Azaphilone and Isocoumarin Derivatives from the Endophytic Fungus *Penicillium sclerotiorum* PSU-A13

Jiraporn Arunpanichlert,[a] Vatcharin Rukachaisirikul,*[,a] Yaowapa Sukpondma,[a] Souwalak Phongpaichit,[b] Supinya Tewtrakul,[c] Nuttawut Rungjindamai,[d] and Jariya Sakayaroj[d]

[a] *Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University;* [b] *Natural Products Research Center and Department of Microbiology, Faculty of Science, Prince of Songkla University;* [c] *Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University; Hat Yai, Songkhla, 90112, Thailand: and* [d] *National Center for Genetic Engineering and Biotechnology (BIOTEC); Thailand Science Park, Klong Luang, Pathumthani 12120, Thailand.*

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Two new azaphilone derivatives, penicilazaphilones A (1) and B (2), and one new isocoumarin, penicilisorin (3), together with six known compounds were isolated from the endophytic fungus *Penicillium sclerotiorum* PSU-A13. Their structures were identified by analysis of spectroscopic data. The antimicrobial activity against *Staphylococcus aureus*, *Candida albicans* and *Cryptococcus neoformans* as well as the inhibitory effect on human immunodeficiency virus (HIV)-1 integrase and protease were examined.

Key words endophytic fungus; *Penicillium sclerotiorum*; azaphilone; antibacterial activity; antifungal activity; anti-human immunodeficiency virus-1 activity

The genus *Penicillium* has the efficiency to produce bioactive substances, for instance, antimicrobial isochromophilone VI,[1] antifungal xestodecalactone B,[2] and anti-human immunodeficiency virus (HIV) atrovenetinone methyl acetal.[3] Our previous investigation on two endophytic fungi isolated from *Garcinia atroviridis* resulted in the isolation of an antifungal pyrone against *Microsporum gypseum* SH-MU-4 and an antioxidant naphthol from *P. paxilli* PSU-A71[4] and the xylariaceous fungus PSU-A80,[5] respectively. These results prompted us to search for bioactive metabolites from the endophytic fungus *P. sclerotiorum* PSU-A13, isolated from this plant, of which the hexane and ethyl acetate extracts from mycelia showed antimicrobial and HIV-1 integrase (IN) and protease (PR) inhibitory activities. Chemical investigation of the culture broth led to the isolation of three new compounds, penicilazaphilones A (**1**) and B (**2**) and penicilisorin (**3**) (Fig. 1), together with two known ones, (5*S*,6*R*)-5,6-dihydro-3,5,6-trimethylpyran-2-one (**8**),[6] and 4-hydroxyacetophenone.[7] Two known compounds, dechloroisochromophilone III (**4**)[8] and 2,4-dihydroxy-6-(5,7*S*-dimethyl-2-oxo-*trans*-3-*trans*-5-nonadienyl)-3-methylbenzaldehyde (**6**),[8] were obtained from the ethyl acetate extract of mycelia while two additional known ones, (+)-sclerotiorin (**5**)[9] and (+)-(2*E*,4*E*,6*S*)-4,6-dimethylocta-2,4-dienoic acid (**7**),[10] were isolated from the corresponding hexane extract. The antimicrobial and anti-HIV-1 activities were evaluated.

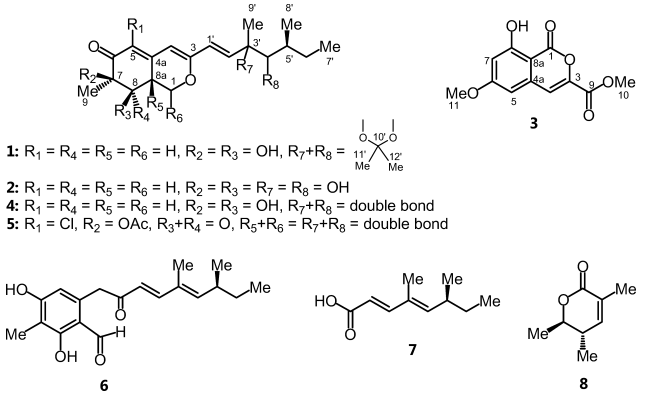

Fig. 1. Structures of Compounds **1**—**8**

Results and Discussion

The endophytic fungus *P. sclerotiorum* PSU-A13 was isolated from the leaves of *Garcinia atroviridis* Griff. *ex.* T. Anderson which were collected from Yala province, South of Thailand. As the fungus (GenBank accession number EF564151) did not produce any conidia or spores, it was identified based on the analysis of the DNA sequences of the internal transcribed spacer (ITS1-5.8S-ITS2) regions of its ribosomal RNA gene. Its ITS sequence (EF564151) matched with three *P. sclerotiorum* sequences from GenBank comprising DQ127231, AY373930 and AF033404 with sequence identity of 100.0, 99.8 and 99.8%, respectively. This endophytic fungus was then identified as *Penicillium sclerotiorum*.

Penicilazaphilone A (**1**) was obtained as a yellow gum with $[\alpha]_D^{26}$ −35.1 (c=0.30, MeOH). The molecular formula was determined to be $C_{22}H_{32}O_6$ on the basis of high resolution-electron ionization-mass spectrum (HR-EI-MS). The UV spectrum showed maximum absorption bands at λ_{max} 243 and 346 nm. The IR spectrum exhibited absorption bands at 3418 and 1717 cm^{-1} for hydroxyl and carbonyl groups, respectively. The ^{1}H-NMR spectral data (Table 1) composed of signals for *trans*-olefinic protons [δ 6.41 (1H, d, J=15.6 Hz) and 6.21 (1H, d, J=15.6 Hz)], one oxymethine proton (δ 3.44, 1H, m), one methine proton (δ 1.64, 1H, m), two nonequivalent methylene protons [δ 1.45 (1H, m) and 1.02 (1H, m)] and five methyl groups [δ 1.46 (3H, s), 1.38 (3H, s), 1.27 (3H, s), 1.03 (3H, d, J=6.6 Hz) and 0.90 (3H, t, J=6.9 Hz)]. In the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum, the cross peaks of H_3-7′ (δ 0.90)/H_{ab}-6′ (δ 1.45, 1.02) and H-5′ (δ 1.64)/H-4′ (δ 3.44), H_{ab}-6′ and H_3-8′ (δ 1.03) together with heteronuclear multiple bond correlations (HMBC) of H-4′/C-2′ (δ 140.2), C-3′ (δ 81.7), C-6′ (δ 25.0), C-8′ (δ 16.4) and C-9′ (δ 20.9) established a 3,5-dimethyl-3,4-dioxyheptenyl unit. In addition, both H_3-11′ (δ

* To whom correspondence should be addressed. e-mail: vatcharin.r@psu.ac.th.

Table 1. The NMR Data of Penicilazaphilones A (**1**) and B (**2**)

Position	**1**		**2**		HMBC correlation
	δ_H, mult. (J in Hz)	δ_C, mult.	δ_H, mult. (J in Hz)	δ_C, mult.	
1	a: 4.82, dd (10.8, 5.4) b: 3.79, dd (13.5, 10.8)	68.8, CH_2	a: 4.78, dd (10.8, 5.4) b: 3.77, dd (13.5, 10.8)	68.8, CH_2	C-3, C-4a, C-8, C-8a
3		160.3, qC		160.4, qC	
4	5.66, s	105.0, CH	5.62, s	104.7, CH	C-3, C-4a, C-5, C-8a, C-1′
4a		151.8, qC		152.3, qC	
5	5.76, d (1.8)	116.3, CH	5.71, d (1.8)	116.3, CH	C-4, C-7, C-8a
6		196.3, qC		197.3, qC	
7		74.2, qC		74.2, qC	
8	3.47, br d (9.6)	74.3, CH	3.38, d (9.6)	74.5, CH	C-1, C-8a, C-9
8a	2.99, m	36.7, CH	3.00, m	36.4, CH	C-1, C-4a, C-8
9	1.48, s	20.6, CH_3	1.43, s	19.8, CH_3	C-6, C-7, C-8
1′	6.21, d (15.6)	123.1, CH	6.16, d (15.3)	122.4, CH	C-3, C-4, C-2′, C-3′
2′	6.41, d (15.6)	140.2, CH	6.48, d (15.3)	143.1, CH	C-3, C-1′, C-3′, C-4′, C-9′
3′		81.7, qC		75.7, qC	
4′	3.44, m	87.2, CH	3.46, d (2.4)	78.2, CH	C-2′, C-3′, C-6′, C-8′, C-9′
5′	1.64, m	34.4, CH	1.68, m	35.3, CH	C-6′, C-7′, C-8′
6′	a: 1.45, m b: 1.02, m	25.0, CH_2	a: 1.45, m b: 1.30, m	28.7, CH_2	C-4′, C-5′, C-7′, C-8′
7′	0.90, t (6.9)	10.8, CH_3	0.91, t (7.2)	11.8, CH_3	C-5′, C-6′
8′	1.03, d (6.6)	16.4, CH_3	0.97, d (6.9)	13.4, CH_3	C-4′, C-5′, C-6′
9′	1.27, s	20.9, CH_3	1.30, s	23.4, CH_3	C-2′, C-3′, C-4′
10′		106.7, qC			
11′	1.46, s	28.4, CH_3			C-10′, C-12′
12′	1.38, s	26.3, CH_3			C-10′, C-11′

1.46) and H_3-12′ (δ 1.38) showed HMBC cross peaks with quaternary carbon C-10′ (δ 106.7). These results together with the chemical shifts of C-3′, C-4′ (δ 87.2) and C-10′ attached an acetonide unit at C-3′ and C-4′.

The remaining proton signals belonged to two olefinic protons [δ 5.76 (1H, d, J=1.8 Hz) and 5.66 (1H, s)], one oxymethine proton (δ 3.47, 1H, br d, J=9.6 Hz), one methine proton (δ 2.99, 1H, m), two nonequivalent methylene protons [δ 4.82 (1H, dd, J=10.8, 5.4 Hz) and 3.79 (1H, dd, J=13.5, 10.8 Hz)] and one methyl group (δ 1.48, 3H, s). These results established an azaphilone unit identical to that of **4**.[8] The HMBC correlations as depicted in Fig. 2 supported this conclusion. The chemical shifts of C-7 (δ 74.2) and C-8 (δ 74.3) confirmed the presence of the hydroxyl groups at these carbons. C-1′ (δ 123.1) of the heptenyl unit was connected with C-3 (δ 160.3) of the azaphilone unit according to a HMBC correlation of H-1′ (δ 6.21) with C-3. Signal enhancement of H-4 (δ 5.66) and H_3-9′ (δ 1.27) upon irradiation of H-1′ in the NOEDIFF experiment supported the assigned location of the heptenyl unit. In addition, irradiation of H-8 in the NOEDIFF experiment enhanced signal intensity of H_3-9, but not H-8a (δ 2.99), indicating that H-8 was *cis* to H_3-9 but *trans* to H-8a. These data revealed that the relative configuration of the azaphilone unit in **1** was identical to that of **4**. As compounds **1** and **4** were co-metabolites, the absolute configuration of this moiety in **1** was proposed to be 7*R*, 8*R* and 8a*R*, identical to that of **4**. In addition, the *S* configuration was assigned for C-5′ on the basis of the known absolute configuration of the corresponding carbon in co-metabolites **4**—**7**. Irradiation of H-4′ in the NOEDIFF experiment enhanced signal intensity of H_3-8′ and H_3-9′ (Fig. 3), thus indicating that they were located at the same side of the molecule. The absolute configuration at C-3′ and C-4′ was then proposed to be *R* and *S*, respectively. Consequently,

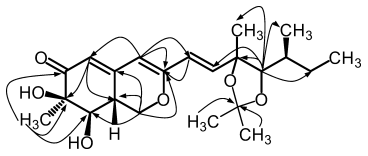

Fig. 2. Key HMBC Correlations of Penicilazaphilone A (**1**)

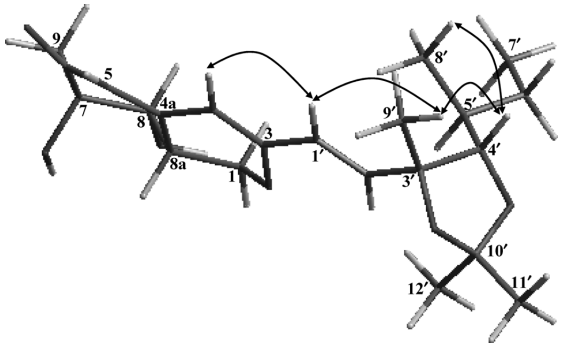

Fig. 3. Selected NOEDIFF Data of Penicilazaphilone A (**1**)

penicilazaphilone A (**1**) was assigned as a new acetonide azaphilone. Since acetone solvent was not used in extraction and purification procedure, compound **1** was not an artifact.

Penicilazaphilone B (**2**) was obtained as a yellow gum. The molecular formula $C_{19}H_{28}O_6$ was analysed on the basis of the HR-EI-MS. The molecular weight was 40 mass units less than that of **1**. The UV and IR spectra were almost identical to those of **1**. The ^{1}H-NMR spectral data (Table 1) were similar to those of **1** except for the disappearance of two methyl singlets of the acetonide unit. These results together with the chemical shifts of C-3′ (δ 75.7) and C-4′ (δ 78.2) confirmed that the substituents at these carbons were hy-

droxyl groups. The absolute configuration of the azaphilone and the side chain was proposed to be identical to that of **1** as they possessed similar optical rotations. Therefore, penicilazaphilone B (**2**) was assigned as a deacetonide derivative of **1**.

Penicilisorin (**3**) was obtained as a colorless gum. The HR-EI-MS showed the molecular formula $C_{12}H_{10}O_6$, corresponding to eight degrees of unsaturation. The UV spectrum showed absorption bands for an isocoumarin chromophore at λ_{max} 252, 273, 303 and 341 nm.[11] The IR spectrum displayed absorption bands at 3385, 1742 and 1675 cm^{-1} for hydroxyl, ester carbonyl and isocoumarin carbonyl groups, respectively. The ^{1}H-NMR spectral data consisted of signals for one chelated hydroxy proton (δ 10.90, 1H, s), two *meta*-coupled aromatic protons [δ 6.54 (1H, d, J=2.4 Hz) and 6.48 (1H, d, J=2.4 Hz)], one olefinic proton of an α,β-unsaturated carbonyl group (δ 7.31, 1H, s) and two methoxyl groups [δ 3.87 (3H, s) and 3.81 (3H, s)]. The ^{13}C-NMR spectrum showed one ester carbonyl (δ 166.9), one isocoumarin carbonyl (δ 160.4), five quaternary (δ 166.9, 164.1, 142.9, 136.5, 101.4), three methine (δ 113.5, 104.7, 103.0) and two methoxyl (δ 55.9, 53.0) carbons. The chelated hydroxy proton was placed at C-8 (δ 164.1) and gave HMBC cross peaks with C-1 (δ 166.9), C-7 (δ 103.0), C-8 and C-8a (δ 101.4). The aromatic proton at δ 6.54 was attributed to H-7 due to a ^{1}H-detected heteronuclear multiple quantum correlation (HMQC) of H-7 to C-7. The other *meta*-aromatic proton (δ 6.48) was then assigned as H-5 on the basis of its multiplicity and coupling constant. Signal enhancement of both H-5 and H-7 upon irradiation of H_3-11 (δ 3.81) in the NOEDIFF experiment as well as a HMBC correlation of H_3-11 and C-6 (δ 166.9) revealed the attachment of the methoxyl group at C-6. H-5 gave HMBC cross peaks with C-1, C-4 (δ 113.5), C-6, C-7 and C-8a, indicating that C-4 was linked with C-4a (δ 136.5) of the benzene ring. The olefinic proton resonating at δ 7.31 was then assigned as H-4 on the basis of its HMQC cross peak with C-4. This assigned location was supported by signal enhancement of H-4 after irradiation of H-5 in the NOEDIFF experiment. HMBC correlations of H-4 (δ 7.31)/C-3 (δ 142.9) and H_3-10 (δ 3.87) with C-3 and C-9 (δ 160.4) established the presence of a methyl ester moiety at C-3. The appearance of H-4 at δ 7.31 supported the presence of an α,β-unsaturated ester moiety. The chemical shift of C-1 together with mass data revealed that penicilisorin had the isocoumarin structure **3**.

Compounds **2**, **5** and **6** were evaluated for antimicrobial activity against *Staphylococcus aureus* (SA) ATCC 25923, methicillin-resistant SA SK1 (MRSA), *Candida albicans* (CA) NCPF 3153 and *Cryptococcus neoformans* (CN) ATCC 90113 as well as HIV-1 IN and PR inhibitory activity. The remaining compounds were not tested due to minute quantity of sample. (+)-Sclerotiorin (**5**), isolated from the crude hexane extract of the mycelia, showed moderate antifungal activity against CA and CN with the minimum inhibitory concentration (MIC) values of 64 and 32 μg/ml, respectively, while **2** and **6** displayed no activity at the initial concentration of 200 μg/ml. All of them displayed no antibacterial activity against SA and MRSA at the same concentration of 200 μg/ml. In addition, **5** exhibited anti-HIV-1 IN and PR activities with the IC_{50} values of 14.5 and 62.7 μg/ml, respectively, whereas **2** and **6** were completely inactive at the concentration of 100 μg/ml. Interestingly, the inactive compound **6** differed from **5** in the absence of an azaphilone unit and a chlorine atom. However, compound **2** having the azaphilone unit was also inactive. The results indicated that the chlorine atom might play an important responsibility for the biological activities. Compound **5** was previously reported to exhibit weak antifungal and antibacterial activities against CA ATCC 18804 and SA ATCC 29213, respectively, with the same MIC value of 128 μg/ml.[1]

Experimental

General Experimental Procedures Infrared spectra (IR) were measured on a FTS165 FT-IR spectrometer or a Perkin Elmer Spectrum GX FT-IR system and recorded on wavenumber (cm^{-1}). ^{1}H- and ^{13}C-nuclear magnetic resonance spectra (^{1}H- and ^{13}C-NMR) were recorded on a FT-NMR, Bruker Avance 300 MHz or 500 MHz spectrometers using tetramethylsilane (TMS) as an internal standard. Spectra were recorded as chemical shift parameter (δ) value in ppm down field from TMS (δ 0.00). Ultraviolet spectra (UV) were measured with an UV-160A SHIMADZU spectrophotometer. Principle bands (λ_{max}) were recorded as wavelengths (nm) and $\log \varepsilon$ in MeOH solution. Optical rotations were measured in methanol solution with sodium D line (590 nm) on an AUTOPOLR® Π automatic polarimeter. Solvents for extraction and chromatography were distilled at their boiling point ranges prior to use except for ethyl acetate which was an analytical grade reagent. Thin-layer chromatography (TLC) and precoated TLC plate were performed on silica gel 60 GF_{254} (Merck). Column chromatography was performed on silica gel (Merck) type 100 (70—230 mesh ASTM) with a gradient of MeOH–CH_2Cl_2, Sephadex LH-20 with MeOH, reverse phase C_{18} silica gel with a gradient of MeOH–H_2O or otherwise stated.

Fungal Material The endophytic fungus *P. sclerotiorum* PSU-A13 was isolated from the leaves of *G. atroviridis*, collected in Yala Province, Thailand. The fungus was deposited as PSU-A13 at the Department of Microbiology, Faculty of Science, Prince of Songkla University and as BCC40796 at the National Center for Genetic Engineering and Biotechnology (BIOTEC) Culture Collection, Thailand.

Fermentation, Extraction and Isolation The flask culture of the fungus PSU-A13 was filtered to separate into the filtrate and wet mycelia. The filtrate was divided into three portions. Each portion was extracted twice with an equal volume of EtOAc (2×500 ml). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark brown gum in 2.24 g. The wet mycelia were extracted twice with 500 ml of MeOH for 2 d. The aqueous MeOH layer was concentrated under reduced pressure. To the extract was added H_2O (50 ml), and the mixture washed with hexane (500 ml). The aqueous residue was extracted three times with an equal amount of EtOAc (3×300 ml). The ethyl acetate and hexane extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness under reduced pressure to obtain both as brown gum in 3.10 g and 1.04 g, respectively. The broth extract was fractioned by CC over Sephadex LH-20 to yield four fractions (A1—A4). Fraction A2 (554.4 mg) was subjected to CC over Sephadex LH-20 to afford four fractions (A21—A24). Fraction A22 (427.5 mg) was further purified by CC over reverse phase silica gel to yield five fractions. The second fraction (31.6 mg) was subjected to CC over Sephadex LH-20 with 50% MeOH–CH_2Cl_2 followed by flash CC over silica gel with a gradient of EtOAc–CH_2Cl_2 to give **2** (8.3 mg). The fourth fraction (67.8 mg) was further separated using the same procedure as the second fraction to yield three subfractions. The second subfraction (24.8 mg) was subjected to flash CC over silica gel with a gradient of EtOAc-light petroleum to give **1** (2.8 mg). Compound **3** (1.8 mg) was obtained from fraction A23 (37.2 mg) after separation by flash CC over silica gel. Fraction A3 (188.2 mg) was purified by CC over silica gel to obtain four fractions (A31—A34). Fraction A32 (4.5 mg), upon PTLC with 100% CH_2Cl_2, and fraction A34 (124.8 mg), upon CC over silica gel with a gradient of EtOAc-light petroleum, gave 4-hydroxyacetophenone (1.8 mg) and **8** (2.7 mg), respectively. The ethyl acetate extract of mycelia was purified by CC over Sephadex LH-20 to yield four fractions (B1—B4). Fraction B2 (906.9 mg) was separated by CC over silica gel to give three subfractions. The second subfraction (97.3 mg) was further subjected to CC over silica gel with a gradient of EtOAc-light petroleum followed by flash CC over silica gel using a gradient of EtOAc-light petroleum and finally with PTLC using 30% EtOAc-light petroleum to obtain **4** (1.7 mg). Fraction B3 (180.3 mg) was purified by CC over silica gel to afford **6** (3.0 mg). Furthermore, the hexane mycelial extract was further separated by CC over

Sephadex LH-20 to give three fractions (C1—C3). Fraction C2 (633.3 mg) was subjected to CC over silica gel to afford four fractions (C21—C24). Fraction C22 (428.7 mg) was separated by CC over silica gel to yield four subfractions. The second subfraction (368.8 mg) was further purified using the same procedure as fraction C22 to afford **5** (18.5 mg). Compound **7** (2.3 mg) was obtained from fraction C23 (52.3 mg) after purification by CC over silica gel followed by PTLC with 15% EtOAc–CH_2Cl_2.

Penicilazaphilone A (**1**): Yellow gum. ^{1}H- ($CDCl_3$, 300 MHz) and ^{13}C-NMR ($CDCl_3$, 75 MHz) data see Table 1. IR (neat) cm^{-1}: 3418, 1717. UV λ_{max} (MeOH) nm (log ε): 243 (3.03), 346 (3.39). HR-EI-MS *m/z*: 392.2185 (Calcd for $C_{22}H_{32}O_6$: 392.2199). EI-MS *m/z*: 392 (M^+), 376, 335, 306, 232, 111. $[\alpha]_D^{26}$ −35.1 (*c*=0.30, MeOH).

Penicilazaphilone B (**2**): Yellow gum. ^{1}H- ($CDCl_3$, 300 MHz) and ^{13}C-NMR ($CDCl_3$, 75 MHz) data see Table 1. IR (neat) cm^{-1}: 3395, 1723. UV λ_{max} (MeOH) nm (log ε): 225 (3.96), 244 (3.88), 345 (4.22). HR-EI-MS *m/z*: 352.1885 (Calcd for $C_{19}H_{28}O_6$: 352.1886). EI-MS *m/z*: 352 (M^+), 334, 266, 149, 98. $[\alpha]_D^{26}$ −44.0 (*c*=0.30, MeOH).

Penicilisorin (**3**): Colorless gum. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 10.90 (1H, s, 8-OH), 7.31 (1H, s, H-4), 6.54 (1H, d, *J*=2.4 Hz, H-7), 6.48 (1H, d, *J*=2.4 Hz, H-5), 3.87 (3H, s, H-10), 3.81 (3H, s, H-11). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 166.9 (s, C-1, C-6), 164.1 (s, C-8), 160.4 (s, C-9), 142.9 (s, C-3), 136.5 (s, C-4a), 113.5 (d, C-4), 104.7 (d, C-5), 103.0 (d, C-7), 101.4 (s, C-8a), 55.9 (q, C-11), 53.0 (q, C-10). IR (neat) cm^{-1}: 3385, 1742, 1675. UV λ_{max} (MeOH) nm (log ε): 252 (4.18), 273 (3.61), 303 (3.46), 341 (3.39). HR-EI-MS *m/z*: 250.0469 (Calcd for $C_{12}H_{10}O_6$: 250.0477). EI-MS *m/z*: 250 (M^+), 191, 135.

Antimicrobial Assays Using a Colorimetric Broth Microdilution Test[12—15] Crude extracts (200 μg/ml) were preliminarily tested against all the test microorganisms by a colorimetric broth microdilution test. The crude extract stock solutions (10 mg/ml) were diluted with Mueller–Hinton broth (MHB) for bacteria and RPMI for fungi to 400 μg/ml and 50 μl of each extract solution was pipetted into 3 wells of a 96 well plate. Fifty microliters of each inoculum was added to the test solution and incubated at 35 °C for 15 h (*S. aureus*, MRSA and *C. albicans*), 25 °C for 45 h (*C. neoformans*), then 10 μl of 0.18% resazurin was added into each well and further incubated for another 2—3 h for bacteria and yeast and 1 d for fungus. Vancomycin and amphotericin B are used as positive controls for bacteria and yeasts, respectively. The color change was then observed visually. Any color changes from purple to pink or colorless was recorded as positive. The crude extracts that showed antimicrobial activity at 200 μg/ml were further assessed for their Minimum Inhibitory Concentrations (MICs). The MICs of extracts were tested over the concentration range of 0.25—128 μg/ml by the above colorimetric broth microdilution test. The lowest concentration at which color change occurred (2 to 3 wells) was taken as the MIC value.

HIV-1 Protease and HIV-1 Integrase Inhibitory Activity Assay of HIV-1 PR inhibitory activity was modified from the previously reported method[16] while the integration reaction was evaluated according to the method previously described.[17] For statistical analysis, the results of anti-HIV-1 PR activity were expressed as mean±S.D. of three determinations, while anti-HIV-1 IN were as mean±S.E.M of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. Dunnett's test was used *versus* control for calculation of statistical significance.

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