Cytotoxic and NF-κB Inhibitory Constituents of *Artocarpus rigida*

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Abstract

Four new prenylated flavonoids (**1–4**), a new stilbenoid (**5**), and nine known compounds were isolated from the twigs of *Artocarpus rigida*, collected in Indonesia. The structures of the new compounds were determined by analysis of their spectroscopic data, and the absolute configuration at C-12 of **1** and **2** and the known compounds artonin O (**6**), artobiloxanthone (**7**), and cycloartobiloxanthone (**8**), was determined by analysis of their CD and NMR spectroscopic data. Several of the compounds were cytotoxic towards HT-29 human colon cancer cells, with the most potent being compound **2** and the known compounds **6** and **8**. Of the substances obtained, compounds **1** and **7** were the most active in the NF-κB p50 and p65 assay, respectively.

The genus *Artocarpus* (Moraceae), consisting of around 50 species, is a rich source of prenylated flavonoids[1–7] and stilbenoids.[8–12] Several of the compound types found in *Artocarpus* have been found to exhibit cytotoxicity against cancer cells,[3,5–7,8–11] including artonin E (ED$_{50}$ value of 0.06 μg/mL against P388 leukemia cells)[5] and 3-hydroxy-4,3′,5′-trimethoxy-*trans*-stilbene (**9**), which showed cytotoxicity with ED$_{50}$ values of 0.01 μg/mL and 0.1 μg/mL in the 9KB and 9PS in vitro test system, respectively.[8]

Artocarpus rigida Blume (Moraceae) is an evergreen tree distributed in tropical regions and Asia, and is a rare plant in Indonesia.[12] Several prenylated flavonoids and stilbenoids have been reported from this plant previously.[13–19] As a continuation of a research program on the discovery of potential new natural product anticancer agents from diverse organisms,[20] it was found that a crude methanol extract of the twigs of *A. rigida*, collected in Indonesia, exhibited cytotoxicity toward HT-29 human colon cancer cells. Using column chromatography, four new prenylated flavonoids (**1–4**), a new stilbenoid (**5**), and several known compounds were isolated

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from this plant. Herein, we report the isolation and structure elucidation of compounds **1**–**5** and the biological evaluation of all metabolites obtained in this investigation.

Results and Discussion

A chloroform-soluble extract of the methanol extract of the dried ground twigs of *A. rigida* was separated by column chromatography over silica gel and yielded nine pooled fractions. Combined fractions three and four were further purified by silica gel column chromatography to afford the new compounds artorigidin B (**2**) and artorigidin C (**3**), along with the known compounds artonin O (**6**),[14] 3-hydroxy-4,3′,5′-trimethoxy-*trans*-stilbene (**9**),[21] artonin K,[2] and betulinic acid.[22] Combined fractions five and six also were further purified by silica gel column chromatography to afford the new compounds artorigidin A (**1**), cyclorigidol (**4**), and artoristilbene (**5**), along with the known compounds artobiloxanthone (**7**),[2, 23] cycloartobiloxanthone (**8**),[24] artonin N,[14] artonin G,[14] and ursolic acid.[25] The known compounds were identified by comparison of their spectroscopic data with literature values.

Compound **1** was isolated as an amorphous yellow powder, and showed a purple color under UV light at 365 nm. The HRESIMS exhibited a sodiated molecular ion peak at *m/z* 527.2051 (calcd 527.2046), consistent with a molecular formula of $C_{30}H_{32}O_7$. The UV (λ_{max} 230, 266, 278, 368 nm) and IR (ν_{max} 3364, 1646, 1615, 1557, 1473 cm^{-1}) spectra showed the typical absorption characteristics of a prenylated flavonoid,[2,13,14,26] similar to analogous data for artonin B.[26] The ^{1}H and ^{13}C NMR spectra gave evidence of **1** being a 3,5,6,7,8,2′,4′,5′,6′-nonasubstituted prenylflavonoid.[26] The ^{1}H NMR spectrum showed a signal due to a hydrogen-bonded phenolic OH group (δ 13.25) and other signals for other groups of a prenylflavonoid (Table 1). The ^{13}C NMR and DEPT spectra contained signals consistent with the presence of a flavonoid unit and three prenyl groups (Table 2). Moreover, in the ^{1}H NMR spectrum, two singlets at δ 4.47 and 4.75, due to two terminal olefinic protons, a singlet at δ 1.72 for a methyl group, together with an ABX system manifesting as a double doublet at δ 2.64 (*J* = 7.2 and 16.5 Hz, H-11), a doublet at δ 3.38 (*J* = 16.2, H-11), and another doublet at δ 3.85 (*J* = 7.2 Hz, H-12). These indicated a partially cyclized isoprenyl group, namely, a D-ring formed by linking C-3 and C-6′.[1,2,14] This was confirmed by the HMBC correlations between H-11 and C-2, C-4, and C-6′ (Figure 1). In addition, the HMBC correlations between two prenyl methylene protons (H_2-16) and C-5 and C-7 and between another two prenyl methylene protons (H_2-21) and C-7 and C-9 (Figure 1) supported the placement of these two prenyl groups at C-6 and C-8, respectively, which was confirmed by NOESY correlations between H_2-16 and H_3-20 and the C-7 hydroxy proton, and between H_2-21 and H_3-25 and the C-7 hydroxy proton (Figure 2). Comparison of the ^{1}H and ^{13}C NMR data of **1** with those of artonin B[26] indicated the same structures for the A, B, C, and D ring systems, but with a different substituent at C-6 for these compounds. The signals at δ 116.4 (CH) and 128.9 (CH) due to a 2,2-dimethylpyran ring in artonin B[26] were replaced by those at δ 120.7 (CH) and 136.1 (C) assignable to a 3,3-dimethylallyl group at C-6 in **1**. Finally, the NOESY correlations between H-17 and H_3-19 and H-22 and H_3-24, together with analysis of the HMQC and HMBC spectra, enabled the assignment of the individual methyl groups in the prenyl substuents of **1** (Tables 1 and 2).

The absolute configuration at C-12 of **1** was determined by comparison of its CD spectrum with that of a model compound, (+)-(5R)-*tert*-butyl-1,3-cyclohexadiene, for which a specific rotation value ($[\alpha]^{25}_D$) of +187 and a positive Cotton effect at 270 nm in its CD spectrum have been reported.[27] Compound **1** contains one stereogenic center, the same as that of (+)-(5R)-*tert*-butyl- 1,3-cyclohexadiene, and showed $[\alpha]^{20}_D$ +80 and a positive Cotton effect at 270 nm. Therefore, the absolute configuration at C-12 was determined as *R*. In the ^{1}H NMR spectrum, the signals at δ 2.64 and δ 3.38 were ascribed to the pseudo-axial H-11a and pseudo-equatorial H-11e,[2] which were supported by the NOESY correlation between H-11a and H-3′ (Figure 2) and H-11e and H-12, and the allyl group at C-12 in **1** was assigned in an equatorial

orientation (Figure 2). Consequently, the structure of **1** was determined as (5*R*)-5,6-dihydro-1,3,4,8,10-pentahydroxy-5-isopropenyl-9,11-bis(3-methyl-2-butenyl)-7*H*-benzo[*c*]xanthen-7-one, and this substance has been accorded the trivial name, artorigidin A.[28]

Compound **2** was isolated as an amorphous yellow powder, and its molecular formula of $C_{30}H_{32}O_7$ was determined by HRESIMS analysis. Similarities of its UV, IR, MS, and NMR spectra with those of **1** indicated that **2** is also a tetraprenylflavonoid. The ^{1}H NMR spectrum showed signals due to a substituted flavonoid unit, two tetra-3,3-dimethylallyl groups, and a furan ring fused with an ABX system (Table 1). Also observed in the ^{13}C NMR and DEPT spectra, were signals due to five methyl, four methylene, five methine, and 16 quaternary carbons, including an oxygenated quaternary carbon (δ 97.5) (Table 2). On comparing the ^{13}C NMR spectrum of **2** with that of **1**, the signals at δ 144.9 (C, C-13) and δ 112.6 (CH_2, C-14), due to a terminal double carbon bond in **1**, were replaced by signals at δ 97.5 (C, C-13, an oxygenated quaternary carbon) and δ 41.5 (CH_2, C-15, a methylene), in **2**. On considering an oxidative cyclization between a hydroxy group at C-5′ and a prenyl group at C-13,[26] a 2,2-dimethyldihydrofuran unit linking C-5′, C-6′, and C-13 could be proposed. Additionally, in the ^{13}C NMR spectrum, the signal at δ 93.7 (CH) was assigned unambiguously to the non-substituted C-8, based on both the assignments for **2** and artonin M[14] and the HMBC correlations between H-8 and C-7 and C-9 (Figure 3). A prenyl group was apparent at C-6, according to the HMBC correlations between H_2-21 and C-5, C-7, and C-23 (Figure 3). Another prenyl group was assumed to be affixed to C-15 because of the observation of HMBC correlations between H_2-15 and C-14 and C-17, and between H-17 and C-19 and 20 (Figure 3). Comparison of the ^{1}H and ^{13}C NMR spectra of **2** with those of artonin M[14] showed that both compounds have similar NMR characteristics, except for the replacement of the signals at δ 116.0 (CH) and 129.2 (CH), due to a 2,2-dimethylpyran ring in artonin M,[14] with the signals at δ 123.3 (CH, C-22) and 136.6 (C, C-23) being assigned to a 3,3-dimethylallyl group at C-6 in **2**. This facilitated an assignment of the planar structure of artorigidin B (**2**).

The NOESY correlations between H_2-21 and H_3-25, and H-22 and H_3-24 (Figure 4) indicated a *cis* arrangement between H-22 and H_3-24. The signals at δ 2.51 and 3.26 were assigned to a pseudo-axial H-11a and a pseudo-equatorial H-11e, respectively, the same as in **1**. A NOESY correlation between H-11e and H-12 suggested their *cis* relationship. A positive Cotton effect at 262 nm in the CD spectrum of **2** indicated the *S* configuration of C-12.[27] Other NOESY correlations between H-11a and H-14 and H-3′ were supportive of an equatorial methyl linked at C-13. Therefore, the absolute configuration at C-13 was determined as *R*, which was substantiated by the NOESY correlations between H-15 and H-12 and H-17, H-14 and H-16, H- 16 and H-20, and H-17 and H-19. Accordingly, compound **2** was determined[28] as (5*S*)-5,6-dihydro-1,3,8,10-tetrahydroxy-9-(3-methyl-2-butenyl)-5-[(1*R*)-1-methyl-1-(3-methyl-2-butenyl)]-7*H*-benzofuro[3,3*a*,4:*ab*]xanthen-7-one.

Compound **3** was isolated as an amorphous red powder. Both the IR spectrum, with absorptions typical of hydroxy (v_{max} 3303 cm^{−1}) and conjugated carbonyl (v_{max} 1646 cm^{−1}) groups, and the UV spectrum, with absorptions at 271 and 362 nm, indicated a quinonoid prenyflavonoid derivative.[2,14,29] The molecular formula of $C_{30}H_{28}O_7$ was determined by HRESIMS (two mass units less than **6**), consistent with a C-15 flavonoid unit and three prenyl groups, which was supported by the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2). HMBC correlations between H-11 and C-2 and C-6′, H_2-16 and C-5, C-7, and C-18, and H_2-21 and C-2′, C-4′, and C-23 (Figure 5) indicated the linkage of three prenyl groups at C-3, C-6, and C-3′ of **3**, respectively. The signals at $δ_H$ 6.70 (s) and δ 8.20 (s) were assigned to H-8 and H-11, respectively, because of the HMBC correlations between H-8 and C-9 and C-10 and H-11 and C-2 and C-6′ (Figure 5). In the ^{13}C NMR spectrum, three signals due to carbonyl groups were observed at δ 179.7, 181.9, and 181.9 (Table 2). One of these could be assigned to the carbonyl carbon of a chromone unit (C-4), with the others attributable to ring B.[14,29] Comparison of the ^{13}C NMR spectrum

of **3** with that of artonin O (**6**)[14] indicated the structures of the A-, B-, and C- ring systems of these compounds to be the same, with the D-ring being different. Also, C-11 and C-12 were found to be unsaturated in **3**, which was implied by the replacement of ^{13}C NMR signals at δ 21.4 (CH$_2$, C-11) and 35.3 (CH, C-12) in artonin O (**6**) by signals at δ 132.3 (CH, C-11) and 140.7 (C, C-12) in **3**. Finally, the correlations between H-11 and C-2 and C-6′, H-14 and C-12, H-15 and C-12 and C-14, H-19 and H-20 and C-17, H-24 and H-25 and C-22 supported the structure, 4,8,10-trihydroxy-5-isopropenyl-2,9-bis(3-methyl-2-butenyl)-7-benzo[*c*]xanthen-2,4,7-trione for **3** (artorigidin C). [28]

Compound **4** was obtained as an amorphous yellow powder. Its similar IR, UV, and NMR spectra to those of related compounds[26,30–32] indicated this compound to be a prenylated flavonoid. The HRESIMS showed a sodiated molecular ion peak at *m/z* 527.2073, consistent with a molecular formula of C$_{30}$H$_{32}$O$_7$. The ^{13}C NMR spectrum revealed the presence of 30 carbons, including six methyl groups and a carbonyl group (δ 178.6), as expected for a triprenylated flavonoid. The ^{1}H NMR spectrum exhibited six singlets at δ 1.68, 1.76, 1.76, 1.83, 1.87, and 1.95 for six vinyl methyl groups and two doublets at δ 6.23 (*J* = 9.0 Hz, H-11) and 5.46 (*J* = 9.0 Hz, H-12), consistent with a substituted 2*H*-benzopyran ring system, that would arise from oxidative cyclization between the prenyl group at C-3 and the C-2′ hydroxy group.[31,32] This was confirmed by long-range cross-peaks of H-11 (δ 6.23) with C-2, C-4, C-2′, and C-13. Moreover, two aromatic singlets were observed, with that at δ 6.50 assigned to H-3′ since it correlated with C-1′ and C-5′, and the second at δ 7.26 assigned to H-6′ because of correlations observed with C-2, C-2′ and C-4′. Comparison of the ^{1}H and ^{13}C NMR spectra of **4** with those of **1** suggested that both compounds have the same substitution pattern at the A-ring. The presence of a prenyl group connected to C-6, and another at C-8, was supported by the HMBC correlations between H$_2$-16 and C-5, C-7, and C-18, and H$_2$-21 and C-7, C-9, and C-23 in the HMBC spectrum of **4** (Figure 6).

A set of NOESY correlations, as described for **1**, was used to confirm the assignments of two prenyl groups at C-6 and C-8 in compound **4**. The signal at δ 6.23 was assigned to an equatorial H-11, and the isobutylene group at C-11 was assigned to an axial form, confirmed by the observation of the NOESY correlations between H-12 and H-14, H-14 and H-6′, which supported a relative configuration of **4**, as shown in Figure 7. The absolute configuration at C-11 was not determined because of the apparent unavailability of relevant reference CD or specific optical rotation data in the literature. Accordingly, the structure of **4** was proposed as 2,3,8,10- tetrahydroxy-6-(2-methylpropenyl)-9,11-bis(3-methyl-2-butenyl)-6*H*[1] benzopyrano[4,3-*b*]benzopyran-7-one, and the compound has been named cyclorigidol.[33]

Compound **5** was isolated as a colorless powder. The UV (λ$_{max}$ 234, 340, and 360 nm) and IR (ν$_{max}$ 3364, 1615, 1456 cm^{-1}) spectra showed absorption characteristics of a stilbenoid derivative.[15–19] The molecular formula of C$_{19}$H$_{16}$O$_4$ was determined by HRESIMS, and was consistent with a 14-carbon stilbene nucleus bearing a prenyl group substituent. The ^{1}H and ^{13}C NMR signals of **5** were assigned by analysis of the HMQC and HMBC spectra. The ^{1}H NMR spectrum (Table 1) showed signals due to a 2,2-dimethylpyran ring (the D ring, connected to the B ring at C-3 and C-4), including two *gem*-dimethyl groups at δ 1.44 (s) and two typical *cis*-olefinic protons at δ 6.64 (dd, *J* = 0.6 and 9.9 Hz, H-15) and δ 5.63 (d, *J* = 9.9 Hz, H-16).[14,26] A COSY correlation between H-15 and H-16 and HMBC correlations between H-15 and C-3, C-5 and C-17, H-16 and C-4 and C-17, and H$_3$-18 or H$_3$-19 and C-16 and C-17 (Figure 8) were observed. The ^{1}H NMR spectrum also displayed resonances at δ 7.38 (1H, d, *J* = 8.4 Hz, H-10), 6.76 (1H, dd, *J* = 2.1 and 8.4 Hz, H-11), and 6.95 (1H, d, *J* = 2.1 Hz, H-13), due to the ABX spin system of a 1,3,4-trisubstituted benzene ring (the A ring, substituted by a hydroxy group at C-12 and connected to the C ring at C-9 and C-14). This was supported by the COSY correlation between H-10 and H-11 and the HMBC correlations between H-10 and C-12 and C-14, H-11 and C-9 and C-13, and H-13 and C-9 and C-11 (Figure 8). Additionally,

the absence of any signal for a pair of *cis* or *trans* vinyl protons[8–11] or any other *ortho* aromatic protons in the ^{1}H NMR spectrum indicated one substituent to be linked to C-8, and the B ring to be connected with the C ring at C-1 and C-6 and with the D ring at C-3 and C-4. Comparison of the NMR and MS data of **5** with those of artocarpol E[16] indicated that these two compounds contain the same structure of a stilbene core. However, artocarpol E has a prenyl group at C-4 and a geranyl group at C-7, which were either absent (geranyl group) or changed to a 2,2-dimethylpyran ring in **5**.[16] These inferences were confirmed by the HMBC correlations between H-5 and C-1, C-3 and C-15, and between H-7 and C-5 and C-9, as well as the appearance of five signals due to oxygen-substituted olefinic or aromatic carbons in the ^{13}C NMR spectrum (Table 2) of **5**, and its molecular weight of 308. Therefore, the structure of **5**, artoristilbene,[34] was determined as 2,2-dimethyl-2*H*-benzo{6,7-oxepino[3,2-*g*]} chromene-7,10-diol.

Previously, prenylflavonoids and stilbenoids have been isolated from *A. rigida*.[13–19] Lin and associates investigated the root bark of this species collected in Taiwan, and reported several stilbenoids and prenylated flavonoids.[15–19] Nomura's group studied the bark of *A. rigida*, as obtained from Java in Indonesia, and reported several prenylated flavonoids,[13,14] but no stilbenoids. In the present study, both prenylated flavonoids and stilbenoids were isolated from the twigs of *A. rigida*, collected in Java. It was found that prenylflavonoids are the main components, with stilbenoids being trace components. Therefore, it may be proposed that *A. rigida* contains different components in its various anatomical parts when obtained from different locations. The absolute configuration at C-12 of the prenylflavonoids has not been determined by previous investigators. The CD and NOESY spectra were used for the determination of the C-12 absolute configuration of five representatives of this class of compounds (**1**, **2**, **6**–**8**) for the first time in this study.

All compounds isolated from *A. rigida* twigs were evaluated for their cytotoxicity against the HT-29 human colon cancer cell line, using paclitaxel as the positive control (Table 3). Compounds **2**, **6** and **8** were found to be cytotoxic with ED_{50} values of 3.4, 3.2, and 3.7 μM, respectively. The compounds isolated from *A. rigida* twigs were also evaluated for their ability to inhibit NF-κB activity in both the NF-κB p50 and NF-κB p65 assays, using rocaglamide as the positive control (Table 3). Compounds **1** and **7** exhibited NF-κB inhibitory activity in the NF-κB p50 assay, with IC_{50} values of 2.0 μM (**1**) and 3.7 μM (**7**), respectively, and compound **7** exhibited NF-κB inhibitory activity in the NF-κB p65 assay, with an IC_{50} value of 2.3 μM.

A number of prenylated oxygen heterocyclic constituents with cytotoxic effects against cancer cell lines have been isolated previously from *Artocarpus* species.[3, 5–7] When the cytotoxicity data against the HT-29 colon cancer cell line, as obtained in the present investigation for the compounds isolated from *A. rigida* (Table 3), are compared with the previous literature, a number of preliminary observations about the structural requirements for activity may be made. In the current study, the known compound, cycloartobiloxanthone (**8**) was found to be cytotoxic (ED_{50} 3.7 μM), but the closely related dihydrobenzoxanthone, artonin K, was inactive (ED_{50} >10 μM), indicating the potentiating effects on resultant biological potency of the two prenyl groups at C-2 and C-15 present in the structure of compound **8**. Compounds **6** (artonin O) and **3** (artorigidin C) have the exact same structure apart from the presence of an olefinic group between C-11 and C-12 in **3**, and exhibited ED_{50} values of 3.2 and >10 μM. Therefore, within this series, the occurrence of a C-12-affixed β-oriented substituent seems to be necessary. Compounds **7** (ED_{50} >10 μM) and **8** are also closely related structurally, with the C-12 isoprenyl group of **7** cyclized with the C-5′ hydroxy group to form a dihydrofuran ring in **8**. Since only compound **8** was active, then this additional cyclization enhances the resultant cytotoxicity. Although not isolated in the present study, artonin E, with a C-3 prenyl group and a tricyclic overall composition, appears to be the most potent cytotoxic prenylated oxygen heterocyclic compound to have been isolated from the genus *Artocarpus*, being approximately

two orders of magnitude more potent than artonin O (**6**) and cycloartobiloxanthone (**8**), when evaluated against the P-388 murine lymphocytic leukemia cell line.[5]

Experimental Section

General Experimental Procedures

Optical rotations were measured with a Perkin-Elmer model 343 polarimeter. UV spectra were recorded on an UV-Hitachi U2910 spectrophotometer. CD measurements were performed using a JASCO J-810 spectropolarimeter. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer. ^{1}H, ^{13}C, DEPT, HMQC, HMBC, NOESY, and COSY NMR spectra were recorded at room temperature on a Bruker Avance DRX-300 MHz NMR spectrometer, with TMS as internal standard. ESIMS and HRESIMS were recorded on a LCT-TOF mass spectrometer. Column chromatography was conducted using silica gel (70–230 mesh, Merck, Darmstadt, Germany). Analytical and preparative thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (Sorbent Technologies, Atlanta, GA; 0.20 mm layer thickness). Sephadex LH-20 was purchased from Amersham Biosciences, Uppsala, Sweden. For visualization of TLC plates, sulfuric acid reagent was used. Fluorescence was tested using a Spectroline (model ENF-260C) UV light source.

Plant Material

The twigs of *Artocarpus rigida* were collected at Gunung Kancana, Cianjur, West Java, Indonesia in August, 2003, and identified by S. R. A voucher specimen (A05753) has been deposited at the Field Museum of Natural History, Chicago, IL, under the accession number SR-CJR 82 (2285411).

Extraction and Isolation

The milled, air-dried twigs of *A. rigida* (800 g) were extracted with MeOH (3 L × 5) at room temperature, and the solvent evaporated in vacuo. The dried MeOH extract (77.6 g, 9.8%) was resuspended in 10% H_2O in MeOH (600 mL) and partitioned with *n*-hexane (500 mL and 3 × 300 mL) to yield a hexane-soluble residue (D001, 9.9 g, 1.3%). The aqueous-MeOH layer was then partitioned with $CHCl_3$ (500, 400, 300, 200 mL) to afford a chloroform-soluble extract (D002, 19.8 g, 2.5%), which was followed by washing with a 1% aqueous solution of NaCl, to partially remove tannins. The chloroform-soluble extract exhibited cytotoxicity towards the HT29 cell line (ED_{50} < 20 μg/mL). Both the *n*-hexane-soluble and aqueous-soluble extracts were inactive in the bioassay system used.

The chloroform-soluble extract (19.0 g) was subjected to silica gel column chromatography (9.0 × 45 cm) and eluted with a gradient of *n*-hexane–acetone (100:1, 80:1, 60:1, 30:1, 20:1, 10:1, 5:1, 3:1, 1:1; 500 mL each). Fractions were pooled by TLC analysis to give nine combined fractions (D002F01-D002F09). Of these, D002F03 and D002F04 (ED_{50} < 20 μg/mL) were combined and further chromatographed over a silica gel column (2.5 × 20 cm), eluted with a gradient of *n*-hexane–acetone (20:1, 15:1, 10:1, 8:1, 5:1, 3:1, 1:1, 300 mL each), to yield seven combined fractions (D002F0301–D002F0307). Fraction D002F0301 was chromatographed over silica gel using *n*-hexane-acetone (10:1) as solvent, and then purified by separation over a Sephadex LH-20 column, eluted with $CHCl_3$-MeOH (1:1), affording **6** (20 mg). Fraction D002F0302 was separated by silica gel chromatography, eluted with *n*-hexane–acetone (10:1), and then purified by passage over a Sephadex LH-20 column, eluted with a mixture of $CHCl_3$- MeOH (1:1), to afford **2** (2.0 mg). Fraction D002F0303 was chromatographed over silica gel, eluted by *n*-hexane–acetone (8:1), and then purified using a Sephadex LH-20 column, eluted with a mixture of $CHCl_3$-MeOH (1:1), to afford **3** (2.0 mg). Fraction D002F0304 was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then purified by separation over a Sephadex LH-20 column, using $CHCl_3$-MeOH (1:1) for elution, affording

9 (2.5 mg). Fraction D002F0305 was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then purified over a Sephadex LH-20 column, eluted by CHCl$_3$-MeOH (1:1), furnishing betulinic acid (25 mg). Fraction D002F0306 was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), yielding artonin K (5.0 mg). D002F05 and D002F06 (ED$_{50}$ < 20 µg/mL) were combined and further chromatographed over a silica gel column, eluted with a gradient of *n*-hexane–acetone (12:1, 10:1, 8:1, 6:1, 5:1, 4:1, 3:1, 2:1, and 1:1, 300 mL each), to yield nine combined fractions (D002F0501-D002F0509). Fraction D002F0501 was chromatographed over silica gel using *n*-hexane–acetone (10:1) as solvent, and then purified by passage over a Sephadex LH-20 column, eluted with CHCl$_3$-MeOH (1:1), affording artonin N (3.5 mg). Fraction D002F0502 was purified by silica gel chromatography, using *n*-hexane– acetone (10:1) for elution, and then purified using Sephadex LH-20, eluted with a mixture of CHCl$_3$-MeOH (1:1), to afford artonin G (5.0 mg). Fraction D002F0503 was chromatographed over silica gel, eluted by *n*-hexane–acetone (8:1), and then purified by passage over Sephadex LH-20, eluted with a mixture of CHCl$_3$-MeOH (1:1), to afford cycloartobiloxanthone (**8**, 6 mg). Fraction D002F0504 was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then purified with a column containing Sephadex LH-20, using CHCl$_3$-MeOH (1:1) for elution, affording **7** (21 mg). Fraction D002F0505 was chromatographed over silica gel, with *n*hexane –acetone (3:1) as solvent, and then purified over a Sephadex LH-20 column, eluted by CHCl$_3$-MeOH (1:1), producing **1** (25 mg). Fractions D002F0506 and D002F0507 were chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then purified over Sephadex LH-20, using CHCl$_3$-MeOH (1:1) as solvent, affording **4** (3.5 mg). Fraction D002F0508 was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then purified over a Sephadex LH-20 column, using CHCl$_3$-MeOH (1:1) as solvent, affording **5** (2 mg). Fraction D002F0509 was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then purified over a column containing Sephadex LH-20, using CHCl$_3$-MeOH (1:1) as solvent, affording ursolic acid (10 mg).

Artorigidin A (1)—Amorphous yellow powder (*n*-hexane), showing a purple color under UV light at 365 nm; $[\alpha]^{20}_D$ +80 (*c* 0.2, CH$_2$Cl$_2$); UV (CH$_2$Cl$_2$) λ_{max} (log ε) 230 (4.18), 266 (4.18), 278 (4.15), 368 (4.08) nm; CD (MeOH, nm) λ_{max} (Δε) 228 (+1.62), 270 (+1.04), 295 (−0.55), 312.5 (−1.07); IR (dried film) ν_{max} 3364, 1646, 1615, 1557, 1473, 1282, 1096 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 527.2 [M + Na]$^+$; positive HRESIMS *m/z* 527.2051, calcd for C$_{30}$H$_{32}$O$_7$Na, 527.2046.

Artorigidin B (2)—Amorphous yellow powder (*n*-hexane), showing a purple color under UV light at 365 nm; $[\alpha]^{20}_D$ +10 (*c* 0.1, CH$_2$Cl$_2$); UV (CH$_2$Cl$_2$) λ_{max} (log ε) 233 (4.33), 268 (4.42), 326 (4.18), 374 (4.30) nm; CD (MeOH, nm) λ_{max} (Δε) 223.5 (+0.87), 243 (−0.47), 262 (+0.30), 294 (−0.68), 314 (−0.38); IR (dried film) ν_{max} 3354, 1645, 1606, 1471, 1272, 1170 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 505.2 [M + H]$^+$; positive HRESIMS *m/z* 505.2222, calcd for C$_{30}$H$_{32}$O$_7$H, 505.2226.

Artorigidin C (3)—Amorphous red powder (*n*-hexane), showing a purple color under UV light at 365 nm; UV (CH$_2$Cl$_2$) λ_{max} (log ε) 271 (4.52), 362 (3.78) nm; IR (dried film) ν_{max} 3303, 1646, 1463, 1268, 1078 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 523.1 [M + Na]$^+$; positive HRESIMS *m/z* 523.1725, calcd for C$_{30}$H$_{28}$O$_7$Na, 523.1733.

Cyclorigidol (4)—Amorphous yellow powder (*n*-hexane), showing a purple color under UV light at 365 nm; $[\alpha]^{20}_D$ +20 (*c* 0.2, CH$_2$Cl$_2$); UV (CH$_2$Cl$_2$) λ_{max} (log ε) 228 (4.28), 282 (4.35), 386 (4.21) nm; CD (MeOH, nm) λ_{max} (Δε) 222 (+1.13), 235 (−0.54), 253.5 (−0.30), 269 (−0.11), 294.5 (−0.39), 310 (−0.33); IR (dried film) ν_{max} 3335, 1647, 1556, 1472, 1290, 1213 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 527.2 [M + Na]$^+$; positive HRESIMS *m/z* 527.2073, calcd for C$_{30}$H$_{32}$O$_7$Na, 527.2046.

Artoristilbene (5)—Amorphous white powder (*n*-hexane), showing a blue color under UV light at 365 nm; UV (CH$_2$Cl$_2$) λ_{max} (log ε) 234 (3.57), 340 (3.99), 360 (3.87) nm; IR (dried film) ν_{max} 3364, 1615, 1558, 1456, 1262, 1065 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive ESIMS *m/z* 331.1 [M + Na]$^+$; positive HRESIMS *m/z* 331.0968, calcd for C$_{19}$H$_{16}$O$_4$Na, 331.0946.

Cytotoxicity Assay

Cytotoxicity of the samples was screened against HT-29 human colon cancer cells by a previously reported procedure.[35] The cells were cultured under standard conditions and trypsinized. Then, the harvested cells were added to 96-well plates and treated by either the test compounds dissolved in DMSO or by DMSO (the negative control). The plates were incubated at 37 °C in 5% CO$_2$ for three days, and then the cells were fixed, then incubated at room temperature for 30 min, washed with water once, dried at room temperature overnight, and then dyed using sulforhodamine B. After the dyed cells were lysed in tris-base buffer, the plates were read at 515 nm with an ELISA reader. Paclitaxel was used as a positive control, and cytotoxicity was calculated based on the values read from the samples and the negative control.

Enzyme-Based ELISA NF-κB Assays

The NF-κB assays were carried out as in a previous procedure with minor modification.[36] Using an EZ-Detect Transcription Factor Assay System ELISA kit (Pierce Biotechnology, Rockford, IL), a nuclear extract of HeLa cells (ATCC, American Type Culture Collection) was treated by positive control and the list samples. The specific binding ability of activated p50 or p65 subunits of NF-κB to the biotinylated-consensus sequence was measured by detecting the chemiluminescent signal in a Fluostar Optima plate reader (BMG Labtech Inc., Durham, NC). Rocaglamide was used as a positive control in both the NF-kB p50 and p65 assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References and Notes

1. Aida M, Shinomiya K, Matsuzawa K, Hano Y, Nomura T. Heterocycles 1994;39:847–858.

2. Aida M, Yamaguchi N, Hano Y, Nomura T. Heterocycles 1997;45:163–175.

3. Hakim EH, Fahriyati A, Kau MS, Achmad SA, Makmur L, Ghisalberti EL, Nomura T. J Nat Prod 1999;62:613–615. [PubMed: 10217722]

4. Makmur L, Syamsurizal, Tukiran, Achmad SA, Aimi N, Hakim EH, Kitajima M, Takayama H. J Nat Prod 2000;63:243–244. [PubMed: 10691717]

5. Suhartati T, Achmad SA, Aimi N, Hakim EH, Kitajima M, Takayama H, Takeya K. Fitoterapia 2001;72:912–918. [PubMed: 11731116]

6. Wang YH, Hou AJ, Chen L, Chen D-F, Sun HD, Zhao QS, Bastow KF, Nakanish Y, Wang XH, Lee KH. J Nat Prod 2004;67:757–761. [PubMed: 15165133]

7. Ko HH, Lu YH, Yang SZ, Won SJ, Lin CN. J Nat Prod 2005;68:1692–1695. [PubMed: 16309328]

8. Gill MT, Bajaj R, Chang CJ, Nichols DE, McLaughlin JL. J Nat Prod 1987;50:36–40. [PubMed: 3598597]

9. Likhitwitayawuid K, Sritularak B. J Nat Prod 2001;64:1457–1459. [PubMed: 11720533]

10. Su BN, Cuendet M, Hawthorne ME, Kardono LBS, Riswan S, Fong HHS, Mehta RG, Pezzuto JM, Kinghorn AD. J Nat Prod 2002;65:163–169. [PubMed: 11858749]

11. Roberti M, Pizzirani D, Simoni D, Rondanin R, Baruchello R, Bonora C, Buscemi F, Grimaudo S, Tolomeo M. J Med Chem 2003;46:3546–3554. [PubMed: 12877593]

12. Suhartati T, Yandri, Hadi S. Eur J Sci Res 2008;23:330–337.

13. Hano Y, Inami R, Nomura T. Heterocycles 1990;31:2173–2179.

14. Hano Y, Inami R, Nomura T. Heterocycles 1993;35:1341–1350.

15. Chung MI, Ko HH, Yen MH, Lin CN, Yang SZ, Tsao LT, Wang JP. Helv Chim Acta 2000;83:1200–1204.

16. Ko HH, Lin CN, Yang SZ. Helv Chim Acta 2000;83:3000–3005.

17. Ko HH, Yang SZ, Lin CN. Tetrahedron Lett 2001;42:5269–5270.

18. Lu YH, Lin CN, Ko HH, Yang SZ, Tsao LT, Wang JP. Helv Chim Acta 2002;85:1626–1632.

19. Lu YH, Lin CN, Ko HH, Yang SZ, Tsao LT, Wang JP. Helv Chim Acta 2003;86:2566–2572.

20. Kinghorn AD, Carcache-Blanco EJ, Chai HB, Orjala J, Farnsworth NR, Soejarto DD, Oberlies NH, Wani MC, Kroll DJ, Pearce CJ, Swanson SM, Kramer RA, Rose WC, Fairchild CR, Vite GD, Emanuel S, Jarjoura D, Cope FO. Pure Appl Chem 2009;81:1051–1063. [PubMed: 20046887]

21. Mannila E, Talvitie A, Kolehmainen E. Phytochemistry 1994;33:813–816.

22. Fujioka T, Kashiwada Y, Kilkuskie RE, Cosentino LM, Ballas LM, Jiang JB, Janzen WP, Chen IS, Lee KH. J Nat Prod 1994;57:243–247. [PubMed: 8176401]

23. Fujimoto Y, Zhang XX, Kirisawa M, Uzawa J, Sumatra M. Chem Pharm Bull 1990;38:1787–1789.

24. Aida M, Shinomiya K, Hano Y, Nomura T. Heterocycles 1993;36:575–583.

25. Ma CM, Cai SQ, Cui JR, Wang RQ, Tu PF, Hattori M, Daneshtalab M. Eur J Med Chem 2005;40:582–589. [PubMed: 15922841]

26. Hano Y, Aida M, Shiina M, Nomura T, Kawai T, Ohe H, Kagei K. Heterocycles 1989;29:1447–1453.

27. Lightner DA, Bouman TD, Gawronski JK, Gawronska K, Chappuis JL, Crist BV, Hansen AE. J Am Chem Soc 1981;103:5314–5327.

28. The systematic names for **1–3** are based on a numbering scheme published in reference [7].

29. Shieh WL, Lin CN. Phytochemistry 1992;31:364–367.

30. Ferrari F, Messana I, Mesquita de Araujo MC. Planta Med 1989;55:70–72. [PubMed: 17262259]

31. Chen CC, Huang YL, Ou J-C, Lin CF, Pan TM. J Nat Prod 1993;56:1594–1597.

32. Achmad SA, Hakim EH, Juliawaty LD, Makmur L, Suyatno, Aimi N, Ghisalberti EL. J Nat Prod 1996;59:878–879.

33. The systematic name for **4** is based on a numbering scheme published in reference [6].

34. The systematic name for **5** is based on a numbering scheme published in reference [15].

35. Seo EK, Kim NC, Mi QW, Chai HB, Wall ME, Wani MC, Navarro HA, Burgess JP, Graham JG, Cabieses F, Tan GT, Farnsworth NR, Pezzuto JM, Kinghorn AD. J Nat Prod 2001;64:1483–1485. [PubMed: 11720542]

36. Deng Y, Balunas MJ, Kim J-A, Lantvit DD, Chin YW, Chai H-B, Sugiarso S, Kardono LBS, Fong HHS, Pezzuto JM, Swanson SM, Carcache de Blanco EJ, Kinghorn AD. J Nat Prod 2009;72:1165–1169. [PubMed: 19422206]
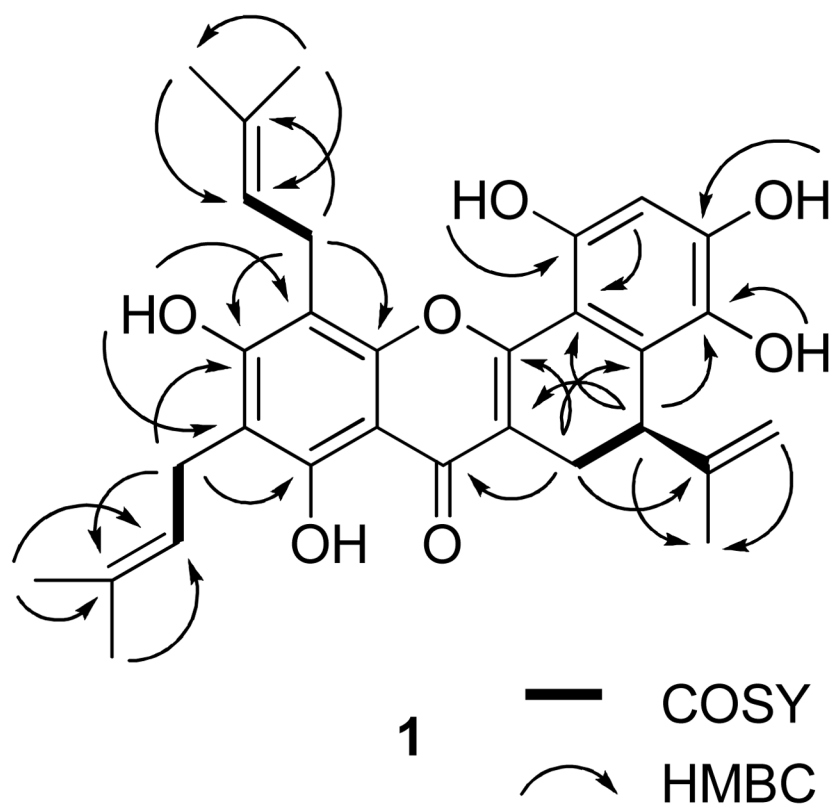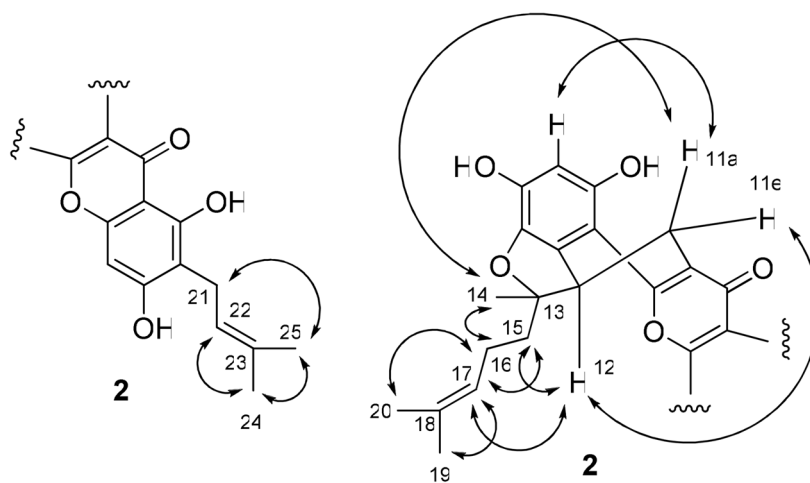
Figure 1.
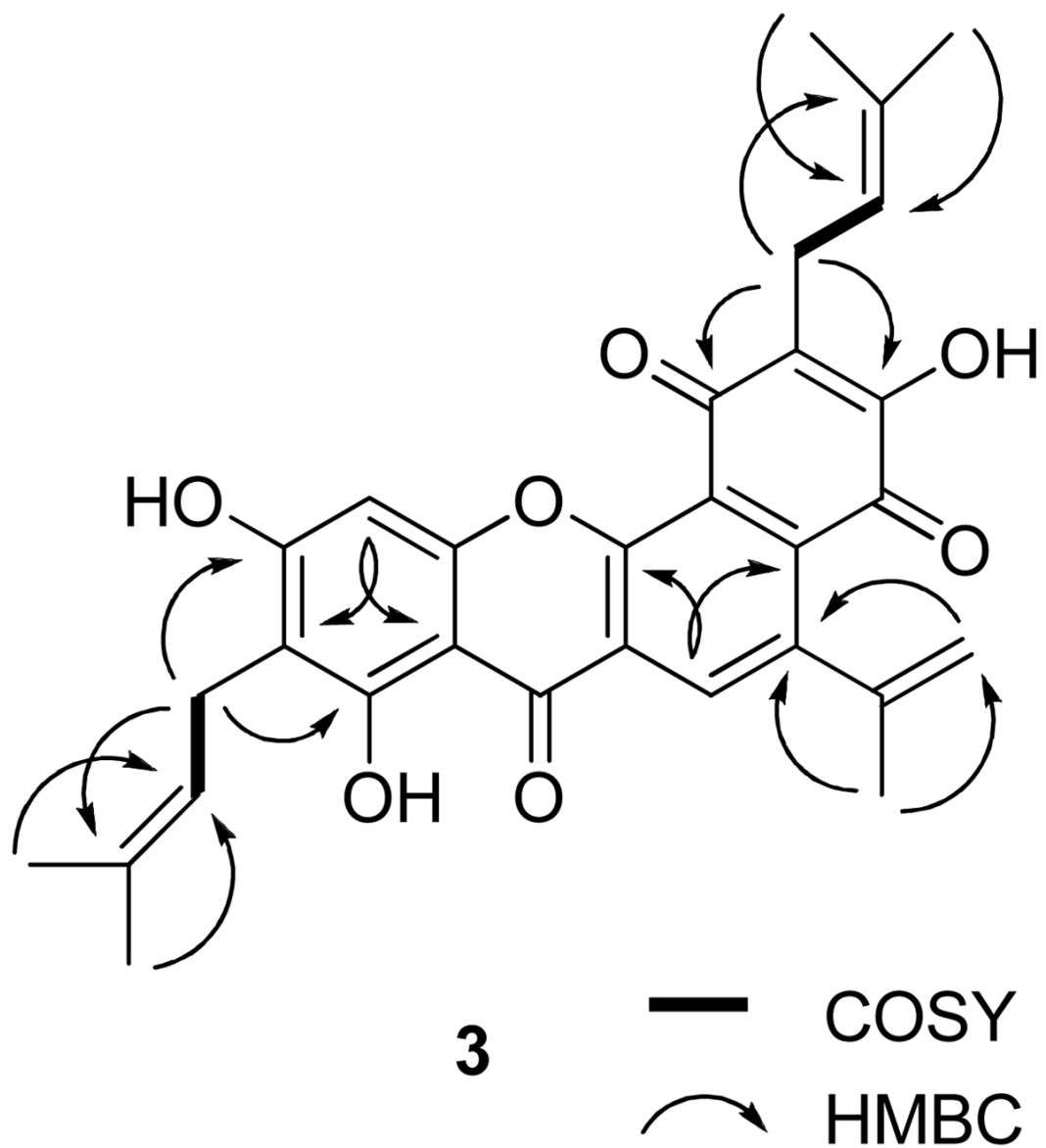COSY and key HMBC correlations of **1**.
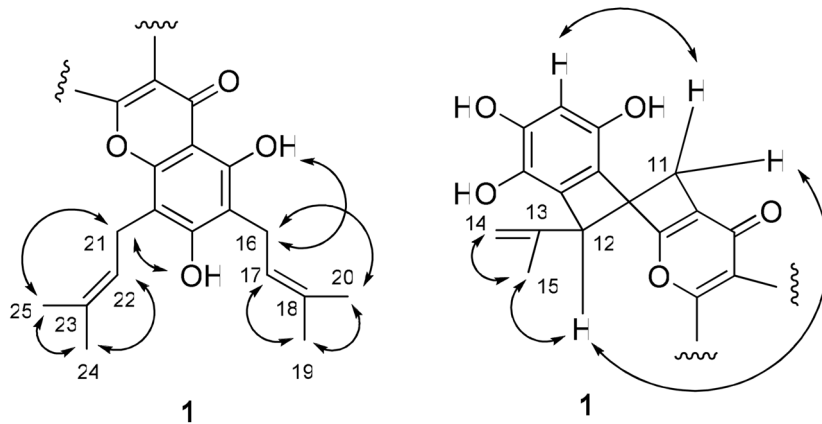
Figure 2.
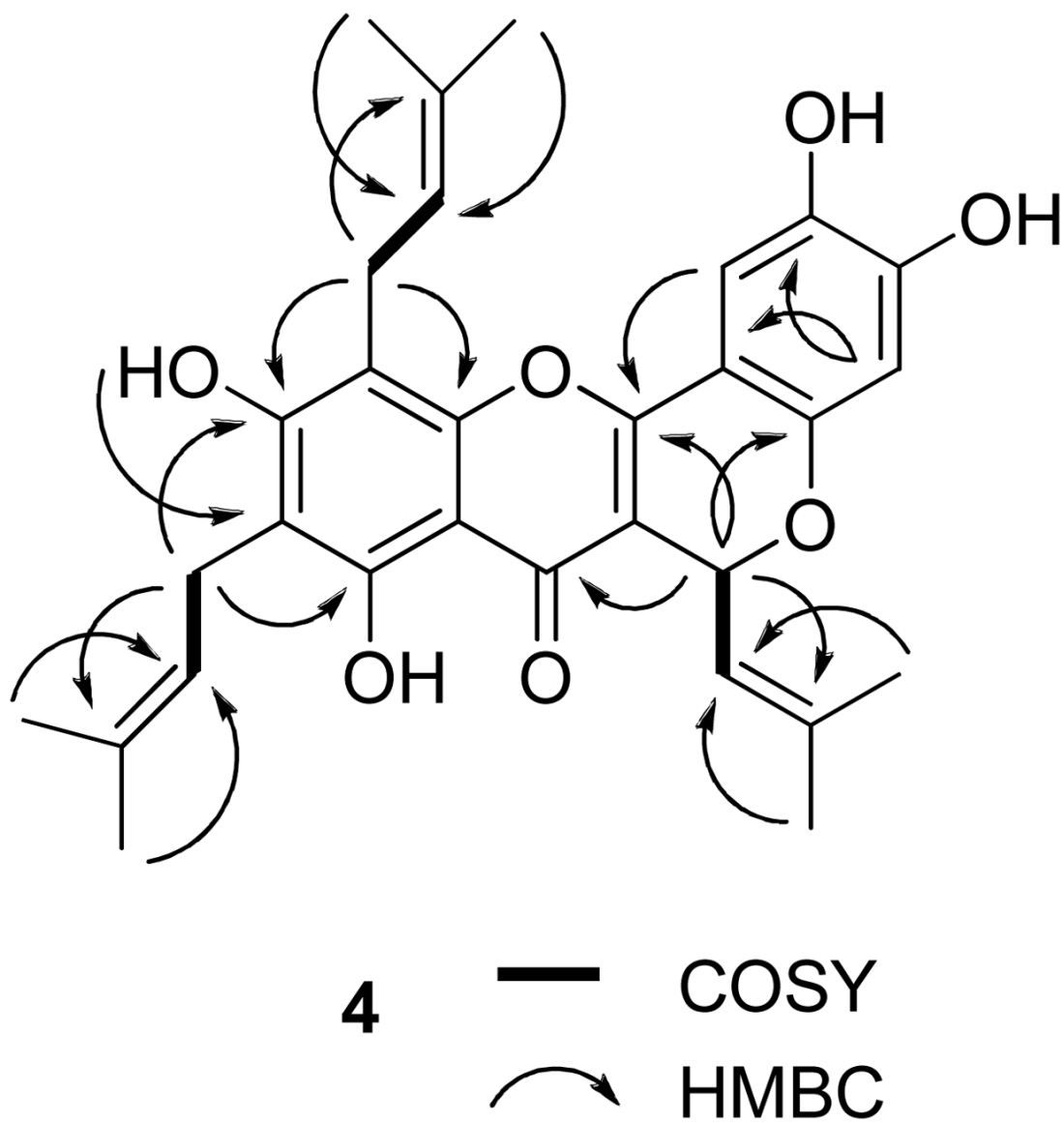Selected NOESY correlations of **1**.
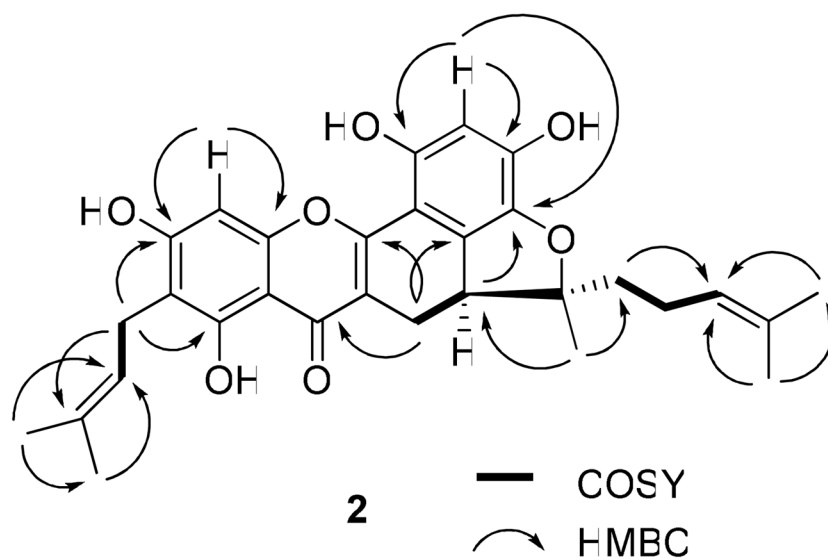
Figure 3.
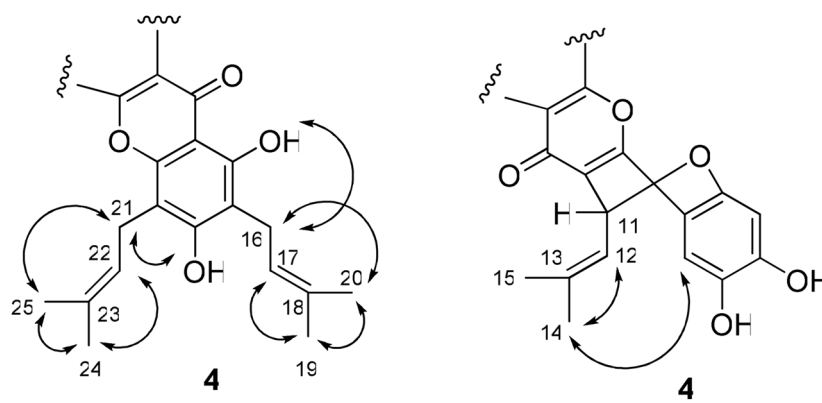COSY and key HMBC correlations of **2**.

Figure 4.
Selected NOESY correlations of **2**.

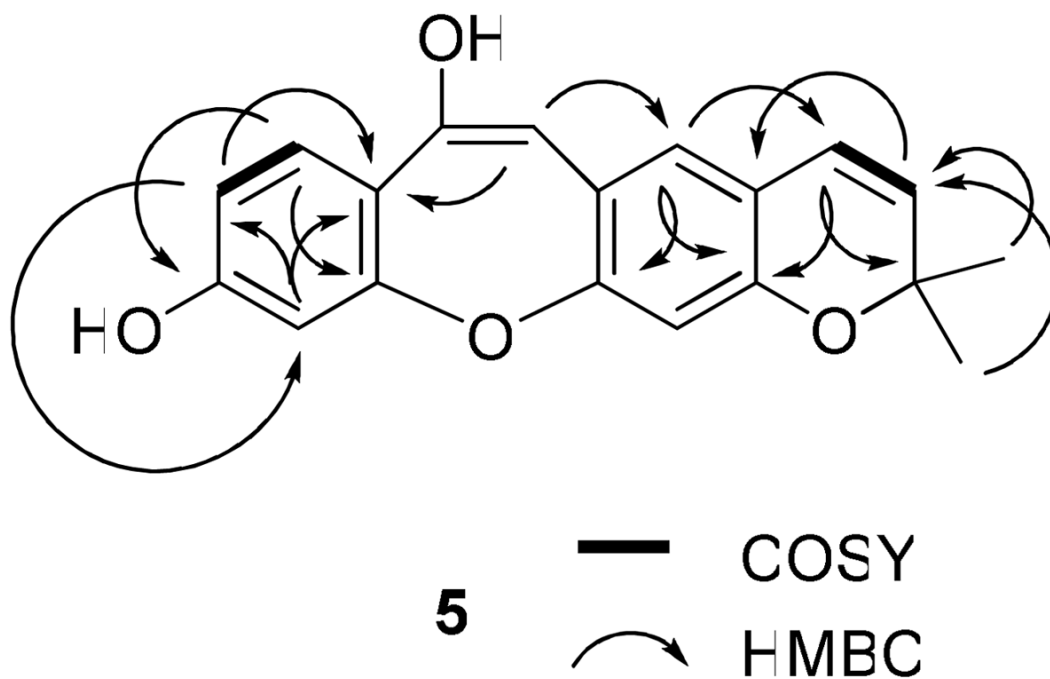**Figure 5.**
COSY and key HMBC correlations of **3**.

Figure 6.
COSY and key HMBC correlations of **4**.

Figure 7.
Selected NOESY correlations of **4**.
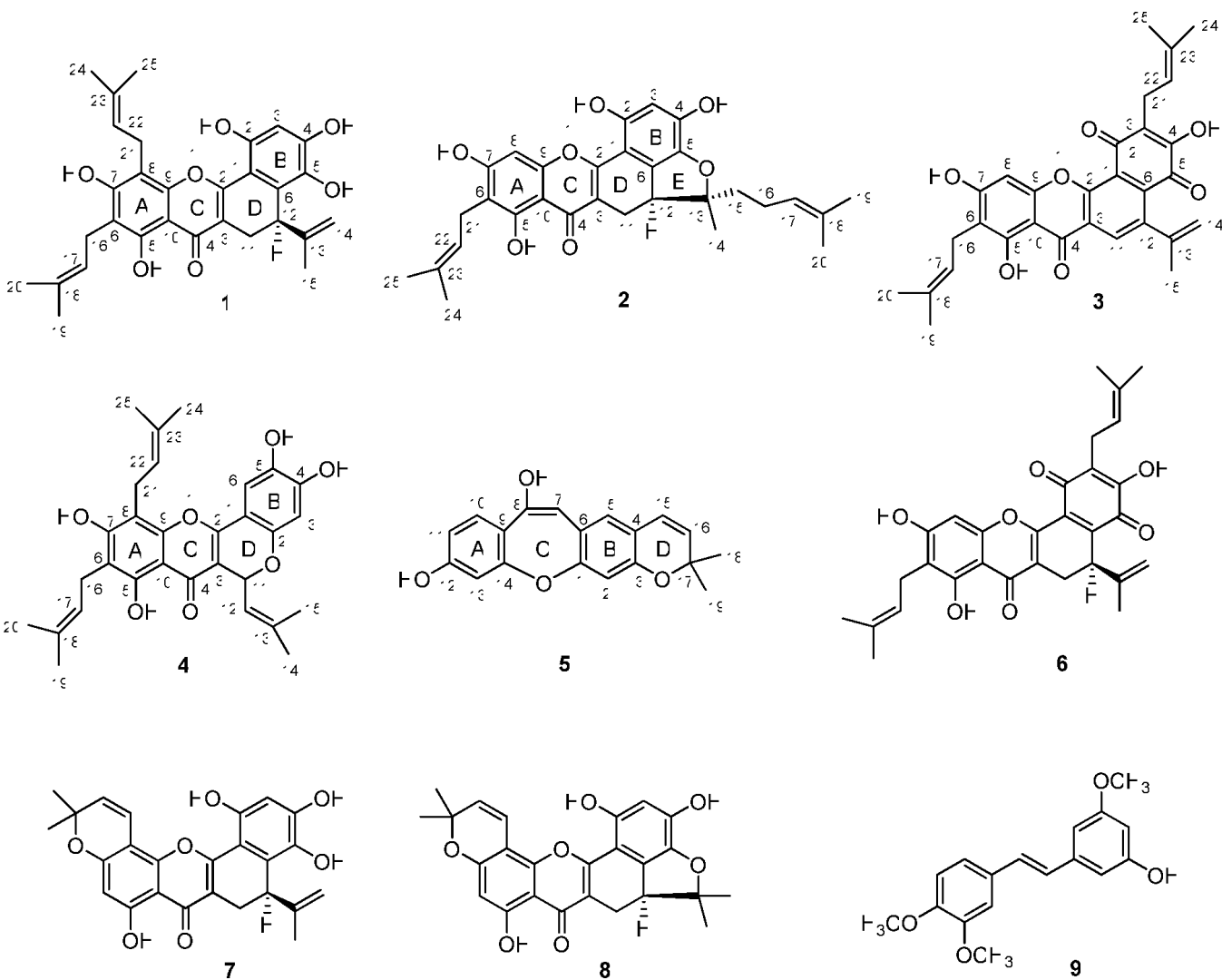
Figure 8.
COSY and key HMBC correlations of **5**.

Table 1

¹H NMR Spectroscopic Data of Compounds **1–5**

position	1[a]	2[a]	3[b]	4[a]	5[a]
2					6.84 d (1.0)
5	13.25 s (OH)	13.35 s (OH)	12.97 s (OH)	13.10 (OH)	6.79 d (1.0)
6					
7	6.36 s (OH)	10.18 s (OH)	6.60 (OH)	6.86 dd (0.6, 1.0)	
8		6.37 s	6.70 s		
10					7.38 d (8.4)
2′	7.54 s (OH)	6.65 s (OH)			
3′	6.50 s	6.34 s		6.50 s	
4′	4.97 s (OH)	6.18 s (OH)	9.25 s (OH)		
5′	6.47 s (OH)				
6′				7.26 s	
11	2.64 dd (7.2, 16.5) 3.38 d (16.5)	2.51 t (15.6) 3.26 dd (7.2, 15.6)	8.20 s	6.23 d (9.0)	6.76 dd (2.1, 8.4)
12	3.85 d (7.2)	3.46 m		5.46 d (9.0)	
13					6.95 d (2.1)
14a	4.47 s	1.32 s	4.90 brs	1.68 s	
14b	4.75 s		5.17 brs		
15	1.72 s	1.94 m	2.09 s	1.95 s	6.64 dd (0.6, 9.9)
16	3.46 m	2.14 m	3.39 d (7.2)	3.44 d (7.2)	5.63 d (9.9)
17	5.09 t (7.0)	5.12 t (7.2)	5.28 t (7.2)	5.27 d (7.2)	
18					1.44 s
19	1.76 s	1.83 s	1.65 s	1.76 s	1.44 s
20	1.83 s	1.77 s	1.79 s	1.83 s	
21	3.46 m	3.46 d (7.2)	3.30 d (7.2)	3.56 d (7.2)	
22	5.23 t (7.0)	5.28 t (7.2)	5.25 t (7.2)	5.27 d (7.2)	
24	1.76 s	1.69 s	1.65 s	1.76 s	
25	1.80 s	1.62 s	1.79 s	1.87 s	

[a]Data were measured in CDCl₃ at 300 MHz.

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[b]Data were measured in acetone-d_6 at 300 MHz. Chemical shifts (δ) are expressed in ppm, with s = singlet, brs = broad singlet, d = doublet, t = triplet, m = multiplet, dd = double doublet. J values are presented in Hz and are omitted if the signals were overlapped as multiplets.

Table 2

^{13}C NMR Spectroscopic Data of Compounds **1–5**

position	1[a]	2[a]	3[b]	4[a]	5[a]
1					151.5 C
2	159.8 C	159.4 C	153.8 C	155.3 C	101.6 CH
3	110.2 C	112.2 C	113.7 C	109.2 C	153.7 C
4	180.4 C	180.5 C	179.7 C	178.6 C	109.7 C
5	157.1 C	157.2 C	161.4 C	156.9 C	103.9 CH
6	110.4 C	110.2 C	112.3 C	110.0 C	131.1 C
7	159.1 C	161.1 C	165.1 C	158.6 C	105.7 CH
8	104.5 C	93.7 CH	95.2 CH	107.4 C	154.3 C
9	152.0 C	154.1 C	157.0 C	151.0 C	122.9 C
10	105.4 C	104.3 C	103.9 C	105.0 C	121.2 CH
1′	105.1 C	104.3 C	124.4 C	105.3 C	
2′	151.2 C	149.6 C	181.9 C	152.0 C	
3′	103.1 CH	104.3 CH	125.9 C	104.2 CH	
4′	149.7 C	145.0 C	153.8 C	150.0 C	
5′	134.7 C	138.0 C	181.9 C	139.5 C	
6′	127.7 C	132.5 C	132.0 C	108.8 CH	
11	21.9 CH$_2$	20.7 CH$_2$	132.3 CH	69.1 CH	112.1 CH
12	38.1 CH	44.7 CH	140.7 C	120.9 CH	155.7 C
13	144.9 C	97.5 C	147.8 C	138.2 C	98.2 CH
14	112.6 CH$_2$	20.7 CH$_3$	113.7 CH$_2$	25.5 CH$_3$	154.8 C
15	20.9 CH$_3$	41.5 CH$_2$	23.8 CH$_3$	18.4 CH$_3$	116.2 CH
16	21.8 CH$_2$	23.0 CH$_2$	22.0 CH$_2$	21.3 CH$_2$	129.6 CH
17	120.7 CH	120.9 CH	122.9 CH	121.4 CH	76.2 C
18	136.1 C	137.9 C	132.3 C	134.3 C	27.9 CH$_3$
19	25.7 CH$_3$	25.8 CH$_3$	25.9 CH$_3$	25.6 CH$_3$	27.9 CH$_3$
20	18.2 CH$_3$	18.0 CH$_3$	18.0 CH$_3$	17.7 CH$_3$	
21	21.7 CH$_2$	21.6 CH$_2$	23.1 CH$_2$	21.7 CH$_2$	
22	121.4 CH	123.3 CH	121.4 CH	121.6 CH	

position	1[a]	2[a]	3[b]	4[a]	5[a]
23	136.3 C	136.6 C	133.4 C	133.5 C	
24	25.9 CH$_3$	25.7 CH$_3$	25.9 CH$_3$	25.6 CH$_3$	
25	18.0 CH$_3$	17.8 CH$_3$	17.9 CH$_3$	17.6 CH$_3$	

[a] Data were measured in CDCl$_3$ at 75.5 MHz.

[b] Data were measured in acetone-d_6 at 75.5 MHz. Chemical shifts (δ) are presented in ppm.

Table 3

Cytotoxicity for HT-29 Cells and NF-κB Inhibition of Compounds **1–9**[a]

compound	cytotoxicity[b]	NF-κB p50 inhibition[c]	NF-κB p65 inhibition[c]
1	9.3	2.0	>10
2	3.4	NT[d]	NT[d]
3	>10	NT[d]	NT[d]
4	>10	>10	>10
5	10.7	>10	>10
6	3.2	>10	>10
7	>10	3.7	2.3
8	3.7	>10	>10
9	8.7	>10	>10

[a]All other compounds isolated in this study were inactive in these assay systems.

[b]Data are represented as ED_{50} values (μM), and paclitaxel was used as positive control (ED_{50}, 0.10 nM).

[c]Data are represented as IC_{50} values (μM), and rocaglamide was used as positive control (IC_{50} = 4.0 μM in the NF-κB p50 assay and IC_{50} = 0.075 μM in the NF-κB p65 assay).

[d]Not tested.