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Hybrid flavan–flavanones from *Friesodielsia desmoides* and their inhibitory activities against nitric oxide production†

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The first phytochemical investigation of *Friesodielsia desmoides* leaves and twigs led to the isolation and identification of three new hybrid flavan–flavanones, friesodielsones A–C (**1–3**), together with 18 known compounds (**4–21**). The structures of compounds **1–3** were elucidated through intensive analysis of spectroscopic data and their absolute configurations at C-2 and C-4 were determined by a combination of ^{1}H NMR and CD spectroscopy. The configuration at C-2″ is tentatively assigned as 2″*S* based on biosynthesis considerations. Compounds **2** and **15** significantly inhibited NO production with IC_{50} values of 10.21 ± 0.074 and 7.56 ± 0.087 μM, respectively, whereas compounds **11** (IC_{50} = 28.14 ± 0.024 μM) and **12** (IC_{50} = 37.21 ± 0.017 μM) were moderate inhibitors.

Introduction

Friesodielsia is a small genus belonging to the Annonaceae family which is distributed in Africa and Asia. Five species, including *F. desmoides*, *F. fornicata*, *F. discolor*, *F. filipes*, and *F. kingii*, are found in Thailand.[1] Many types of secondary metabolites are produced from this genus including flavonoids,[1,2] chalcones,[1,3] alkaloids,[3] benzyl benzoate derivatives[2] and sesquiterpenes.[2] Some of these compounds show interesting biological properties, such as antiplasmodial[1,3] and cytotoxicity activities.[1,3] *F. desmoides* (Craib) Steenis (Fig. 1) is a small tree or shrub that is grown as an ornamental plant in Thailand. This plant has two synonymous names, *Goniothalamus desmoides* Craib and *Oxymitra desmoides*. To the best of our knowledge, this is the first report of phytochemical investigations of this plant. Three new hybrid flavan–flavanones (**1–3**) along with 18 known compounds (**4–21**) (Fig. 2) were isolated and identified from *F. desmoides* leaves and twigs that were collected from Mae Fah Luang University Health Park, Chiang Rai Province, Thailand. Most of the isolated compounds were evaluated for their NO inhibitory activities.

Fig. 1 *Friesodielsia desmoides* (these photos were taken by Surat Laphookhieo).

Results and discussion

The crude extracts of *F. desmoides* leaves and twigs were separated by column chromatography using various stationary phases to yield three new hybrid flavan–flavanones, friesodielsones A–C (**1–3**), along with 18 known compounds (**4–21**). The known compounds were identified as desmosflavan A (**4**),[4] desmosflavan B (**5**),[4] (2*S*)-8-formyl-5,7-dihydroxyflavanone (**6**),[1] alpinetin (**7**),[5] pinocembrin (**8**),[5] 5,6,7-trihydroxy-flavanone (**9**),[6] 5,6-dihydroxy-7-methoxy-flavanone (**10**),[7] cardamonin (**11**),[5] 2′,4′-dihydroxy-3′,6′-dimethoxychalcone (**12**),[8] *trans*-dihydroquercetin (**13**),[9] quercetin (**14**),[10] chrysin (**15**),[11] *O*-methylmoschatoline (**16**),[12] (−)-epicatechin (**17**),[13] 3′-formyl-2′,4′-dihydroxy-6′-methoxychalcone (**18**),[1] *O*-aristololactam BII (**19**),[14] aristololactam AIa (**20**)[14] and goniothalamin (**21**).[15]

Friesodielsone A (**1**) was obtained as a yellow solid. It showed a pseudomolecular ion peak at *m*/*z* $[M - H]^-$ 523.1393 (calcd 523.1393) in the HRESIMS corresponding to a molecular formula of $C_{31}H_{24}O_8$. The ^{1}H, ^{13}C, DEPT and 2D NMR spectroscopic data of **1** (Table 1) suggested this compound contained two moieties, a flavan unit and a flavanone unit.[16] The flavan unit displayed ^{1}H and ^{13}C NMR signals for a hydrogen-bonded hydroxyl proton [δ_H 12.35 (1H, s, 7-OH)], a formyl group [δ_H 10.10 (1H, s, 8-CHO)/δ_C 192.1], a monosubstituted aromatic ring

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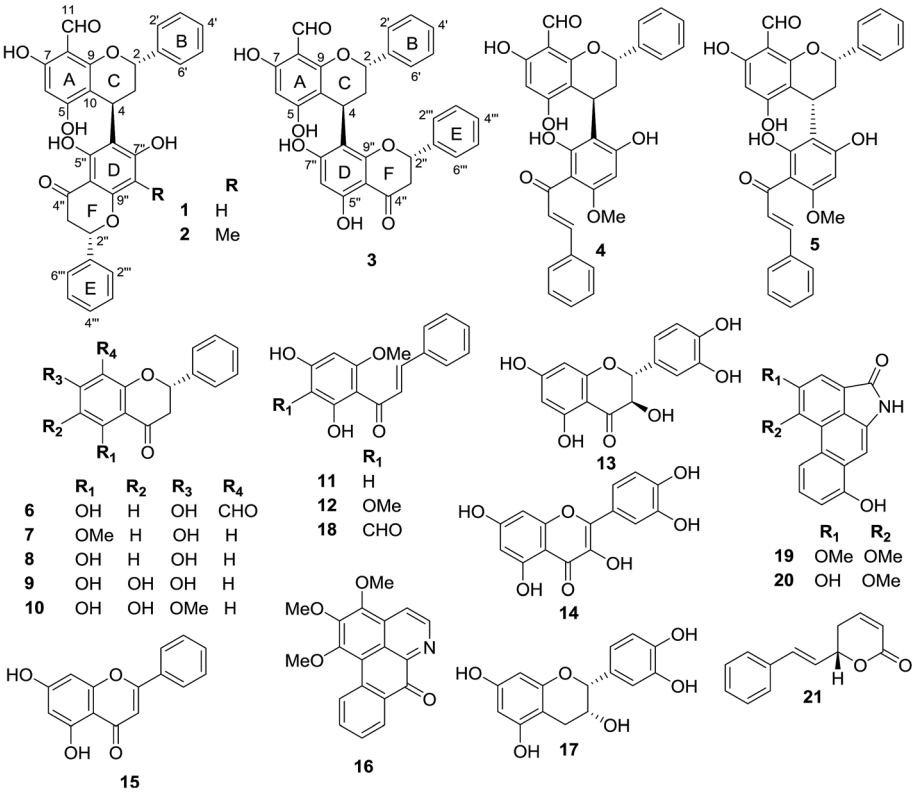


Fig. 2 Compounds isolated from *F. desmoides* leaves and twigs.

[δ_H 7.44–7.47 (2H, m, H-2′, H-6′)/δ_C 126.8, 7.40–7.42 (2H, m, H-3′, H-5′)/δ_C 129.4, and 7.30–7.34 (1H, m, H-4′)/δ_C 128.6], an isolated aromatic proton [δ_H 5.90 (1H, s, H-6)/δ_C 94.9], and an AB_2C proton spin system (deduced from COSY spectrum) [δ_H 5.53 (1H, dd, J = 3.5, 10.0, H-2)/δ_C 76.7, 2.24–2.33 (2H, m, H-3)/δ_C 37.9, and 4.67 (1H, dd, J = 2.6, 5.4 Hz, H-4)/δ_C 26.1]. The formyl group was located at C-8 (δ_C 105.9) from the HMBC correlations (Fig. 3) of C-8 (δ_C 105.9) to the hydrogen-bonded hydroxyl proton (δ_H 12.35), the formyl proton (δ_H 10.10) and the isolated aromatic proton H-6 (δ_H 5.90).

The second fragment, a flavanone unit, showed ^{1}H and ^{13}C NMR resonances for a hydrogen-bonded hydroxyl [δ_H 12.73 (1H, s, 5″-OH)], a monosubstituted aromatic ring [δ_H 7.56–7.58 (2H, m, H-2‴, H-6‴)/δ_C 127.8, 7.43–7.47 (2H, m, H-3‴, H-5‴)/δ_C 129.5, and 7.40–7.43 (1H, m, H-4‴)/δ_C 129.4], an isolated aromatic proton [δ_H 6.08 (1H, s, H-8″)/δ_C 95.9] and an AB_2 proton spin system (deduced from COSY spectrum) [δ_H 5.57 (1H, dd, J = 3.0, 13.5 Hz, H-2″)/δ_C 79.9, 2.80 (1H, dd, J = 3.0, 17.1 Hz, H-3″)/δ_C 43.7 and 3.16 (1H, dd, J = 13.5, 17.1 Hz, H-3″)/δ_C 43.7].

The flavan and flavanone units of **1** had a C–C bond linkage between C-4 of ring C and C-6″ of ring D which was deduced from the following HMBC correlations: δ_H 4.67 (H-4) with C-5″ (δ_C 163.1), C-6″ (δ_C 111.9) and C-7″ (δ_C 165.2); and δ_H 2.24–2.33 (H-3) with C-6″ (δ_C 111.9). The assignments of the NMR spectroscopic data of **1** are summarized in Table 1. Therefore, friesodielsone A was identified as structure **1**. The relative configuration of the C-ring of **1** was deduced to be the same as that of desmosflavan A (**4**),[4] from the similarity of their ^{1}H NMR coupling constants for the protons H-2, H-3 and H-4. The absolute 4*S* configuration of **1** was evident from the positive Cotton effect (Fig. 4) at λ_{max} ($\Delta\varepsilon$) 225.5 (2.92×10^4) nm.[17–19] This allowed the assignment of the 2*S* configuration of **1** based on the aforementioned ^{1}H NMR comparisons. The configuration at C-2″ could not be unequivocally determined but is based on the biosynthetic consideration outline in Scheme 1.

Friesodielsone B (**2**) was obtained as a yellow solid. Its molecular formula, $C_{32}H_{26}O_8$, was obtained from HRESIMS analysis which showed a $[M - H]^-$ at *m*/*z* 537.1549 (calcd for $C_{32}H_{25}O_8$, 537.1549). The ^{1}H and ^{13}C NMR spectroscopic data of **2** (Table 1) were similar to those of **1**. The main differences were the ring D resonances of the flavanone unit. Compound **2** displayed an additional resonance for methyl protons at δ_H 2.09 (3H, s) and the absence of the C-8″ aromatic proton resonance at δ_H 6.08 (1H, s, H-8″) as was observed in **1**. The HMBC correlation (Fig. 3) between these methyl protons (δ_H 2.09) and C-8″ (δ_C 159.1) further supported the position of this methyl group at C-8″. The assignments of the ^{1}H and ^{13}C spectroscopic data of **2** are summarized in Table 1. The CD spectrum of **2** (Fig. 4) and ^{1}H NMR coupling constants of H-2, H-3 and H-4 were similar to that of **1** indicating that the absolute configuration at C-2 and C-4 of **2** and **1** were the same. This was further supported by their similar and negative specific optical rotations, $[\alpha]_D^{25}$ −57.6 (*c* 0.03, MeOH) for **1** and $[\alpha]_D^{26}$ −45.1 (c 0.006, MeOH) for **2**. Thus, friesodielsone B was assigned the structure **2**.

Friesodielsone C (**3**) was obtained as a yellow solid. Its HRESIMS spectrum showed a $[M - H]^-$ at *m*/*z* 523.1393 (calcd

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) spectroscopic data for friesodielsones A–C

	Friesodielsone A (**1**)			Friesodielsone B (**2**)		Friesodielsone C (**3**)	
Position	δ_C [a]	δ_H [a] (J in Hz)	δ_H [b] (J in Hz)	δ_C [a]	δ_H [a] (J in Hz)	δ_C [a]	δ_H [a] (J in Hz)
2	76.7	5.53 (dd, 3.5, 10.0)	5.40 (dd, 2.6, 11.0)	76.7	5.52 (dd, 3.0, 10.8)	76.7	5.53 (dd, 3.2, 10.7)
3	37.9	2.24–2.33 (m)	2.19 (ddd, 5.4, 11.0, 14.3) 2.13 (dt, 2.6, 14.3)	37.9	2.25–2.30 (m)	37.9	2.25–2.32 (m)
4	26.1	4.67 (dd, 2.6, 5.4)	4.55 (dd, 2.6, 5.4)	26.5	4.70 (dd, 2.4, 5.6)	26.1	4.67 (dd, 2.6, 5.5)
5	162.1	—	—	159.1	—	162.8	—
6	94.9	5.90 (s)	5.80 (s)	94.9	5.91 (s)	94.8	5.90 (s)
7	164.8	—	—	164.9	—	165.2	—
8	105.9	—	—	106.0	—	105.8	—
9	160.6	—	—	160.8	—	164.7	—
10	104.7	—	—	105.9	—	104.7	—
11	192.1	10.10 (s)	10.05 (s)	192.1	10.16 (s)	192.1	10.15 (s)
1′	142.5	—	—	142.3	—	142.4	—
2′,6′	126.8	7.44–7.47 (m)	7.34–7.37 (m)	126.8	7.45–7.47 (m)	126.8	7.44–7.48 (m)
3′,5′	129.4	7.40–7.42 (m)	7.37–7.41 (m)	129.4	7.38–7.42 (m)	129.4	7.39–7.42 (m)
4′	128.6	7.30–7.34 (m)	7.26–7.30 (m)	128.6	7.32–7.34 (m)	128.6	7.33–7.35 (m)
2″	79.9	5.57 (dd, 3.0, 13.5)	5.49 (dd, 3.0, 13.5)	79.6	5.61 (dd, 3.0, 13.0)	79.9	5.59 (dd, 3.0, 13.0)
3″	43.7	2.80 (dd, 3.0, 17.1) 3.16 (dd, 13.5, 17.1)	2.73 (dd, 3.0, 17.1) 3.06 (dd, 13.5, 17.1)	43.6	2.82 (dd, 3.0, 17.0) 3.16 (dd, 13.0, 17.0)	43.7	2.81 (dd, 3.0, 17.0) 3.22 (dd, 13.0, 17.0)
4″	197.0	—	—	197.7	—	197.1	—
5″	163.1	—	—	160.6	—	162.0	—
6″	111.9	—	—	111.8	—	95.8	6.08 (s)
7″	165.2	—	—	160.6	—	162.8	—
8″	95.9	6.08 (s)	5.98 (s)	159.1	—	111.8	—
9″	162.1	—	—	164.5	—	160.6	—
10″	103.1	—	—	103.8	—	103.5	—
11″				8.32	2.09 (s)		
1‴	140.2	—	—	140.4	—	140.1	—
2‴,6‴	127.8	7.56–7.58 (m)	7.46–7.48 (m)	127.2	7.60–7.61 (m)	129.4	7.44–7.46 (m)
3‴,5‴	129.5	7.43–7.47 (m)	7.34–7.37 (m)	129.5	7.45–7.47 (m)	129.4	7.57–7.58 (m)
4‴	129.4	7.40–7.43 (m)	7.37–7.41 (m)	129.3	7.38–7.42 (m)	128.6	7.39–7.42 (m)
7-OH	—	12.35 (s)	—	—	12.35 (s)	—	12.35 (s)
5″-OH	—	12.73 (s)	—			—	12.72 (s)

[a] Spectrum measured in acetone-d_6. [b] Spectrum measured in methanol-d_4.

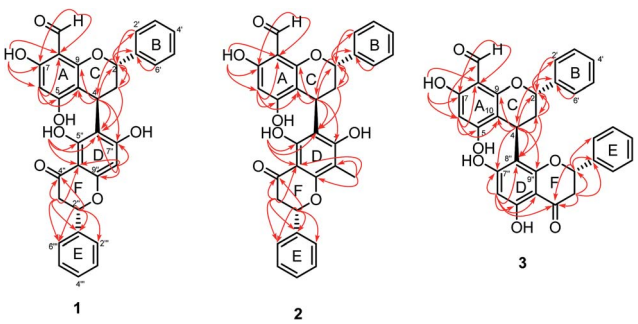


Fig. 3 Selected HMBC correlations of compounds **1–3**.

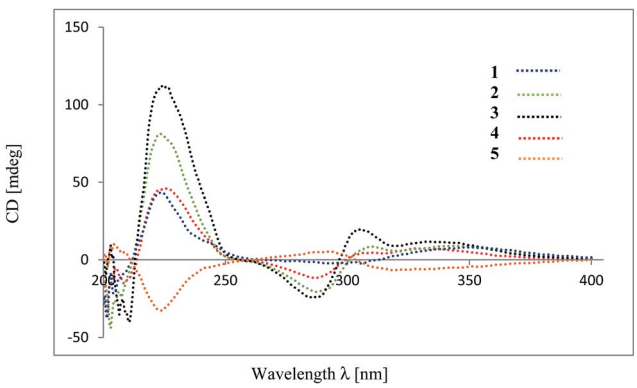
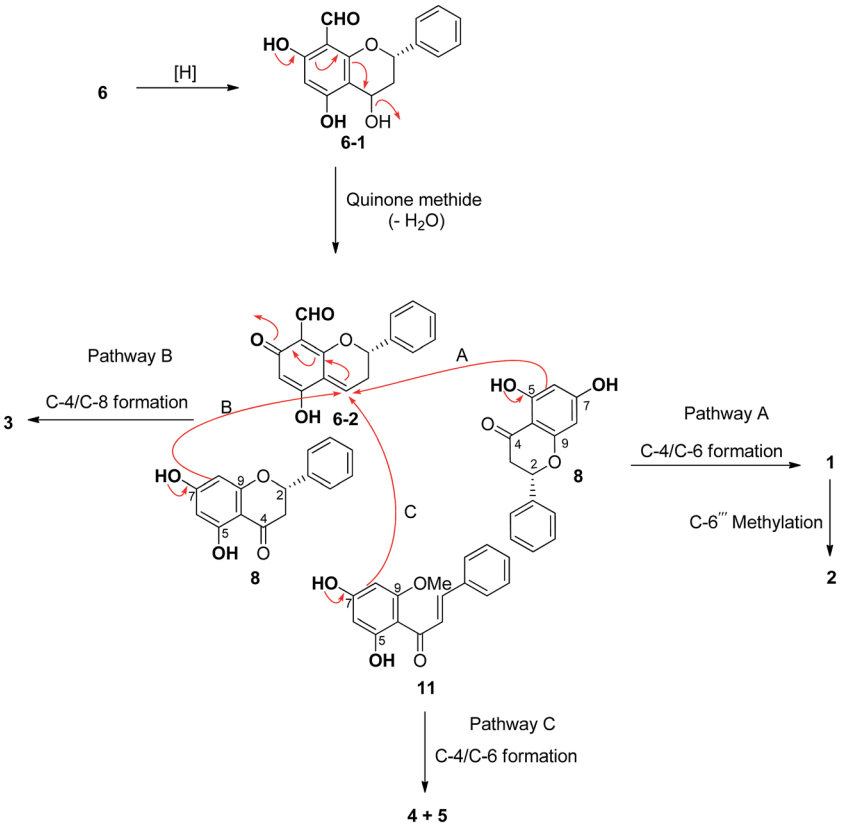


Fig. 4 CD spectra (MeOH) of compounds **1–5**.

523.1393) corresponding to the molecular formula of $C_{31}H_{24}O_8$. The ^{1}H and ^{13}C NMR spectroscopic data of **3** (Table 1) were similar to those of **1** and **2**. The significant difference in the structure of **3** was the position of flavan–flavanone linkage. Compounds **1** and **2** were linked at C-4/C-6″ whereas compound **3** was linked at C-4/C-8″. The HMBC correlations of δ_H 4.67 (H-4) to C-7″ (δ_C 162.0), C-8″ (δ_C 111.8) and C-9″ (δ_C 162.8) supported this C-4/C-8″ linkage (Fig. 3). The absolute configuration at C-4 of **3** was determined by comparison of its CD spectrum with that of compounds **1** and **2**. The absolute configuration at C-4 of **3** was the same as that of **1** and **2** from the positive Cotton effect seen in its CD spectrum (Fig. 4) at λ 224 nm, which was similar to those of compounds **1** and **2**. Therefore, friesodielsone C was assigned the structure **3**.



Scheme 1 Plausible biosynthetic pathway of compounds **1–5**.

Hybrid biflavonoids comprising a linked flavan and flavanone units are rarely found as natural products.[16,20–23] Biosynthetically, compounds **1–3** could be derived from compound **6** (Scheme 1) *via* reduction of the C-4 carbonyl group and then formation of the *para*-quinone methide **6–2** by dehydration.[24] Intermediate **6–2** could couple with compound **8** at C-4/C-6 to provide compound **3** (*via* pathway B) and at C-4/C-6 to produce compounds **1** and **2** (*via* pathway A). The latter compound obtained from **1** *via* methylation at C-6‴. Similarly, the biosynthetic pathway to compounds **4** and **5** could be derived from the coupling of compounds **6–2** and **11** (Scheme 1). The isolation of compounds **6**, **8** and **11** in this study supports this biosynthetic hypothesis. The absolute configuration tentatively assigned as 2″*S* at C-2″ in compounds **1–3** is based on this biosynthetic hypothesis.

Table 2 NO production inhibition effect and cytotoxicity on J774.A1 cells

Compounds	NO production IC_{50}, μM	MTT% cell viability at 10 μM
1	Inactive	116.2
2	10.21 ± 0.074	126.6
5	Inactive	102.5
6	Inactive	72.80
7	Inactive	73.00
8	Inactive	90.90
9	Inactive	97.40
10	Inactive	78.10
11	28.14 ± 0.024	98.30
12	37.21 ± 0.017	97.80
13	Inactive	82.20
15	7.56 ± 0.087	84.30
16	Inactive	76.60
17	Inactive	82.90
21	Inactive	64.40
Indomethacin	28.4 ± 3.5	—

Most of the isolated compounds were evaluated for their NO inhibitory activities in J774.A1 macrophage cells. Compounds **2** and **15** significantly exhibited NO production with IC_{50} values of 10.21 ± 0.074 and 7.56 ± 0.087 μM, respectively, whereas compounds **11** (IC_{50} = 28.14 ± 0.024 μM) and **12** (IC_{50} = 37.21 ± 0.017 μM) were more moderate inhibitors (Table 2). Significantly, the active compounds, **2**, **11**, **12** and **15**, did not show cytotoxicity at 10 μM against J774.A1 cells (Table 2).

Conclusion

Friesodielsia is a small genus in the Annonaceae family. To the best of our knowledge, only three species have been investigated phytochemically and the major compounds are flavonoids.[1–3] However, a few chalcones,[1,3] alkaloids,[3] benzyl benzoate derivatives[2] and sesquiterpenes[2] were also been found. In this study, 21 compounds were isolated and identified including three new unique hybrid flavan–flavones (**1–3**), two hybrid flavan–chalcones (**4** and **5**), nine flavonoids (**6–10**, **13–15**, **17**), three

chalcones (**11–12**, **18**), three alkaloids and one styryl lactone (**21**). A hypothesis for the biosynthesis of compounds **1–5** from a *para*-quinone methide intermediate derived from compound **6** is proposed. This is the first reported isolation of compounds **4–10**, **13–17**, and **19–21** from this genus. Compounds **2**, **11**, **12** and **15** inhibited NO production indicating that they might be potential lead compounds for further study and development as anti-inflammatory agents.

Experimental

General experimental procedures

Melting points were determined on a Stuart SMP3 Melting Point Apparatus. The $[\alpha]_D$ values were measured with a Bellingham and Stanley ADP400 polarimeter. UV-vis spectra were recorded with a BMG LABTECH/SPECTROstar Nano spectrometer. The circular dichroism (CD) spectra were measured on a JASCO J-815 apparatus. The IR spectra were recorded using a PerkinElmer FTS FT-IR spectrometer. The NMR spectra were recorded using a 400 MHz Bruker AM 400 spectrometer in acetone-d_6 with TMS as an internal standard. The HRESIMS were obtained on a Bruker microTOF mass spectrometer. Silica gel C_{60} (0–20 μm, SiliCycle® Inc.) and silica gel G60 (60–200 μm, SiliCycle® Inc.) were used to perform quick column chromatography (QCC) and column chromatography (CC), respectively. Analytical thin-layer chromatography (TLC) was performed with the precoated plates of silica gel 60 F_{254}. The macrophage J774.A1 cells were purchased from CLS (Cell Line Service, Germany).

Plant material

The twigs and leaves of *F. desmoides* were collected in August 2015 from an authentically identified plant growing at Mae Fah Luang University Health Park, Chiang Rai Province, Thailand. The plant specimen (no. MFU-NPR0102) was deposited at the Natural Products Research Laboratory, School of Science, Mae Fah Luang University.

Extraction and isolation

Air-dried leaves of *F. desmoides* (564.3 g) were extracted with EtOAc (5 L) over a period of 3 days at room temperature. Removal of the solvent provided the crude EtOAc extract (49.42 g), which was subjected to QCC over silica gel, eluting with a gradient of hexanes–EtOAc (100% hexanes to 100% EtOAc) to give compound **6** (5.2 mg), **14** (6.5 mg) and nine fractions (A–I). Fractions C (1.59 g) was further separated by CC (100% DCM) to give compounds **9** (17.1 mg) and **10** (6.3 mg). Fraction D (2.38 g) was subjected to CC using reverse phase silica gel (4 : 1 MeOH/H_2O) to afford four subfractions (DA–DD). Compound **7** (14.7 mg) was obtained from subfraction DA (31.4 mg) by Sephadex LH-20 (100% MeOH). Subfraction DC (173.4 mg) was further separated by CC (100% DCM) to yield compounds **11** (13.6 mg) and **12** (14.2 mg). Fraction F (1.30 g) was subjected to CC using reverse phase silica gel (4 : 1 MeOH/H_2O) to afford three subfractions (FA–FC). Purification of subfraction FC (526.9 mg) by CC (1 : 4 acetone/hexanes) gave compounds **1** (16.8 mg) and **3** (5.3 mg). Fraction G (1.09 g) was further separated by CC using reverse phase silica gel (4 : 1 MeOH/H_2O) to obtained seven subfractions (GA–GG). Subfractions GC (376.5 mg) was further purified by CC (100% DCM), yielding compounds **2** (7.0 mg), **4** (3.1 mg) and **5** (4.5 mg). Compounds **6** (7.4 mg) and **14** (5.5 mg) were obtained from fraction H (1.21 g) by repeated CC (3 : 7 acetone/hexanes). Fraction I (1.84 g) was further separated by CC using reverse phase silica gel (4 : 1 MeOH/H_2O) to obtained four subfractions (IA–ID). Subfraction IC (451.8 mg) was further purified by CC (2 : 3 acetone/hexanes) to afford compounds **13** (12.4 mg) and **16** (4.3 mg).

Air-dried twigs of *F. desmoides* (1.26 kg) were extracted with EtOAc (5 L) over a period of 3 days at room temperature. Removal of the solvent provided the crude EtOAc extract (52.02 g), which was subjected to QCC over silica gel, eluting with a gradient of hexanes–EtOAc (100% hexanes to 100% EtOAc) to give five fractions (A–E). Fraction B (396.1 mg) was separated on Sephadex LH-20 (100% MeOH) to obtained four subfractions (BA–BD). Subfractions BC (165.9 mg) was further purified by CC (100% DCM), yielding compound **21** (2.8 mg). Compounds **11** (10.1 mg) and **15** (1.4 mg) were obtained from subfraction BD (93.7 mg) by CC (100% DCM). Purification of fraction C (745.9 mg) by CC (1 : 4 acetone/hexanes) yielded compounds **17** (6.8 mg) and **18** (6.0 mg). Fractions D (325.0 mg) was further purified by CC (1 : 9 EtOAc/DCM) to give compound **16** (5.3 mg). Compounds **19** (1.3 mg) and **20** (1.4 mg) were obtained from fraction E (212.5 mg) by CC (0.5 : 9.5 MeOH/DCM).

Friesodielsone A (1). Yellow powder; mp 244–246 °C; $[\alpha]_D^{25}$ −57.6 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 245 (3.51), 313 (3.40) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 328 (4.72×10^3), 311 (2.99×10^3), 286 (-7.41×10^3) and 225.5 (2.92×10^4) nm; IR (neat) ν_{max} 3087, 2924, 2851, 1652, 1614, 1580, 1501, 1449, 1167, 766 cm^{-1}; see Table 1 for ^1H NMR (acetone-d_6, 400 MHz) and ^{13}C NMR (acetone-d_6, 100 MHz); HRESIMS *m*/*z* 523.1393 $[M - H]^-$ (calcd for $C_{31}H_{23}O_8$, 523.1393).

Friesodielsone B (2). Yellow powder: mp 167–169 °C; $[\alpha]_D^{26}$ −45.1 (*c* 0.006, MeOH); UV (MeOH) λ_{max} (log ε) 235 (3.61), 298 (3.64), 340 (3.33) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 322 (3.52×10^4), 311 (4.92×10^4), 289 (-1.19×10^5), and 225 (4.70×10^5) nm; IR (neat) ν_{max} 3434, 2920, 2851, 1634, 1441, 1373, 1275, 1111, 618 cm^{-1}; see Table 1 for ^1H NMR (acetone-d_6, 400 MHz) and ^{13}C NMR (acetone-d_6, 100 MHz); HRESIMS *m*/*z* 537.1549 $[M - H]^-$ (calcd for $C_{32}H_{25}O_8$, 537.1549).

Friesodielsone C (3). Yellow powder: mp 178–181 °C; $[\alpha]_D^{25}$ −63.2 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 240 (4.27), 298 (4.33), 340 (3.75) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 344 (7.00×10^3), 305 (1.24×10^4), 286 (-1.56×10^4), and 224 (7.15×10^4); IR (neat) ν_{max} 3200, 2928, 2854, 1634, 1443, 1371, 1275, 1161, 1167, 766 cm^{-1}; see Table 1 for ^1H NMR (acetone-d_6, 400 MHz) and ^{13}C NMR (acetone-d_6, 100 MHz); HRESIMS *m*/*z* 523.1393 $[M - H]^-$ (calcd for $C_{31}H_{23}O_8$, 523.1393).

Nitric oxide production inhibitory assay

The effects of the isolated compounds on nitric oxide production in murine macrophage J774.A1 cells (Cell Line Service, Germany) in supernatant were determined using the previously reported method.[22] In brief, J774.A1 cells were

added in 96-well plate with 5×10^5 cells per well and incubated for 1 h at 37 °C and 5% CO_2. After that, cells were treated with various concentrations of sample or vehicle (DMSO) for 2 h, followed by LPS 10 μg mL^{-1}. After 18 h incubation, NO production in the culture medium was determined using the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) for 10 min and the absorbance was measured at 540 nm. Indomethacin was used as a positive control.

Cell viability assay

The measurement of cell viability of the tested compounds was performed using the 3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide (MTT) assay against unstimulated J774.A1 cells.[25] Briefly, 10 μL of fresh MTT solution (5 mg mL^{-1} in saline) was added to each well, incubated at 37 °C in CO_2 for 3 h. The media was discarded and DMSO was added to dissolve the formazan crystals and the absorbance value at 540 nm was measured. The percentages of cell survival were calculated from the absorbance value of the tested compounds and control (LPS) using the equation below.

$$\%\ \text{cell viability} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{LPS}}} \times 100$$

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