6-hydroxyramulosin (**8**),[7)] griseofulvin (**9**),[8)] 3-(2-hydroxypropyl)benzene-1,2-diol (**10**),[9)] desoxybostrycin (**11**)[10)] and austrocortinin (**12**).[11)] Their isolation, structure determination and biological activities including antibacterial, antifungal, antimalarial and antimycobacterial activities are described in this paper.



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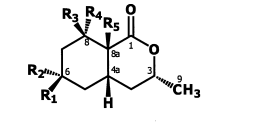
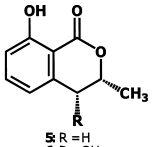
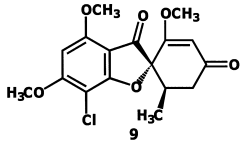
3: $R_1 = OH$, $R_2 = R_3 = R_5 = H$, $R_4 = \alpha$-H, $R_6 = OH$
11: $R_1 = R_2 = H$, $R_3 = R_4$ = double bond, $R_5 + R_6 = O$
13: $R_1 = OH$, $R_2 = R_3 = R_6 = H$, $R_4 = \alpha$-H, $R_5 = OH$



4: $R_1 = R_4 = OH$, $R_2 = R_3 = R_5 = H$
7: $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OH$
8: $R_1 = R_4 = OH$, $R_2 = H$, $R_3 = R_5$ = double bond



5: R = H
6: R = OH



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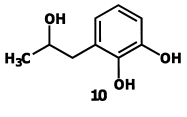

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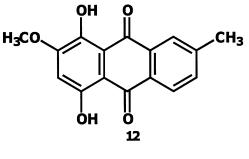

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Results and Discussion

The fungal endophyte PSU-N24 was identified on the basis of the rDNA sequence analysis. The nuclear large subunit ribosomal DNA (LSU rDNA) region was carried out for higher taxonomic levels. The LSU sequence of the endophyte PSU-N24 (1119 base pairs, GenBank accession number EU541479) was closely related to *Nigrospora* sp. AY234934 (Trichosphaeriales, Sordariomycetes *Incertae sedis*) and various species of the genera *Arthrinium* and *Apiospora* (Apiosporaceae, Xylariales, Sordariomycetes).[12—14)] The internal transcribed spacer (ITS) sequence analysis was further performed in order to identify it to a lower taxonomic levels. The results showed that the endophytic fungus PSU-N24 (513 base pairs, GenBank accession number DQ480361) displayed the closest relationship to three unknown fungal endophytes (AF413047, AF413048 and AF413049) with 18 base pairs difference (97.2% similarity) and to three *Nigrospora* sequences (*N. oryzae* DQ219433, *N. oryzae* EU196745 and *Nigrospora* sp. AM262341) with sequence similarity ranging from 92.7—94.8%. Moreover, within the same subclade, the fungus N24 had affinity with various strains of the genera *Apiospora* and *Arthrinium* (sequence similarity 77.6—80.6%). Because of inadequate sequences from the genera of *Nigrospora*, *Arthrinium* and *Apiospora* in GenBank for identification, this fungus was named the endophyte PSU-N24.

Compound **1** with the molecular formula $C_{16}H_{20}O_7$ from HR-EI-MS, was obtained as a red gum with $[\alpha]_D^{25}$ −5.6 (c=0.16, MeOH). UV absorption bands at 244, 283 and 354 nm revealed the presence of a conjugated carbonyl chromophore. Hydroxyl and carbonyl absorption bands were observed at 3365 and 1697 cm^{-1}, respectively, in the IR spectrum. The ^{1}H-NMR spectrum (Table 1) was similar to that of **11** except for additional signals of two coupled methine protons (δ 2.25, m and 2.48, td, J=11.4, 3.9 Hz) and one oxymethine proton (δ 4.84, dd, J=9.9, 5.4 Hz). In addition, **1** displayed one singlet of a hydrogen-bonded hydroxy proton at δ 12.72, indicating that **1** contained only one conjugated

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Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1** and **3** in Acetone-d_6 and **2** in $CDCl_3$

Position	**1**		**2**		**3**	
	^{1}H, δ (mult. J in Hz)	^{13}C, δ (mult.)	^{1}H, δ (mult. J in Hz)	^{13}C, δ (mult.)	^{1}H, δ (mult. J in Hz)	^{13}C, δ (mult.)
1	a: 2.43 (m) b: 1.38 (dd, 13.2, 11.7)	40.8 (t)	a: 2.09 (dd, 12.0, 4.2) b: 1.67 (d, 12.0)	41.4 (t)	a: 2.38 (dm, 12.0) b: 1.45 (t, 12.0)	39.5 (t)
2		69.8 (s)		84.6 (s)		70.3 (s)
3	3.50 (ddd, 11.4, 6.3, 4.8)	73.7 (d)	3.49 (br m)	74.7 (d)	3.31 (dd, 9.0, 5.0)	78.1 (d)
3-OH	3.78 (d, 6.3)				3.27 (d, 5.0)	
4	a: 2.33 (m) b: 1.64 (q, 11.4)	29.3 (d)	a: 2.29 (m) b: 1.36 (dt, 13.5, 9.9)	35.4 (t)	4.06 (td, 9.0, 3.0)	71.4 (d)
4-OH					4.41 (d, 3.0)	
4a	2.48 (td, 11.4, 3.9)	46.1 (d)	2.68 (m)	45.7 (d)	2.44 (t, 9.0)	51.6 (d)
5		158.7 (s)		158.8 (s)		158.9 (s)
5-OH	12.72 (s)		11.95 (s)		12.28 (s)	
6	6.44 (s)	99.0 (d)	6.40 (s)	100.1 (d)	6.48 (s)	99.1 (d)
7		155.8 (s)		154.4 (s)		156.5 (s)
8		137.8 (s)		137.5 (s)		137.9 (s)
8-OH	8.94 (s)		5.57 (br s)		8.96 (s)	
8a		127.5 (s)		121.9 (s)		127.5 (s)
9	4.84 (dd, 9.9, 5.4)	72.5 (d)	5.26 (dd, 3.6, 1.5)	72.6 (d)	4.91 (dd, 9.0, 6.0)	72.2 (d)
9-OH	5.54 (d, 5.4)				5.61 (d, 6.0)	
9a	2.25 (m)	40.9 (d)	2.63 (m)	39.8 (d)	2.38 (m)	39.5 (d)
10		202.5 (s)		205.1 (s)		205.1 (s)
10a		108.0 (s)		106.2 (s)		108.1 (s)
11	3.90 (s)	55.5 (q)	3.88 (s)	56.4 (q)	3.92 (s)	55.7 (q)
12	1.28 (s)	26.5 (q)	1.44 (s)	21.5 (q)	1.29 (s)	26.7 (q)

ketone functionality. The ^{13}C-NMR spectrum supported that one carbonyl carbon and two quaternary olefinic carbons in **11** were replaced in **1**, by one oxymethine carbon (δ 72.5) and two methine carbons (δ 40.9, 46.1). ^3J HMBC correlations (Table 2) of the oxymethine proton (δ 4.84) with C-4a (δ 46.1), C-8 (δ 137.8) and C-10a (δ 108.0) established the attachment of this oxymethine proton at C-9 (δ 72.5). One of the coupled methine protons resonating at δ 2.25 was attributed to H-9a on the basis of its ^{1}H–^{1}H COSY correlation with H-9. The remaining coupled methine proton resonating at δ 2.48 was then assigned as H-4a. This assignment was supported by HMBC correlations of H-4a/C-4 (δ 29.3), C-9a (δ 40.9) and C-10 (δ 202.5) and those of H-9a/C-2 (δ 69.8) and C-10. Both H-9 and H-9a were placed at axial positions as they were coupled to each other with a large coupling constant of 9.9 Hz. Irradiation of H-9 in the NOEDIFF experiment affected the intensity of Hb-1 (δ 1.38, dd, J=13.2, 11.7 Hz) and H-4a (Fig. 1), indicating their *cis* arrangement. Signal enhancement of Hb-1, H-4a and Me-12 (δ 1.28, s) upon irradiation of H-3 (δ 3.50, ddd, J=11.4, 6.3, 4.8 Hz), established the orientation of H-3 and Me-12 at axial and equatorial, respectively. The large coupling constant of 11.4 Hz between H-3 and axial Hb-4 (δ 1.64, q, J=11.4 Hz) supported the orientation of H-3 at axial position. The absolute configurations of C-2 and C-3 in **1** were proposed to be identical to those of (2*S*,3*R*)-**11**[10] because of their co-occurrence. The remaining absolute configurations were then established on the basis of the relative configuration obtained from the NOEDIFF results. Consequently, **1** was identified as 9α-hydroxydihydrodesoxybostrycin, a new hydroxynaphthalenone derivative.

Compound **2** with the same molecular formula as **1**, was obtained as a pale yellow gum with $[\alpha]_D^{26}$ +8.10 (c=0.16, MeOH). The ^{1}H-NMR data (Table 1) were almost identical to those of **1**, except for signals of Hab-1, H-3 and H-9. Both

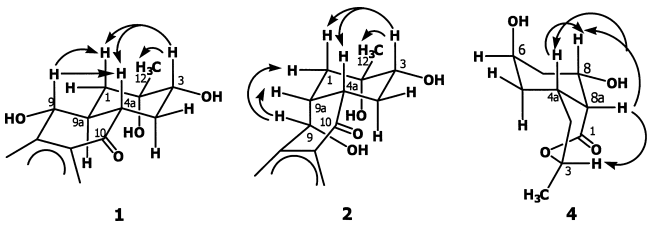

Fig. 1. Selected NOEDIFF Results of Compounds **1**, **2** and **4**

H-3 (δ 3.49, brm) and H-4a (δ 2.68, m) were placed at axial positions, the same as those in **1**, as they were coupled with axial Hb-4 (δ 1.36, dt, J=13.5, 9.9 Hz) with large coupling constant. This was confirmed by signal enhancement of axial H-4a and axial Hb-1 (δ 1.67, d, J=12.0 Hz) after irradiation of axial H-3 in the NOEDIFF experiment (Fig. 1). The multiplicity and coupling constants of equatorial Ha-1 (δ 2.09, dd, J=12.0, 4.2 Hz) and axial Hb-1 in **2** indicated that only equatorial Ha-1 was coupled with H-9a (δ 2.63, m) with a small coupling constant of 4.2 Hz. Consequently, H-9a had an equatorial orientation, not axial as that in **1**. Irradiation of H-9 (δ 5.26, dd, J=3.6, 1.5 Hz) affected signal intensity of equatorial H-9a and equatorial Ha-1 in the NOEDIFF experiment, thus indicating the location of H-9 at equatorial position. Therefore, **2** differed from **1** only in the configuration at C-9a.

Compound **3** was obtained as a red gum with the molecular formula $C_{16}H_{20}O_8$ from HR-EI-MS. Compounds **1** and **3** gave similar ^{1}H- and ^{13}C-NMR data except for the replacement of the methylene protons, Hab-4, in **1** with an oxymethine proton (δ 4.06, td, J=9.0, 3.0 Hz) in **3**. This methine proton was coupled with both H-3 (δ 3.31, dd, J=9.0, 5.0 Hz) and H-4a (δ 2.44, t, J=9.0 Hz) by equal coupling constant of 9.0 Hz and with 4-OH by coupling constant of 3.0 Hz. These results established the location of 4-OH at an

Table 2. ^{1}H-, ^{13}C-NMR and Selected HMBC Data of Compound **4** in $CDCl_3$

Position	^{1}H, δ (mult. J in Hz)	^{13}C, δ (mult.)	HMBC correlation
1		175.1 (s)	
3	4.47 (m)	74.1 (d)	C-1, C-4a, C-9
4	a: 1.31 (m)	36.0 (t)	C-3, C-4a, C-5, C-8a, C-9
	b: 2.25 (ddd, 14.4, 9.9, 3.9)		
4a	2.71 (m)	26.3 (d)	C-1, C-3, C-4, C-5, C-6, C-8a
5	a: 1.76 (dt, 14.1, 3.9)	38.9 (t)	C-4, C-4a, C-6, C-7, C-8a
	b: 1.28 (td, 14.1, 2.4)		
6	4.19 (m)	66.1 (d)	C-4a, C-5, C-7, C-8
7	1.99 (dd, 8.4, 3.3)	38.1 (t)	C-5, C-6, C-8, C-8a
8	4.16 (m)	65.7 (d)	C-1, C-7, C-8a
8-OH	3.60 (d, 11.4)		C-8, C-8a
8a	3.00 (t, 6.0)	44.0 (d)	C-1, C-4, C-4a, C-5, C-7, C-8
9	1.37 (d, 6.0)	20.83 (q)	C-1, C-3, C-4

equatorial position. The remaining NOEDIFF results were the same as those in **1**. Thus, **3** was assigned as 9α-hydroxyhalorosellinia A, a 4-hydroxy derivative of **1** and a C-9 epimer of halorosellinia A (**13**) which was isolated from the fungus *Halorosellinia* sp.[15,16]

Compound **4**, with the molecular formula $C_{10}H_{16}O_4$ from HR-EI-MS, was obtained as a colorless gum with $[\alpha]_D^{26}$ +65.5 (c=0.60, MeOH). Hydroxyl and carbonyl absorption bands were found at 3564 and 1736 cm^{-1}, respectively, in the IR spectrum. The ^{1}H- and ^{13}C-NMR data (Table 2) suggested that the structure of **4** was related to that of **7**. The significant difference in the ^{1}H-NMR spectrum was the replacement of one set of methylene protons in **7** with one oxymethine proton (δ 4.19, m) in **4**. This was supported by the presence of three methylene carbons (δ 36.0, 38.1, 38.9) and three oxymethine carbons (δ 66.1, 65.7, 74.1) in the ^{13}C-NMR spectrum of **4**. The oxymethine proton was attributed to H-6 as it displayed ^{3}J HMBC correlations with C-4a (δ 26.3) and C-8 (δ 65.7). The relative configuration of **4** was established by the following NOEDIFF results. Irradiation of H-8a (δ 3.00, t, J=6.0 Hz) enhanced the signal intensity of H-3 (δ 4.47, m) and H-4a (δ 2.71, m), suggesting their *cis* arrangement (Fig. 1). Signal enhancement of H-8 (δ 4.16, m), but not H-6 (δ 4.19, m), upon irradiation of axial H-4a, thus indicating the location of H-8 at axial position and also suggesting equatorial orientation for H-6. The multiplicity and coupling constant of Hab-5 (δ 1.28, td, J=14.1, 2.4 Hz and δ 1.76, dt, J=14.1, 3.9 Hz) confirmed that H-4a and H-6 had axial and equatorial orientations, respectively. Consequently, **4** was identified as 6β-hydroxy-8-dihydroramulosin which differed from **7** in the presence of the hydroxyl substituent at C-6. In addition, the absolute configurations at C-3, C-4a, C-6, C-8 and C-8a were proposed to be *R*, *S*, *R*, *S* and *R*, respectively, on the basis of known absolute configurations of its cometabolites **7** and **8**[7] as well as the relative configurations of these carbons obtained from the NOEDIFF results.

All metabolites were evaluated for antibacterial, antifungal, antimalarial and antimycobacterial activities. They showed very weak antibacterial activity against the standard *Staphylococcus aureus* ATCC 25923 (SA) and methicillin-resistant *S. aureus* (MRSA) with a MIC of >128 μg/ml, except for 3-(2-hydroxypropyl)benzene-1,2-diol (**10**) and desoxybostrycin (**11**) which showed an identical MIC value of 128 μg/ml for both strains. For antifungal activity against *Microsporum gypseum* SH-MU-4, griseofulvin (**9**), one of the known antifungal drugs, displayed potent activity with the MIC value of 2 μg/ml. The remaining metabolites were inactive. Compound **10** showed the best antimalarial activity against *Plasmodium falciparum* with an IC_{50} of 6.68 μg/ml while **3** and **11** were less active with the IC_{50} values of 7.94 and $\leq$10 μg/ml, respectively. Compound **3** was active against *Mycobacterium tuberculosis* with the MIC value of 12.50 μg/ml whilst compounds **2** and **11** were much less active with the MIC values of 25 and 50 μg/ml, respectively. For both antimalarial and antimycobacterial activities, the remaining compounds were inactive at the respective concentrations of 10 and 50 μg/ml.

We have isolated and determined the structures of three new hydronaphthalenone derivatives (**1**—**3**) and one new dihydroramulosin derivative (**4**) from the endophytic fungus PSU-N24 together with eight known compounds. So far, there have been only three fungi which produced compounds of bostrycin type.[10,15—17] Apart from their antitumor activity,[15] this is the first report on their antimalarial and antimycobacterial activities.

Experimental

General Procedures Infrared spectra (IR) were recorded on a Perkin Elmer 783 FTS165 FT-IR spectrometer. Ultraviolet (UV) absorption spectra were measured in MeOH on a SHIMADZU UV-160A spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a 300 MHz Bruker FTNMR Ultra Shield^{TM} spectrometer. Mass spectra were obtained on a MAT 95 XL Mass Spectrometer (Thermofinnigan). Optical rotations were measured in MeOH on a JASCO P-1020 polarimeter. Thin-layer chromatography (TLC) and precoated TLC were performed on silica gel GF_{254} (Merck). Column chromatography (CC) was carried out on Sephadex LH-20 or silica gel (Merck) type 100 (70—230 Mesh ASTM).

Isolation of the Fungal Endophyte The fungal endophyte PSU-N24 was isolated from a branch of *Garcinia nigrolineata* collected from the Ton Nga Chang wildlife sanctuary, Songkhla province, southern Thailand in 2005.[1] The surface sterilization method was applied in order to remove the contaminant and fungal epiphyte from plant sample. This fungus was grown in potato dextrose agar (PDA) and the agar plug with mycelium was kept in 15% glycerol at −70 °C. The isolate PSU-N24 was maintained in culture collection of Department of Microbiology, Prince of Songkla University, and BIOTEC Culture Collection (BCC29635), National Center for Genetic Engineering and Biotechnology, Thailand.

Molecular Identification of Fungal Endophyte The fungal endophyte PSU-N24 was grown on different media consisting of potato dextrose agar (PDA), corn meal agar (CMA), malt extract agar (MEA) and Czapek agar (Cz) in order to induce the sporulation. The endophytic fungus PSU-N24 was inoculated in potato dextrose broth (PDB) for two weeks at 25 °C, then fresh mycelium was harvested and ground into fine powder. Genomic DNA was extracted using CTAB method.[18] ITS and LSU rDNA were amplified using gene specific primers: ITS5/ITS4 and LROR/LR7, respectively.[19,20] PCR products were then sequenced by Macrogen Inc. using the same primer as for amplification. A BLAST search was employed to obtain the closest matched sequences from GenBank database. Sequence similarity of the endophytic fungus PSU-N24 and relatedness was calculated under sequence identity matrix using BioEdit 7.5.0.3.[21]

Fermentation and Isolation The endophytic fungus PSU-N24 was grown on PDA at 25 °C for 5 d. Three pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into 500 ml Erlenmeyer flasks containing 300 ml PDB at room temperature for 3 weeks. The culture (5 l) was filtered to give the filtrate and mycelia. The filtrate was extracted with EtOAc (3×800 ml) to afford a broth extract (1.4 g) as a brown gum after evaporation to dryness under reduced pressure. The crude EtOAc extract was separated by CC over Sephadex LH-20 using MeOH to yield four fractions (A—D). Fraction B (701.2 mg) was further purified by silica gel CC using a gradient of MeOH–CH_2Cl_2 to give nine subfractions (B1—B9). Subfraction B1 contained **5** (95.8 mg). Subfraction B3 (23.1 mg) was subjected to Sephadex LH-20 CC using MeOH to give **6** (10.4 mg). Subfraction B4 (160.2 mg) was further purified by silica gel CC using a gradient of EtOAc–light petroleum

to give **7** (35.0 mg), **8** (6.5 mg) and **9** (10.9 mg). Subfraction B5 gave **10** (17.5 mg). Subfraction B6 (14.6 mg) was subjected to precoated TLC with 30% EtOAc–light petroleum to give **2** (4.0 mg). Subfraction B7 (71.1 mg) was purified by CC over reverse phase C_{18} silica gel using a gradient system of MeOH–H_2O to give **4** (27.9 mg). Subfraction B8 (68.2 mg) was further purified by silica gel CC using a gradient of MeOH–EtOAc to give **1** (11.0 mg) and **3** (37.0 mg). Fraction C (340.9 mg) was subjected to Sephadex LH-20 CC using 50% MeOH in CH_2Cl_2 to yield **11** (2.0 mg). Fraction D (142.3 mg) was subjected to precoated TLC using 5% MeOH in CH_2Cl_2 (5 runs) to afford **12** (8.7 mg).

Compound **1**: Red gum. ^{1}H- and ^{13}C-NMR data see Table 1. IR (neat) cm^{-1}: 3365, 1697. UV λ_{max} (MeOH) nm (log ε): 244 (4.43), 283 (4.31), 354 (4.16). HR-EI-MS *m/z*: 324.1198 (Calcd for $C_{16}H_{20}O_7$: 324.1209). EI-MS *m/z*: 324 (M^+), 304, 288, 286, 271, 243, 218. $[\alpha]_D^{25}$ −5.6 (*c*=0.16, MeOH).

Compound **2**: Colorless gum. ^{1}H- and ^{13}C-NMR data see Table 1. IR (neat) cm^{-1}: 3392, 1696. UV λ_{max} (MeOH) nm (log ε): 243 (4.20), 282 (4.06), 357 (4.00). HR-EI-MS *m/z*: 306.1115 (Calcd for $C_{16}H_{18}O_6$: 306.1103). EI-MS *m/z*: 306 ($M-H_2O^+$), 249, 248, 219. $[\alpha]_D^{26}$ +8.1 (*c*=0.16, MeOH).

Compound **3**: Red gum. ^{1}H- and ^{13}C-NMR data see Table 1. IR (neat) cm^{-1}: 3364, 1696. UV λ_{max} (MeOH) nm (log ε): 244 (3.04), 284 (2.92), 358 (2.68). HR-EI-MS *m/z*: 340.1154 (Calcd for $C_{16}H_{20}O_8$: 340.1158). EI-MS *m/z*: 340 (M^+), 322, 304, 275, 218. $[\alpha]_D^{25}$ −95.8 (*c*=0.15, MeOH).

Compound **4**: Colorless gum. ^{1}H- and ^{13}C-NMR data see Table 2. IR (neat) cm^{-1}: 3564, 1736. UV λ_{max} (MeOH) nm (log ε): 211 (2.72), 284 (1.99). HR-EI-MS *m/z*: 182.0935 (Calcd for $C_{10}H_{14}O_3$: 182.0943). EI-MS *m/z*: 182 ($M-H_2O^+$), 154, 113, 97, 96, 95. $[\alpha]_D^{26}$ +65.5 (*c*=0.60, MeOH).

Antibacterial Assay The minimum inhibitory concentrations (MICs) were determined by the agar microdilution method.[22] The test substances were dissolved in DMSO (Merck, Germany). Serial 2-fold dilutions of the test substances were mixed with melted Mueller-Hinton agar (Difco) in the ratio of 1 : 100 in microtiter plates with flat-bottomed wells (Nunc, Germany). Final concentration of the test substances in agar ranged from 128 to 0.03 μg/ml. SA and MRSA were used as test strains. Inoculum suspensions (10 μl) were spotted on agar-filled wells. The inoculated plates were incubated at 35 °C for 18 h. MICs were recorded by reading the lowest substance concentration that inhibited visible growth. Vancomycin was used as a positive control drug and displayed the MIC value of 1 μg/ml. Growth controls were performed on agar containing DMSO.

Antifungal Assay The hyphal extension-inhibition assay[23] was used. A modification of the CLSI M38-A broth microdilution test (Clinical and Laboratory Standards Institute)[24] was performed against *M. gypseum* SH-MU-4. Equal volumes of a suspension of conidia (approximately 4×10^3 conidia/ml) were added to each test dilution to make final concentrations of 1—128 μg/ml in triplicate. Plates were incubated at 25 °C for 72 h. Miconazole, a standard antifungal agent, gave the MIC value of 1 μg/ml. The MICs were recorded for the lowest concentration that resulted in a reduction approximately 50% of the fungal growth.

Antimycobacterial Assay Antimycobacterial activity was performed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[25] Standard drugs, rifampicin, kanamycin sulfate and isoniazid, exhibited MIC values of 0.047, 1.25 and 0.25 μg/ml, respectively.

Antimalarial Activity The activity was evaluated against the parasite *P. falciparum* (K1, multidrug-resistant strain), using the microculture radioisotope technique based on the method described.[26] The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*. The standard compound was dihydroartimisinin (IC_{50} 0.0012 μg/ml).

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