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Rare flavonoids and sesquiterpenoids isolated from the leaves of *Goniotalamus gracilipes*

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ABSTRACT

Three previously undescribed benzopyranyl sesquiterpenes gracilipins B-D (**1-3**) and two flavonoids 5,4'-dihydroxy-6-(2-hydroxybenzyl)-3,7,3'-trimethoxyflavone (**4**), and 5,4'-dihydroxy-8-(2-hydroxybenzyl)-3,7-dimethoxyflavone (**5**) were isolated from the leaves of *Goniothalamus gracilipes* (Annonaceae). Their structures were determined by analyses of MS and 2D NMR data. The absolute configurations of **1** were established by analysis of X-ray diffraction data. Cytotoxic evaluation of the compounds **1-5** against four cancer cell lines (KB, LU-1, HepG-2 and MCF-7) indicated that compound **5** had inhibitory activity against HepG-2 cell line with IC₅₀ value of 16.7 μ M. All new compounds (**1-5**) were evaluated for their antimicrobial activity against a panel of clinically significant microorganisms. Compound **2** showed significant antimicrobial effect on *Staphylococcus aureus* with MIC value of 32 μ g/mL.

Keywords: Annonaceae, *Goniothalamus gracilipes*, benzopyran-sesquiterpene, flavonoid, cytotoxic activity, antimicrobial

1. Introduction

The *Goniothalamus* genus is the largest Annonaceae genus in Asia (with c. 130 species) [1,2]. The plants of this genus had attracted much attention of many research groups and are considered as an interesting potential source of drugs for the treatment of cancers and bacterial infections [2-6]. They are known to produce mainly three classes of specialized metabolites: acetogenins, alkaloids and styryllactones, some of which had shown potent cytotoxic activities against several human tumors cell lines [7-11]. *Goniothalamus gracilipes* Ban (Annonaceae) is a shrub or small tree growing up to 4 m high in the central and northern Vietnam, and is one of the 19 Vietnamese species [12]. This species was selected for further chemical study because the ethyl acetate extract of the leaves exhibited significant cytotoxic activity against KB cells (30% inhibition at 1 $\mu\text{g/mL}$). Herein we report the isolation, characterization and biological activities of three previously undescribed benzopyran-sesquiterpenes (**1-3**) and two flavonoids (**4-5**) from the leaves of *Goniothalamus gracilipes* (Annonaceae) (Fig 1).

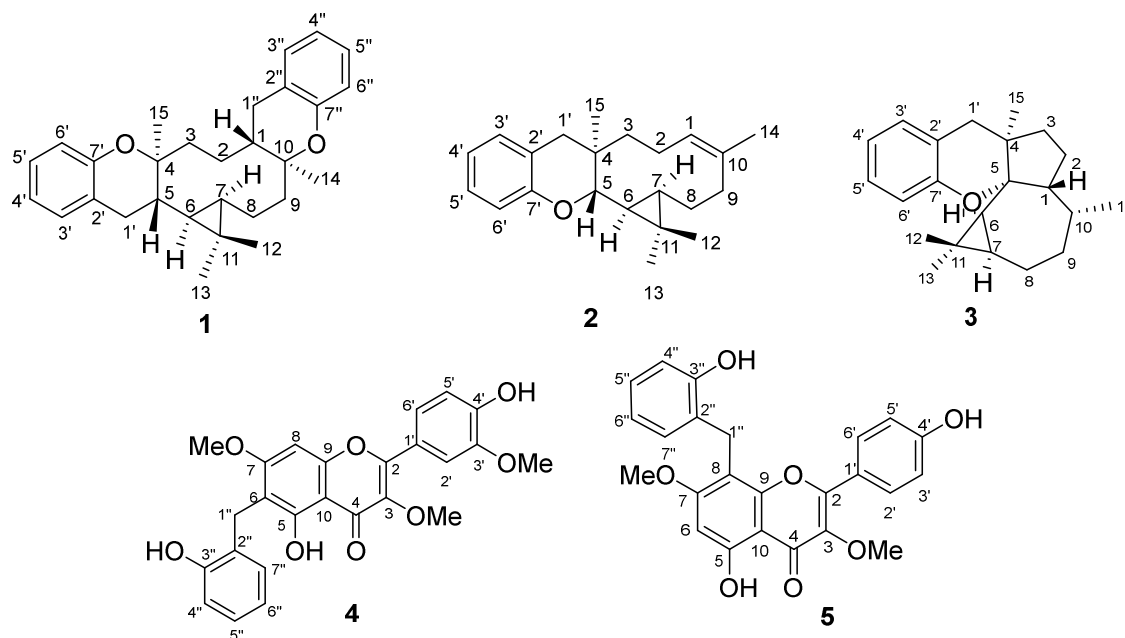


Fig. 1. Structures of compounds **1-5** from *Goniothalamus gracilipes*

2. Experimental

2.1. General Experimental Procedures

Optical rotations were recorded on a Polax-2L polarimeter in CHCl_3 . HR-ESI-MS were recorded on a FT-ICR 910-MS TQFTMS-7 T mass spectrometer. IR spectra were recorded on a Nicolet Impact 410 FT-IR spectrometer, and NMR spectra on a Bruker AM 500 MHz spectrometer operating at 125.76 MHz for ^{13}C NMR, and at 500.13 MHz for ^1H NMR. ^1H chemical shifts were referenced to CD_3OD and CDCl_3 at δ 3.31 and 7.27 ppm, respectively, while the ^{13}C chemical shifts were referenced to the central peak at δ 49.0 (CD_3OD), and 77.1 ppm (CDCl_3). The HMBC measurements were optimized to 7.0 Hz long-range couplings, and NOESY experiments were run with 150 ms mixing time.

2.2. Plant Material

Leaves of *G. gracilipes* Ban were collected in Thai Nguyen, Vietnam, in November 2000. The plant material was identified by Dr. Nguyen Quoc Binh, and a voucher specimen (VN-0759) has been deposited at the Herbarium of the Institute of Ecology and Biological Resources of the Vietnam Academy of Science and Technology, Hanoi, Vietnam.

2.3. Extraction and Isolation

Dried and ground leaves (3.16 kg) of *G. gracilipes* Ban were extracted with MeOH (5 x 5 L x 24h) at room temperature. The MeOH solution was evaporated to dryness under reduced pressure to give a crude extract (889 g). The crude residue was dissolved in a 1:1 MeOH/water solution and subjected to a liquid-liquid partition using *n*-hexane, and then EtOAc (5 times, 1 L/time). The *n*-hexane solution was concentrated under reduced pressure to give a residue of 110 g, which was subjected to a silica gel column chromatography using a gradient of *n*-hexane/EtOAc of increasing polarity (0 to 60% EtOAc) to give 5 fractions (F1-F5). Fraction F1

(53 g) was subjected to a silica gel column chromatography (*n*-hexane 100%) to yield 7 subfractions (F1.1-F1.7). Fraction F1.1 was then subjected to a silica gel column chromatography (from 0% to 4% of EtOAc in *n*-hexane) to yield 6 sub-fractions (F1.1A- F1.1F). Purification of fraction F1.1A by Sephadex LH-20 column chromatography (CH₂Cl₂/MeOH 3:7) afforded compound **3** (113 mg). Fraction F1.1E was subjected to a silica gel column chromatography, using *n*-hexane 100% to yield compound **2** (21 mg). Fraction F1.4 was crystallized from a EtOAc/*n*-hexane solution (1/9, v/v), affording compound **1** (834 mg).

The EtOAc extract were concentrated under reduced pressure to give a residue of 197 g. This residue was subjected to silica gel column chromatography using a gradient of *n*-hexane/EtOAc of increasing polarity (17 to 50% EtOAc) to give 13 fractions (F1-F13). Fraction F9 (9.3 g) was subjected to a silica gel column chromatography using the same gradient to yield seven subfractions (F9.1-F9.7). Fraction F9.5 was subjected to a silica gel column chromatography using a gradient of CH₂Cl₂/EtOAc of increasing polarity (from 0% to 35% of EtOAc) to yield 16 subfractions (F9.1A to F9.1P). F9.1D was then subjected to a silica gel column chromatography using a gradient of *n*-hexane/EtOAc of increasing polarity (25 to 30% EtOAc) to yield compound **4** (12 mg), and a gradient of 2 to 10% of EtOAc in CH₂Cl₂ was used for the isolation of compound **5** (21 mg) from fraction F9.5.J .

2.3.1. *Gracilipin B (1)*

White crystals; mp 189-190°C; $[\alpha]_D^{27} +30.6$ (*c* 0.17, EtOH); UV (EtOH) λ_{\max} (log ϵ) 204 (4.53), 219 (4.31), 277 (3.85) nm; IR (KBr) ν_{\max} : 3488, 2990, 2934, 1610, 1583, 1489, 1373, 1247, 1190, 1061, 958, 756, 610, 549, 489 cm⁻¹; ¹H- and ¹³C-NMR see Table 1; HRESIMS *m/z* 417.2793 [M+H]⁺ (calcd for C₂₉H₃₆O₂, 417.2788).

2.3.2. *Gracilipin C (2)*

Colorless oil; $[\alpha]_D^{25} -42.9$ (*c* 0.275, EtOH); UV (EtOH) λ_{\max} (log ϵ) 205 (4.24), 226 (3.79), 277 (3.31) nm; IR (KBr) ν_{\max} 3450, 3133, 2936, 1645, 1554, 1487, 1383, 1194, 1067, 997, 829, 759, 645, 541, 477 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 1; (–)-ESI-MS m/z 309 $[\text{M-H}]^-$, (+)-ESI-MS m/z 311 $[\text{M+H}]^+$ ($\text{C}_{22}\text{H}_{30}\text{O}$); HRESIMS m/z 311.2374 $[\text{M+H}]^+$ (calcd for $\text{C}_{22}\text{H}_{30}\text{O}$, 311.2369).

2.3.3. *Gracilipin D (3)*

Colorless oil; $[\alpha]_D^{25} -3.8$ (*c* 0.4, EtOH); UV (EtOH) λ_{\max} (log ϵ) 204 (4.32), 221 (3.89), 277 (3.38) nm; IR (KBr) ν_{\max} 2959, 1586, 1489, 1455, 1239, 1114, 1017, 823, 757, 628, 547, 482 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 1; HRESIMS m/z 311.2385 $[\text{M+H}]^+$ (calcd for $\text{C}_{22}\text{H}_{30}\text{O}$, 311.2369).

2.3.4. *5,4'-dihydroxy-6-(2-hydroxybenzyl)-3,7,3'-trimethoxyflavone (4)*

Yellow microcrystalline solid; mp 240–242 °C; UV (MeOH) λ_{\max} (log ϵ) 210 (5.01), 258 (4.68), 272 (4.70), 305 (4.37), 355 (4.09), 366 (4.63) nm; IR (KBr) ν_{\max} 3416, 2955, 2918, 2851, 1732, 1653, 1605, 1516, 1479, 1429, 1335, 1287, 1194, 1161, 1051, 986, 862, 812, 754, 610, 557, 411 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 2; HRESIMS m/z 451.1383 $[\text{M+H}]^+$ (calcd for $\text{C}_{25}\text{H}_{22}\text{O}_8$, 451.1387).

2.3.5. *5,4'-dihydroxy-8-(2-hydroxybenzyl)-3,7-dimethoxyflavone (5)*

Yellow microcrystalline solid; mp 225–226 °C; UV (MeOH) λ_{\max} (log ϵ) 226 (4.60), 271 (4.23), 305 (3.95), 360 (3.48), 366 (4.03) nm; IR (KBr) ν_{\max} 3408, 3171, 2938, 1732, 1653, 1601, 1539, 1454, 1344, 1279, 1227, 1182, 1163, 1098, 995, 941, 883, 823, 754, 664, 565, 517, 438 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 2; HRESIMS m/z 421.1271 $[\text{M+H}]^+$ (calcd for $\text{C}_{24}\text{H}_{20}\text{O}_7$, 421.1282).

2.4. X-ray Crystallographic Analysis of Compound 1.

The intensity data set of compound **1** was collected at low temperature (193 K) with with Cu K α from a Micromax007HF rotating anode generator (Rigaku) equipped with CMF Osmics mirrors and a Rapid II detector (Rigaku). Reflections with average redundancy of 17.1 were integrated and scaled with Fs_process part of the CrystalClearSM package [13] and the structure was solved by direct methods using SHELX-S 97 [14], with two independent copies in the asymmetric unit of the P2₁ unit cell, and refined by full-matrix least-squares calculations on F² using SHELXL-2018/3 [15]. Non-H atoms were refined with anisotropic displacement parameters. Hydrogen atoms were constrained to parent sites, using a riding model. The absolute configuration of **1** was confirmed by extensive exploitation of the weak anomalous signal carefully detected through the meaningful value of the Flack parameter [16], $x = -0.03(3)$, determined using 3738 quotients $[(I^+)-(I^-)]/[(I^+)+(I^-)]$ and likelihood methods providing both Hooft absolute structure parameter $y = -0.03(3)$ and unambiguous probabilities (P2(true), P3(true) = 1.000, P3(rac-twin) $8.0 \cdot 10^{-60}$, P3(false) $8 \cdot 10^{-213}$) in favor of C1(R), C4(S), C5(S), C6 (R), C7(R), and C10(S) [17]. Crystal data, data collection and structure refinement details are summarized in Table S1.

CCDC 2094623 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

2.5. Antimicrobial assay

Antimicrobial assays of compounds **1-5** were carried out using *Enterococcus faecalis* (ATCC299212), *Staphylococcus aureus* (ATCC25923), *Bacillus cereus* (ATCC14579), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella enterica* (ATCC14579) and *Candida albicans* (ATCC10231). Stock solutions of samples were prepared in

DMSO, and the antimicrobial assays were carried out in 96-well microtiter plates against the microbial strains (5×10^5 CFU/mL) using a modification of the published method [18]. After incubation for 24 hours at 37 °C, the absorbance at 650 nm was measured using a microplate reader. Streptomycin and cycloheximide were used as positive reference compounds.

2.6. Cancer cell line cytotoxicity assay

The cytotoxic activities of compounds **1-5** were evaluated in vitro against KB, LU-1, HepG-2, and MCF-7 cell lines. The cells were cultured at 37 °C in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in a 5% CO₂ incubator. Cells between 5 and 20 passages were used for the assays. The cytotoxic activity was measured by using a modified MTT assay [18]. Viable cells were seeded in 96-well plates at a density of 3×10^4 cells/mL. Cells were treated with various concentrations of essential oils (0.3, 3, 10, and 30 µg/mL) and then incubated at 37°C for 72 h in fresh DMEM medium. Cells were subsequently incubated at 37 °C with MTT (0.5 mg/mL) for 4 h. After removal of supernatant, formazan crystals were dissolved in DMSO and the optical density was measured at 540 nm. Ellipticine was used as a positive control.

3. Results and discussion

MeOH extract was prepared from dried leaves of *G. gracilipes* and subjected to solvent-solvent partition, yielding *n*-hexane, EtOAc and remaining aqueous extracts. Further purification of the *n*-hexane, EtOAc extracts with combination of different chromatographic techniques (prep TLC, silica gel and Sephadex LH-20 gel chromatography) resulted in the isolation of five new compounds (**1-5**) (Fig. 1).

Compound **1** was isolated as white crystals, $[\alpha]_D^{27} +30.6^\circ$ (*c* 0.17; EtOH). The positive HR-ESI-MS of **1** gave a protonated molecular ion at m/z 417.2793 $[M+H]^+$, calcd 417.2788,

suggesting the molecular formula of $C_{29}H_{36}O_2$. The UV (λ_{max} (EtOH) ($\log \epsilon$) 204 (4.53); 219 (4.31); 277 (3.85) and IR spectra (KBr) 1610, 1583 (C=C), 1247 (C-O), 756 cm^{-1} (*ortho*-substituted phenyl) are in agreement with benzopyran moieties in **1** [20]. The ^1H NMR spectrum of **1** showed the presence of two *ortho*-substituted phenyl rings, four tertiary methyl groups at δ_{H} 1.00 (CH₃-12), 1.10 (CH₃-13), 1.14 (CH₃-14), 1.24 (CH₃-15), and two cyclopropyl protons at δ_{H} 0.65 (H-7), 0.37 (H-6). The ^{13}C NMR and DEPT spectrum of **1** revealed 29 carbon resonances including four methyls, six methylenes, eight sp^2 methines, four sp^3 methines, four sp^2 quaternary carbons, one sp^3 quaternary carbons, two sp^3 oxygenated tertiary carbons. The COSY spectrum of **1** indicated four spin-spin coupling systems as follows: i) H-1' (δ_{H} 2.70)/H-5 (δ_{H} 2.07)/H-6 (δ_{H} 0.37)/H-7 (δ_{H} 0.65)/H-8(δ_{H} 1.20 and 1.86)/H-9 (δ_{H} 1.86 and 2.20), ii) H-1''(δ_{H} 2.49 and 2.63)/H-1 (δ_{H} 2.28)/H-2(δ_{H} 1.36 and 2.07)/H-3 (δ_{H} 1.77 and 2.63), iii) H-3' (δ_{H} 7.02)/H-4' (δ_{H} 6.82)/H-5' (δ_{H} 7.08)/H-6' (δ_{H} 6.82), and iv) H-3'' (δ_{H} 6.97)/H-4''(δ_{H} 6.75)/H-5'' (δ_{H} 7.02)/H-6'' (δ_{H} 6.75) (Fig. 2). Additionally, the key HMBC correlations of **1** suggested a sesquiterpene fused to two benzopyran units according to figure 1.

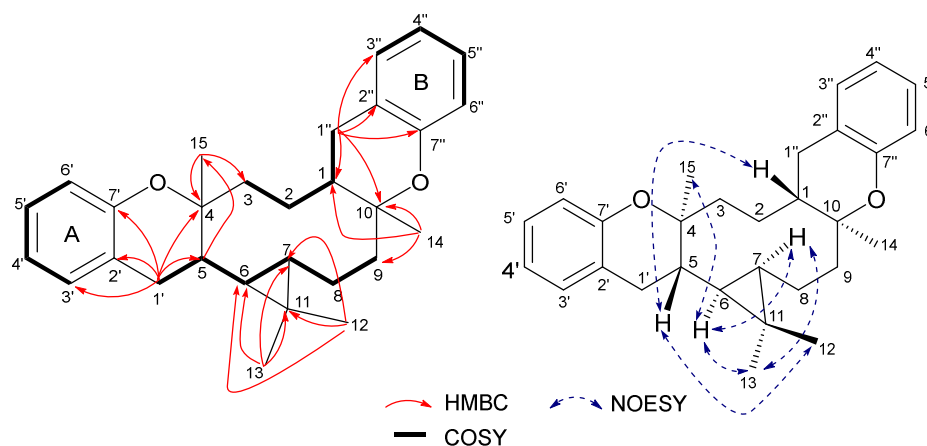


Fig. 2. Key COSY, HMBC and NOESY correlations of **1**

In the HMBC spectrum of **1**, correlations from Me-12 and Me-13 to carbons C-6 (δ_c 29.0), C-7 (δ_c 27.7) and C-11 (δ_c 16.7)) suggested that the dimethylcyclopropyl ring is located at C-6 and C-7 as shown in Fig. 1. Moreover, cross-peaks from H-1' to C-2' (δ_c 122.1), C-3' (δ_c 129.3), C-7' (δ_c 153.9), C-4 (δ_c 78.3) and C-5 (δ_c 35.0) on one side, and from H-1'' to C-2'' (δ_c 121.9), C-3'' (δ_c 128.9), C-7'' (δ_c 153.8), C-1 (δ_c 32.2) and C-10 (δ_c 80.8) on the other side, allowed benzene rings A and B to be connected with carbons C-1' and C-1'' through bonds C-1'/C-2' and C-1''/C-2'', respectively (Fig. 2). All the foregoing information in addition to the other data of HMBC, COSY, HSQC confirm the planar structure of **1** as shown.

The relative configuration of **1** was determined from the NOESY experiment. A correlation was observed between H-6 and H-7, indicating the *cis* arrangement of the two protons of the cyclopropane ring. The cross-peaks between protons of CH₃-12, H-5 and H-1 indicated that they were cofacial, and NOE correlation of CH₃-15 with H-6 indicated the *trans* configuration of CH₃-15 and H-5.

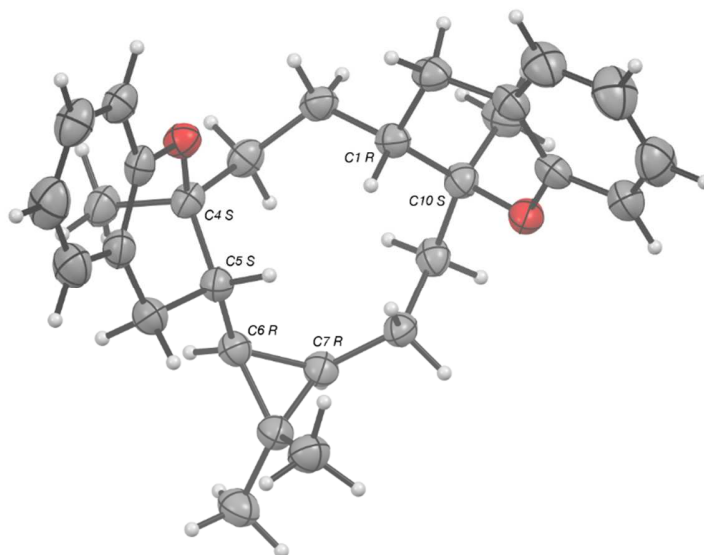


Fig. 3. X-ray crystal structure for **1**

The structure and (1*R*, 4*S*, 5*S*, 6*R*, 7*R*, 10*S*) absolute configuration of compound **1** was confirmed by X-ray diffraction analysis (Fig. 3). This newly described bis(benzopyranyl) bicyclogermacrane sesquiterpene was named gracilipin B. The structure of compound **1** is closely comparable to lucidene, a bis(benzopyranyl) isolated from *Uvaria lucida* ssp *lucida*, which possesses an humulane moiety instead of a bicyclogermacrane [20].

Table 1. NMR Data for **1-3** (CDCl₃, ¹H, 500 MHz; ¹³C, 125 MHz)

1			2		3	
position	δ_{C}	δ_{H} mult (J in Hz)	δ_{C}	δ_{H} mult (J in Hz)	δ_{C}	δ_{H} mult (J in Hz)
1	32.2	2.28, m	127.6	5.22, dd (10.5, 4.0)	51.8	2.35, m
2	27.5	2.07, m	23.2	2.32, m	17.1	1.65, m
		1.36, m		2.00, m		
3	36.1	2.63, m	39.3	1.85, m	35.7	1.69, m
		1.77, m		1.59, m		
4	78.3		36.8		39.1	
5	35.0	2.07, m	80.0	3.53, d (7.5)	87.5	
6	29.0	0.37, m	26.8	0.78, dd (8.0, 7.5)	27.7	0.59, d (10.0)
7	27.7	0.65, m	30.7	0.52, m	30.0	0.76, m
8	17.0	1.86, m	22.6	1.85, m	24.7	1.96, m
		1.20, m		1.08, m		1.44, m
9	38.5	2.20 m	38.2	2.07, m	32.8	1.55, m
		1.86, m		2.00, m		1.44, m
10	80.8		131.2		34.2	1.88, m
11	16.7		18.9		21.4	
12	16.6	1.00, s	18.5	1.16, s	17.4	1.32, s

13	29.9	1.10, s	30.5	1.18, s	30.8	1.07, s
14	20.0	1.14, s	17.7	1.61, s	22.3	0.84, d (7.0)
15	22.9	1.24, s	21.4	0.97, s	22.4	1.04, s
1'	29.0	2.70, d (9.0)	37.7	3.20, d (16.0)	38.9	2.90, d (16.5)
				2.17, d (16.0)		2.47, d (16.5)
2'	122.1		122.3		120.3	
3'	129.3	7.02, m	129.9	7.06, m	129.8	7.00, br d (7.5)
4'	119.6	6.82, m	119.8	6.83, m	119.1	6.80, m
5'	127.1	7.08, m	126.5	7.06, m	127.0	7.08, m
6'	117.0	6.82, m	116.2	6.73, m	116.1	6.80, m
7'	153.9		155.0		153.0	
1''	31.6	2.63, m				
		2.49, m				
2''	121.9					
3''	128.9	6.97, m				
4''	119.4	6.75, m				
5''	127.0	7.02, m				
6''	116.9	6.75, m				
7''	153.8					

Compound **2** was isolated as an optically active colorless oil, $[\alpha]_D^{25} -42.9^\circ$ (*c* 0.275, EtOH). The positive HR-ESI-MS of **2** gave a protonated molecular ion at m/z 311.2374 $[M+H]^+$, suggesting the molecular formula $C_{22}H_{30}O$ (calcd for 311.2369). The 1H and ^{13}C NMR (Table 1) data of **2** were close to those of **1**. However, these data suggested the presence of only one set of resonances corresponding to the benzopyran ring, and the presence of additional olefinic methin signals [δ_H 5.22 (1H, dd, $J=10.5, 4.0$ Hz, H-1), δ_C 127,6]. In addition, one oxymethine carbon was

noted at [δ_{H} 3.53 (1H, d, J = 6.5 Hz); δ_{C} 80.0], suggesting a different ring arrangement for compound **2**. The 2D structure of **2** was established from 2D NMR data analysis. The planar structure of the terpene moiety, which is reminiscent of a bicyclogermacrane skeleton can be deduced from the analysis of COSY and HMBC spectra. We note in particular the presence of two spin systems H-1 to H₂-3 and H-5 to H₂-9, a methyl-substituted ethylenic function and a cyclopropane ring similar to compound **1**, these elements being linked according to the structure depicted in Fig. 4. From HMBC correlations of CH₃-15 (δ_{H} 0.97) with C-3 (δ_{C} 39.3), C-4 (δ_{C} 36.8), C-5 (δ_{C} 80.0), and C-1' (δ_{C} 37.7), it can be deduced that the methyl group CH₃-15 was attached to C-4. Finally, HMBC correlations from H₂-1' to C-2', C-3', C-7', C-3, C-4 and C-15, allowed the benzopyran and germacrene-like moieties to be linked as shown Fig. 4.

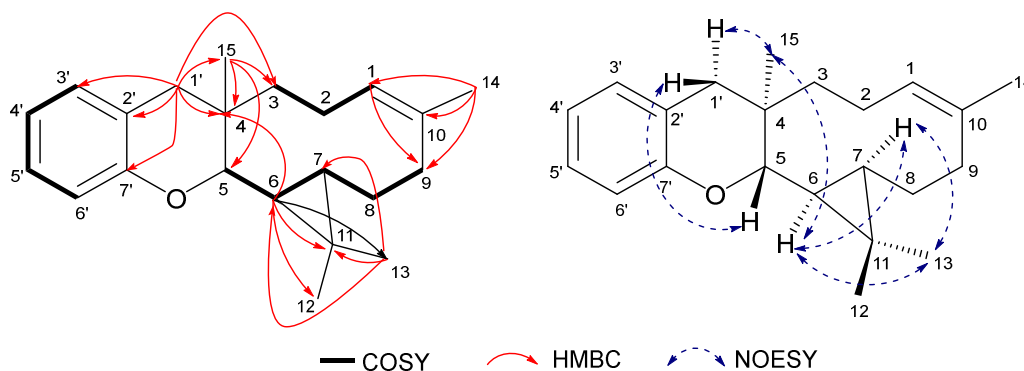


Fig. 4. Key COSY, HMBC and NOESY correlations of **2**

The relative configuration of **2** was determined by NOESY experiment. Accordingly, the relative configurations were found to be similar with those of **1**. Additional, NOE correlation of CH₃-15/ H-1'b (δ_{H} 2.17, d, J = 16.0 Hz) and H-1'a (δ_{H} 3.20, d, J = 16.0 Hz)/H-5 (δ_{H} 3.53) indicated that CH₃-15 and H-5 were *trans*-diaxial oriented. This newly described benzopyranyl bicyclogermacrane sesquiterpene was named gracilipin C.

Compound **3** was isolated as an optically active colorless oil, $[\alpha]_D^{25} -3.8^\circ$ (c 0.4, EtOH). The positive HR-ESI-MS of **3** gave a protonated molecular ion at m/z 311.2385 $[M+H]^+$ (calcd 311.2369), suggesting the molecular formula of $C_{22}H_{30}O$. ^{13}C -NMR and DEPT NMR spectra revealed 22 carbon signals due to two sp^2 quaternary carbons, two sp^3 quaternary carbons, one oxygenated tertiary carbon, four sp^2 methines, four sp^3 methines, five sp^3 methylenes, and four methyl groups. Among them carbons at δ_C 87.5 (C-5) at δ_C 153.0 (C-7') were suggested to be attached to an oxygen atom. The 1H NMR spectrum of **3** indicated the presence of 4 protons of ortho-substituted phenyl ring at δ_H 7.08 (H-5'), 7.00 (H-3'); 6.80 (H-4'+H-6'), three tertiary methyl groups at δ_H 1.32 (CH₃-12), 1.07 (CH₃-13); 1.04 (CH₃-15), one secondary methyl group at δ_H 0.84 (CH₃-14) and two cyclopropyl protons at δ_H 0.76 (H-7), 0.59 (H-6). These observations together with 1H - 1H COSY and HMBC data (Table 1 and Fig. 5) indicated that compounds **3** should possess an aromadendrane sesquiterpene moiety and a benzopyran moiety.

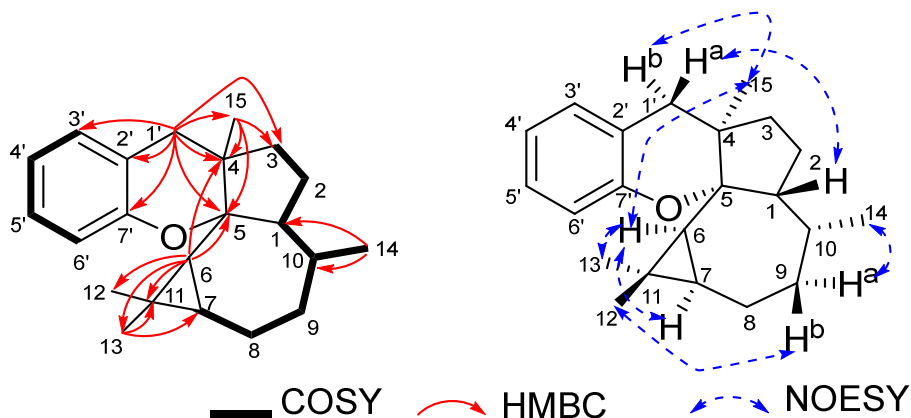


Fig. 5. Key COSY, HMBC and NOESY correlations of **3**

Connectivity of CH₃-14 to C-1 (δ_C 51.8) through C-10 (δ_C 34.2) was indicated by HMBC correlation from CH₃-14 to C-1 and C-10. Moreover, the cross-peaks from H-1' to C-2', C-3', C-7', C-5, CH₃-15 and C-4 were observed, indicating the connectivity of the benzene ring and C-1'

through the bond C-1'/C-2'. The presence of a dimethylcyclopropyl ring composed of CH₃-12, CH₃-13, C-11, C-6 and C-7 was suggested by HMBC cross-peaks for H-6 to C-11, C-12, C-13 and CH₃-13 to C-7, C-11. The relative stereochemistry of **3** was determined by NOESY as shown in Fig. 5. Cross-peaks between H-6 and H-7 suggested a *cis* arrangement of the two protons of the cyclopropane ring. Furthermore, cross-peaks between protons H-1'b/H₃-15/H-6 and H₃-13 on one hand and H-1'a and H-1 on the other hand indicated that these protons are co-facial, and they were arbitrarily oriented as α - and β -, respectively. Finally, from NOE correlations between H₃-12 and H-9b, and between H-9a and H₃-14, it can be determined that the methyl groups CH₃-14 and CH₃-13 are α - and β -oriented, respectively. The newly described compound was named gracilipin D.

Compound **4** was obtained as a yellow microcrystalline solid (mp 240-242 °C). The IR spectrum of **4** indicated the presence of conjugated carbonyl (1653 cm⁻¹) and OH (3416 cm⁻¹) groups. Its HRESI mass spectrum gave a protonated molecular ion [M+H]⁺ at *m/z* 451.1383 (calcd 451.1387 for C₂₅H₂₂O₈) consistent with the molecular formula C₂₅H₂₂O₈. Analysis of the ¹H NMR spectrum revealed three methoxy groups at δ_H 3.85 (3-OCH₃), 3.97 (3'-OCH₃), and 3.99 (7-OCH₃), a methylene at δ_H 3.95 (s) (CH₂-1''), one singlet aromatic proton at δ_H 6.48 (H-8), three aromatic proton signals for ABX system at δ_H 7.03 (d, *J* = 8.0 Hz, H-5'), 7.65 (dd, *J* = 8.0, 2.0 Hz, H-6'), 7.69 (d, *J* = 2.0 Hz, H-2'), four aromatic proton signals for an A₂B₂ system [δ_H 6.82 (ddd, *J* = 7.5, 7.5, 1.5 Hz, H-6''), 6.87 (dd, *J* = 7.5, 1.5 Hz, H-4''), 7.09 (ddd, *J* = 7.5, 7.5, 1.5 Hz, H-5''), and 7.43 (dd, *J* = 7.5, 1.5 Hz, H-7'')], and a chelated OH proton at δ_H 13.73 (OH-5). However, analysis of the ¹³C NMR and DEPT with the aid of HSQC spectra revealed the presence of 25 carbon signals including three methoxy groups, one sp³ methylene, eight sp² methines, one carbonyl and 11 sp² quaternary carbons.

The signals from 1D NMR spectra strongly suggested that **4** was a flavonol derivative substituted by a 2-hydroxybenzyl moiety, 2 hydroxy and 3 methoxy groups. The linkage of a 2-hydroxybenzyl group at C-6 was established by cross-peaks from the protons H-8 and H₂-1'' and the chelated OH-5 to carbon C-6 at δ_C 111.5, and cross-peaks from H₂-1'' protons to C-5 (δ_C 156.8), C-3'' (δ_C 154.7), C-2'' (δ_C 125.6), and C-7'' (δ_C 131.7) in HMBC spectrum (Fig. 6). Careful analysis of 2D NMR spectra established the structure of **4** as 5,4'-dihydroxy-6-(2-hydroxybenzyl)-3,7,3'-trimethoxyflavone.

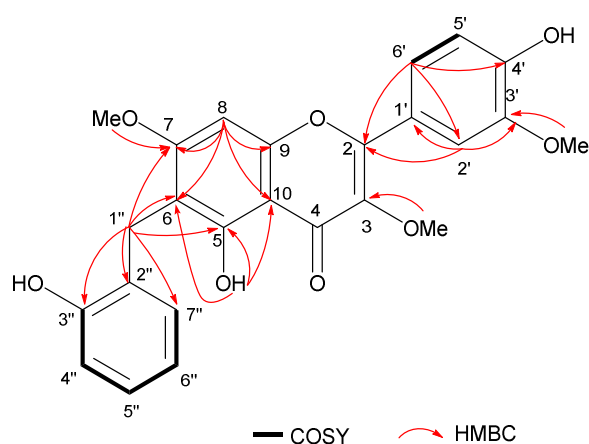


Fig. 6. Key COSY, HMBC correlations of **4**

Table 2. NMR Data for **4** and **5** (¹H, 500 MHz; ¹³C, 125 MHz)

4^a			5^b	
position	δ_C	δ_H mult (<i>J</i> in Hz)	δ_C	δ_H mult (<i>J</i> in Hz)
2	156.2		155.6	
3	138.9		137.4	
4	178.6		178.4	
5	156.8		159.9	
6	111.5		95.0	6.61, s
7	163.0		163.1	

8	90.4	6.48, s	105.7	
9	155.3		153.0	
10	105.8		104.7	
1'	122.3		120.6	
2'	111.0	7.69, d (2.0)	130.0	7.76, d (8.5)
3'	146.4		115.5	6.84, d (8.5)
4'	148.5		160.2	
5'	114.6	7.03, d (8.0)	115.5	6.84, d (8.5)
6'	122.7	7.65, dd (8.0, 2.0)	130.0	7.76, d (8.5)
1''	22.9	3.95, s	21.5	4.01, s
2''	125.6		125.7	
3''	154.7		155.0	
4''	116.6	6.87, dd (7.5, 1.5)	114.5	6.83, d (7.0)
5''	127.9	7.09, ddd (7.5, 7.5, 1.5)	126.6	6.96, m
6''	120.0	6.82, ddd (7.5, 7.5, 1.5)	118.8	6.57, m
7''	131.7	7.43, dd (7.5, 1.5)	127.8	6.57, m
5-OH		13.73, s		12.81
3-OCH ₃	60.2	3.85, s	59.6	3.78, s
7-OCH ₃	56.2	3.99, s	56.4	3.87, s
3'-OCH ₃	56.1	3.97, s		

^a in CDCl₃, ^b in DMSO

Compound **5** was isolated as a yellow microcrystalline material (mp. 225-226 °C). The IR spectrum had conjugated carbonyl (1653 cm⁻¹) and OH (3408 cm⁻¹) bands, and the HRESI mass spectrum yielded the molecular formula of C₂₄H₂₀O₇. The 1D NMR spectrum of **5** was similar to those of **4**, but the 2-hydroxybenzyl moiety is attached at C-8 and the B ring B lacks a methoxy group at C-3'. In the HMBC spectrum of **5**, cross-peaks from H₂-1'' (δ_{H} 4.01) to C-7 (δ_{C} 163.1), C-

8 (δ_C 105.7), C-9 (δ_C 153.0), C-2'' (δ_C 125.7), C-3'' (δ_C 155.0), and C-7'' (δ_C 127.8) indicated linkage of a 2-hydroxybenzyl group to C-8 of the flavonol skeleton (Fig. 7). Similarly, the two methoxy groups were determined to be linked to C-3 and C-7 by 3J -HMBC correlations of methoxy protons at δ_H 3.78 and 3.87 to C-3 (δ_C 137.4), and C-7 (δ_C 163.1), respectively. Analyses of 2D NMR data confirmed the structure of **5** as 5,4'-dihydroxy-8-(2-hydroxybenzyl)-3,7-dimethoxyflavone. Within flavonoids, C-benzyl flavanols and C-benzyl flavonols are scarce and come from a limited number of species. In the family Annonaceae, C-benzyl flavanols have mainly been described in species of the genera *Uvaria* [21] and *Cleistochlamys* [22], whereas several examples of C-benzyl flavonols were isolated from species of the genera *Desmos* [23-24], *Sphaerocoryne* [25] and *Miliusa* [26]. Flavonoids **4** and **5** are the first examples of C-benzylated analogues reported in the genus *Goniothalamus*, a genus member of the tribe Annoneae and the subfamily Annonoideae [27].

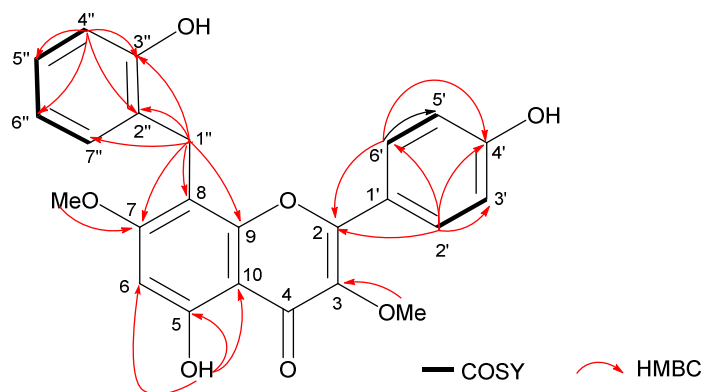


Fig. 7. Key COSY, HMBC correlations of **5**

Gracilipins B-D (**1-3**) are rare benzopyranyl sesquiterpenes. They could be formed via an hetero Diels-Alder cycloaddition reaction between bicyclogermacrene (compounds **1** and **2**) or α -gurjunene (**3**) sesquiterpene with *o*-quinone methide as depicted in Fig. 8. The formation of such Diels-Alder adducts has been supported by the biomimetic synthesis of (\pm)-alboatrin, a close

benzopyranyl analogue [28]. Similar compounds have been reported from a few members of the genus *Uvaria* (Annonaceae), namely *Uvaria angolensis* [29], *U. lucida* ssp. *lucida* [30-31] and *U. tanzaniae* [32]. However, to our knowledge, this is the first report describing benzopyranyl bicyclogermacrene-type and benzopyranyl α -gurjunene-type sesquiterpenes in Annonaceae.

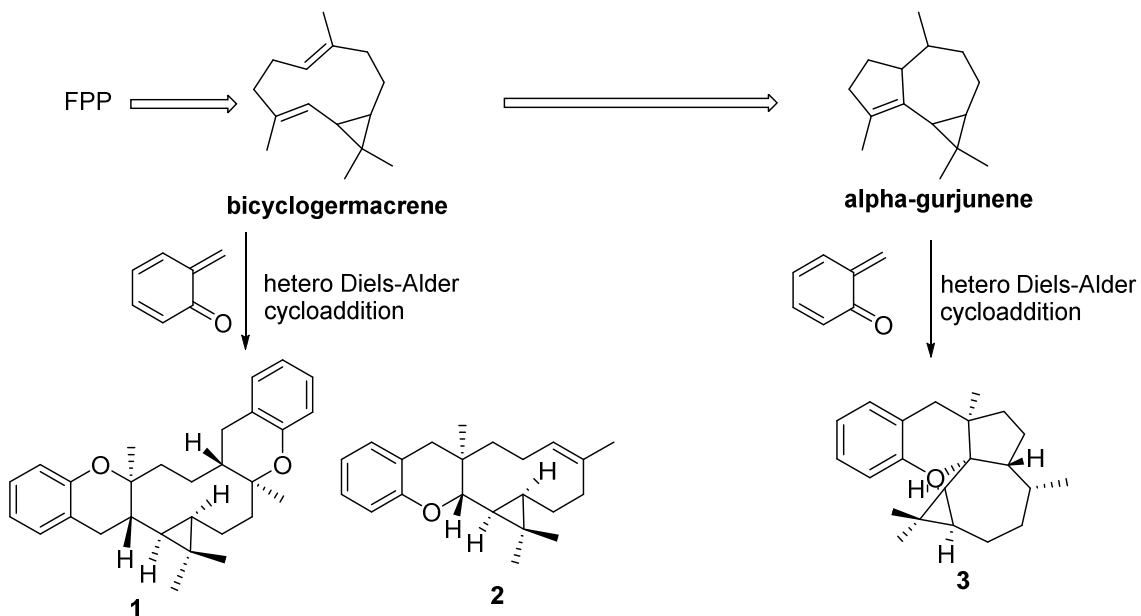


Fig. 8. Plausible biosynthetic pathway of **1-3**

Many natural sesquiterpenoids containing a gem-dimethylcyclopropane unit, such as compounds **1-3** have interesting biological properties, including anti-viral, anti-microbial and cytotoxic activities [33]. Thus, sesquiterpenoids **1-3**, along with flavonoids **4** and **5**, were evaluated for their cytotoxic activity against four cancer cell lines KB (mouth epidermal carcinoma cells), HepG-2 (human liver hepatocellular carcinoma cells), LU-1 (human lung adenocarcinoma cells), and MCF-7 (human breastcancer cells), and their antibacterial activities against *Enterococcus faecalis* (ATCC299212), *Staphylococcus aureus* (ATCC25923), *Bacillus cereus* (ATCC14579), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella enterica* (ATCC14579) and *Candida albicans* (ATCC10231).

Only compound **5** exhibited a weak cytotoxic activity against HepG-2 with an IC₅₀ value of 16.7 μ M. The cytotoxic activity of compound **5** alone cannot explain the strong activity found in the crude EtOAc extract of the leaves studied. It is likely that this activity is due to the presence of low amounts of acetogenins in the extract. Indeed, strong cytotoxic activities of acetogenins isolated from various *Goniothalamus* species have been yet reported [34]. In our case we could not identify new acetogenins from the leaves extract but the chemical study carried out from the fruits of *G. gracilipes* had led to the characterization of two new acetogenins possessing moderate cytotoxic activity [35]. The other compounds were not cytotoxic up to a concentration of 20 μ M. Compound **2** selectively inhibited *Staphylococcus aureus* with a MIC value of 32.00 μ g/mL, identical with the MIC of the reference compound, streptomycin.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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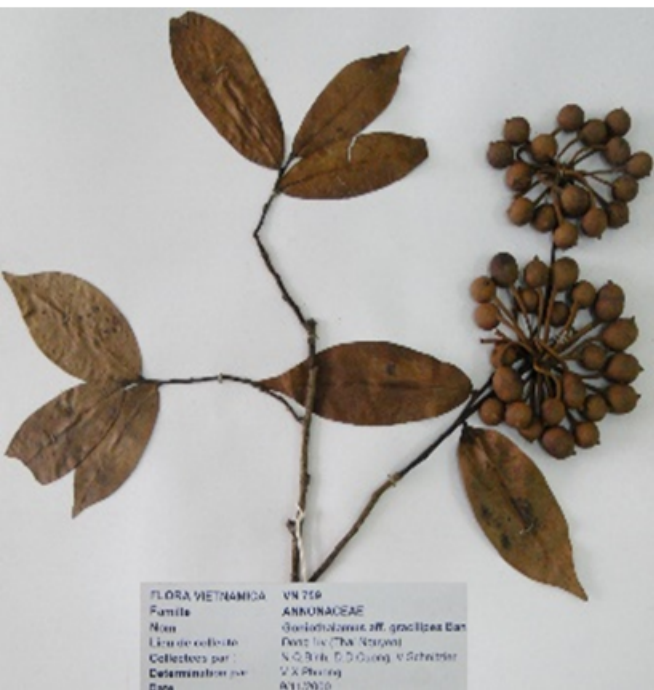
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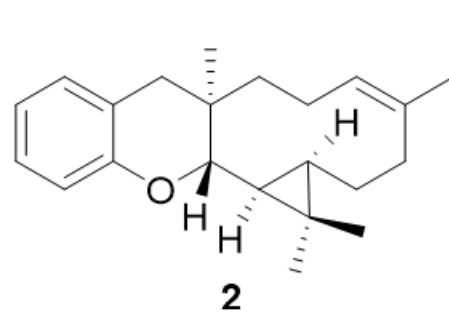
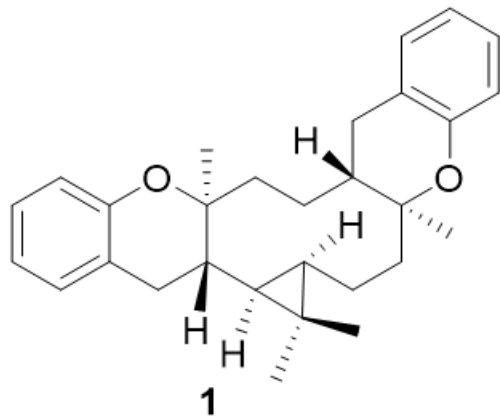
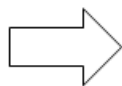
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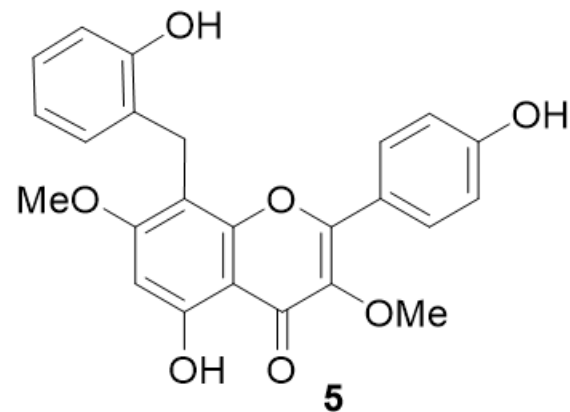
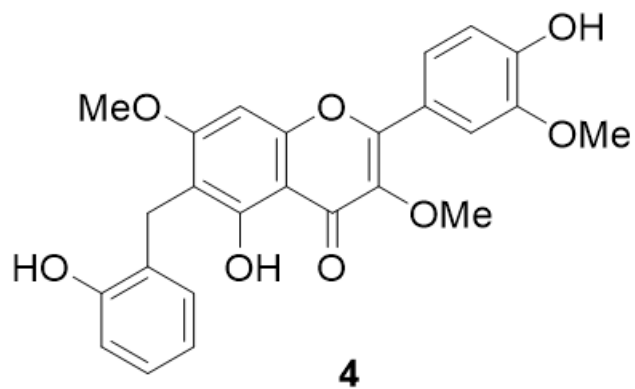
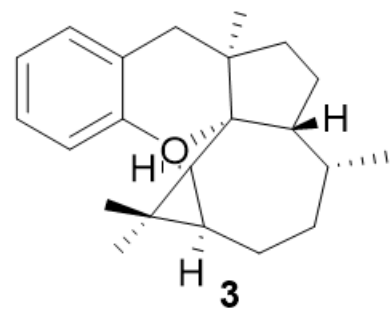
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G. gracilipes Ban



MIC = 32 $\mu\text{g/mL}$ (*S. aureus*)



IC₅₀ = 16.7 μM (HepG-2)