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Phytochemical Study of *Fagraea* spp. Uncovers a New Terpene Alkaloid with Anti-Inflammatory Properties[1]

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Phytochemical investigation of the stem bark of *F. racemosa* JACK *ex* WALL (Loganiaceae) from East Java, Indonesia, has resulted in the isolation of a new alkaloid fagraeoside along with the iridoid glycoside secologanoside. Fagraeoside may be derived from the condensation of secologanin with L-asparagine, and represents a rare example of a terpene alkaloid in which the amino acid component is non-aromatic. Investigation of three additional species of *Fagraea* provided known lignans, iridoid or secoiridoid glycosides, and flavanol-6-*C*-glucosides, thus it is likely that iridoid and secoiridoid glucosides are chemotaxonomic markers for the *Fagraea* genus. Fagraeoside inhibited the production of prostaglandin E_2 in 3T3 murine fibroblasts (IC_{50} ~5.1 μM), and was not cytotoxic to this cell line or to a P388 murine leukaemia cell line. Selected isolated compounds, including fagraeoside, showed low to moderate activity in anti-acetylcholinesterase screening.

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Introduction

Fagraea (Loganiaceae) is a genus of shrubs or small trees consisting of ~50 species, which are distributed throughout Indo-Malaya, China, and Australia. Species of this genus often have large flowers and can live as an epiphyte in host trees with roots eventually reaching to the ground.[1,2] *Fagraea* has been used as a traditional medicine in many parts of South East Asia, including in Cambodia, Malaysia, the Philippines, and in Vietnam. The leaves have been used mainly for their analgesic and antipyretic effects, while a decoction of the bark is used as an antimalarial, an antipyretic, or as an anti-inflammatory agent.[3,4]

Previous studies on the chemistry of *Fagraea* spp. have identified lignans, including (+)-pinoresinol, (+)-epipinoresinol, (+)-lariciresinol, and (+)-isolariciresinol, and phenols from the root of *F. racemosa* JACK *ex* WALL collected in Sabah, Borneo, Malaysia.[5] The methanolic extract of *F. obovata* leaves obtained from Jogfalls, Karnataka, India, contained the glycosyl flavone fagovatin.[4] Other compounds including a benzyl alcohol derivative, di-*O*-methylcrenatin, the flavone *C*-glucoside swertisin and some iridoid glucosides, namely blumeosides A–D, were isolated from *F. blumei* G Don,[6] while the alkaloid gentianine was isolated from the leaves and fruits of *F. fragrans*.[7] Over 50 volatile compounds have been isolated from the white flowers of *F. berteriana*, with these compounds responsible for the characteristic odor of the flower.[8] We now report a study on constituents of stem bark from *Fagraea* spp. collected in East Java, Indonesia which gave a new alkaloid, named by us as fagraeoside, together with eleven known compounds. The structures of the isolated compounds were deduced on the basis of NMR spectroscopic data and mass spectrometry. The anti-inflammatory, cytotoxic, and anti-acetylcholinesterase effects of selected metabolites have also been investigated.

Results and Discussion

Powdered, air-dried stem bark samples from four species of *Fagraea* were collected in East Java, Indonesia and extracted sequentially with hexanes, then DCM and finally MeOH. The methanol extract from *F. racemosa* was purified by reverse phase flash chromatography followed by C_{18} HPLC yielding fagraeoside (**1**), whose structure was established by 1D and 2D NMR spectroscopy, and secologanoside (**2**), identified by comparison with literature data.[9]

For compound (**1**), HRESIMS revealed an intense ion at *m*/*z* 469.1468 $[M-H]^-$, corresponding to a molecular formula of

[1]Dedicated to the memory of the late Professor Athel Beckwith, an outstanding Australian chemist.

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Table 1. NMR data (D_2O) for fagraeoside

Position	δ_C[A]	δ_H, m (J in Hz)[B]	COSY[C]	^{1}H-^{13}C HMBC[D]
1	99.8	5.53, d (1.7)	9	3, 5, 8, 1′
3	151.2	7.43, d (2.2)	5	1, 4, 5, 11
4	110.4	–	–	–
5	26.6	3.10, m	3, 6b	3, 6, 7, 9
6	33.2	a 2.22, td (12.8, 4.0)	6b, 7	4[E], 5, 7[E], 9
		b 1.58, ddd (12.8, 11.5, 10.6)	6a, 5, 7	4[E], 5, 7[E], 9
7	66.2	5.24, dd (10.6, 4.0)	6a, 6b	5, 6[E], 11[E], 2″[E]
8	134.3	5.58, ddd (17.1, 10.1, 9.9)	9, 10a, 10b	1, 5, 9
9	44.8	2.80, m	1, 8	1, 4, 5, 8, 10
10	123.5	a 5.37, dd (17.1, 1.4)	8	8, 9
		b 5.30, dd (10.1, 1.4)	8	9
11	168.3	–	–	–
1′	100.9	4.83, d (8.0)	2′	1, 3′, 5′
2′	75.4	3.28, dd (9.4, 8.0)	1′, 3′	–
3′	78.2	3.50, t (9.4)	2′, 4′	2′, 4′
4′	72.4	3.40, dd (9.8, 9.4)	3′, 5′	3′, 5′, 6′
5′	79.1	3.51, ddd (9.8, 6.0, 2.1)	4′, 6′b	4′
6′	63.5	a 3.92, dd (12.4, 2.1)	6′b	4′
		b 3.72, dd (12.4, 6.0)	6′a, 5′	5′
2″	175.5	–	–	–
3″	36.0	a 2.83, dd (16.6, 7.3)	3″b, 4″	2″, 4″, 5″
		b 2.70, dd (16.6, 6.2)	3″a, 4″	2″, 4″, 5″
4″	56.3	4.89, dd (7.3, 6.2)	3″a, 3″b	7, 11, 2″, 3″, 5″
5″	178.9	–	–	–

[A]Data recorded at 100 MHz, referenced to TSP at 0.0 ppm.
[B]Data recorded at 750 MHz, referenced to H_2O at 4.77 ppm.
[C]Data recorded at 500 MHz.
[D]Data recorded at 750 MHz with $^nJ_{CH}$ 8 Hz.
[E]Additional correlations from data recorded at 750 MHz with $^nJ_{CH}$ 4 Hz.

$C_{20}H_{26}N_2O_{11}$. The ^{13}C NMR data (Table 1) were acquired in D_2O and revealed 20 carbons, including three carbonyl signals at δ_C 168.3, 175.5, and 178.9. The ^{1}H NMR data, also in D_2O, showed features typical of a β-glucose residue, including an anomeric proton at δ_H 4.83 (H-1′) with the corresponding carbon at δ_C 100.9 by HSQC, a pair of multiplets (dd) at δ_H 3.92 and δ_H 3.72 for H-6′, and resonances for four methine protons between δ_H 3.51 and 3.28. A full assignment of the glucose moiety was provided from TOCSY, DQF-COSY, and nOe data.

Other structural features resembled a secoiridoid fragment, and included an alkene proton at δ_H 7.43 (H-3) with the corresponding carbons at δ_C 151.2 (C-3) and 110.4 (C-4), an acetal proton at δ_H 5.53 (H-1) correlated with a carbon at δ_C 99.8 by HSQC, and resonances for three vinylic protons at δ_H 5.58 (H-8), 5.37 (H-10a), and 5.30 (H-10b), with the corresponding alkene carbons at δ_C 134.3 (C-8) and 123.5 (C-10).[2] A proton signal at δ_H 5.24 (1H, dd, J = 10.6, 4.0, H-7) linked to a carbon at δ_C 66.2 (d, C-7) supported either an α-aminoacyl centre or an *N,N* acetal. The H-7 signal could be linked by DQFCOSY and/or TOCSY correlations to signals at δ_H 2.22 (H-6a), δ_H 1.58 (H-6b), δ_H 3.10 (H-5), and δ_H 2.80 (H-9). HSQC correlations then located C-5, C-6, C-7, and C-9, while geHMBC correlations (Fig. 1a) from H-3 and from H-7 to an amide-like carbonyl at δ_C 168.3 identified the remaining 'secoiridoid' carbon C-11. The HMBC correlation between H-1′ and C-1 confirmed the position of the glucose moiety. When the ^{1}H NMR spectrum of fagraeoside was run in DMSO-d_6, a broad NH singlet at δ_H 7.82 was detected which gave HMBC correlations to C-7 and NOESY correlations to H-7.

The four remaining carbons were two carbonyls (δ_C 175.5, and 178.9), a methylene (δ_C 36.0) and a methine (δ_C 56.3). There were three unassigned proton signals: an α-aminoacyl proton at δ_H 4.89 (1H, dd, J = 7.3, 6.2, H-4″) linked to the carbon at δ_C 56.3 by HSQC, and methylene protons at δ_H 2.83 (1H, dd, J = 16.6, 7.3, H-3″a) and 2.70 (1H, dd, J = 16.6, 6.2, H-3″b), linked to the signal at δ_C 36.0 by HSQC. HMBC correlations were from H-4″ to C-3″, to C-7, and notably to the amide C-11 as well as to the two carbonyls at δ 175.5 (C-2″) and 178.9 (C-5″). H-3″ showed correlations to C-2″, C-5″, as well as to C-4″, and H-7 at δ_H 5.24 showed correlations to C-2″ and, as mentioned, to C-11, while the NH signal showed a correlation to C-3″. These data fitted for a tetrahydropyrimid-4(3*H*)-one ring constructed biosynthetically from the condensation of an asparagine unit onto the secoiridoid framework (Fig. 2). An alternative tetrahydropyrimid-4(3*H*)-one structure with the free carboxyl group at C-3″ instead of at C-4″ was both inconsistent with the ^{1}H and ^{13}C chemical shifts of C-3″ and C-4″ and would be biosynthetically-unprecedented. The tetrahydropyrimid-4(3*H*)-one jadomycin N with δ_C 66.95 for the *N,N* acetal centre, a value comparable to that shown by C-7 of **1**, has recently been isolated from cultures of *Streptomyces venezuelae* ISP5230 grown on L-asparagine as sole nitrogen source.[10]

The relative configuration was deduced from NOESY data (Fig. 1b) which showed correlations from H-5 to H-7 and to H-9, and between H-7 and the diastereotopic proton at δ_H 2.83 (H-3″a). Modelling of fagraeoside was undertaken using Chem Bio 3D ultra 12.0 (Cambridge), using MM2 software for energy minimization to an RMS gradient of 0.100, and revealed a

[2]Standard secoiridoid numbering used throughout.

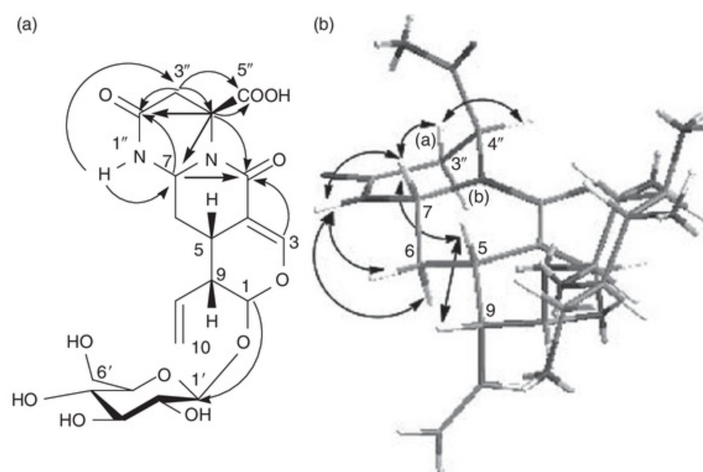

Fig. 1. Structure of fagraeoside (**1**) showing (a) important HMBC correlations observed in d_6-DMSO; (b) MM2 minimised model showing selected nOe or NOESY correlations observed in d_6-DMSO (double-headed arrows).

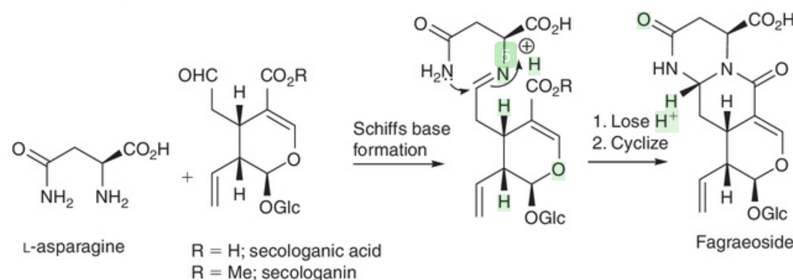

Fig. 2. Proposed biosynthesis of fagraeoside from a secologanin-derived precursor and from L-asparagine.

flattened chair for the tetrahydropyrimidinone ring. In the ^{1}H NMR in D_2O, H-4″ appeared as a doublet of doublets with J values of 7.3 Hz to H-3″a and of 6.2 Hz to H-3″b, matching the observed dihedral angles in **1** between H-4″ and H-3″a (56.7°) and H-3″b (61.8°). When the C-4″ epimer was modelled, the measured dihedral angles were respectively 64.6° and 175.9° which would have led to one large J value for the H-4″ signal. Although such data must be used with care, the absence of a NOESY correlation between H-7 and H-4″ was also consistent with the proposed relative configuration. In the C-4 epimer these protons are on the same face with an inter proton distance of 2.6 Å, whereas in **1**, they are on the opposite face, and thus 3.9 Å apart.

Another metabolite isolated from *F. racemosa* was (−)-secologanoside (**2**)[9] which was easily recognised by comparison of its NMR data with the literature. The presence of a D-glucose moiety in **1** and in **2** was confirmed by acid hydrolysis, derivatization with TFAA, and enantioselective gas chromatography.[11] The absolute configuration shown for **1** is further supported by its co-isolation with secologanoside, and by the proposed role of secologanin in the biosynthetic pathway of **1**. Consequently, L-asparagine is implicated in the biosynthesis of **1**.

Three other species of *Fagraea* were investigated for their chemistry. Stem bark from the species *F. blumei* yielded first, from the methanolic fraction, di-*O*-methylcrenatin (**3**),[12] potalioside B (**4**),[12] the iridoid glucoside adoxosidic acid (**5**),[6,13] and its methyl ester derivative adoxoside (**6**);[14] second, from the DCM extract, (+)-pinoresinol (**7**)[5,15,16] and salicifoliol (**8**).[16] Di-*O*-methylcrenatin (**3**), adoxosidic acid (**5**), and adoxoside (**6**) have all been previously reported from *F. blumei*.[6] The iridoid glucoside loganic acid (**9**)[17] and the secoiridoid glucoside sweroside (**10**)[18] were isolated from *F. auriculata* together with potalioside B (**4**). An extract from *F. ceilanica* yielded two hydroxyflavanol C-glycosides whose NMR data matched those of taxifolin-6-*C*-glucoside (**11**)[19] and aromadendrin-6-*C*-glucoside (**12**)[20] together with secologanoside (**2**) and loganic acid (**9**) (Fig. 3).

In view of the traditional usage of *Fagraea* bark as an anti-inflammatory and antipyretic agent, compound **1** was screened for cytotoxicity and anti-inflammatory activity.[21] Although **1** inhibited the production of PGE_2 in 3T3 murine fibroblasts (IC_{50} 5.1 μM), an atypical dose response curve was obtained (Fig. 4), even on repeat screening. Acetyl salicylic acid (IC_{50} 2.6 μM) was used as a positive control. The observed anti-inflammatory activity did not appear to be a cytotoxic response since the compound lacked cytotoxicity to the 3T3 cells, and also to a P388 murine leukaemia cell line, when compared with chlorambucil as a reference compound. Additional testing is required to understand these effects. Although several iridoid glycosides show useful anti-inflammatory activity, there are few such reports for seco-iridoids.[22,23] Compounds **1**, **2**, **4**, **7–9**, **11**,

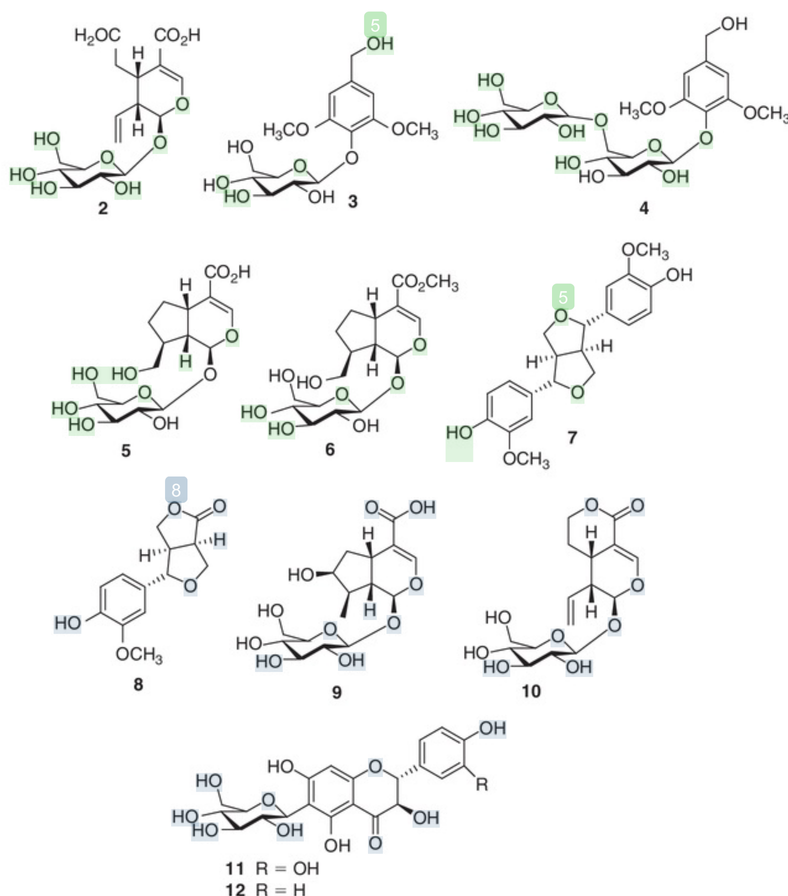

Fig. 3. Secoiridoid glycoside, iridioid glycoside, and lignan metabolites from *Fagraea* spp.

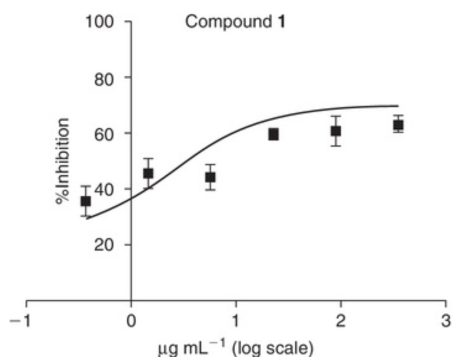

Fig. 4. Anti-inflammatory activity of fagraeoside against 3T3 murine fibroblast cells.

and **12** were also screened for anti-acetylcholinesterase activity in an assay using Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB),[24] with the data quantified by detection at 415 nm using a microplate reader.[24,25] The inhibition observed for all eight metabolites at $100\,\mu g\,mL^{-1}$ was similar to the inhibition found for galanthamine HBr salt at $0.184\,\mu g\,mL^{-1}$, which indicated that the metabolites are relatively weak inhibitors of acetylcholinesterase.

Conclusion

One of the largest group of plant alkaloids are the indole alkaloids formed from the condensation of secologanin with the aromatic amino acid tryptophan. This paper has described the structure and stereochemistry of a novel alkaloid that may be derived in Nature from the condensation of secologanin with the amino acid L-asparagine. There are remarkably few reports of terpene alkaloids in the literature in which the amino acid component is non-aromatic. Machida et al. (2002)[26] have described L-phenylalaninosecologanin, presumably formed by enzymatic reduction of the Schiffs base product of secologanin and L-phenylalanine, from *Lonicera japonica* Thunb. Our investigations on the secondary metabolites of the genus *Fagraea* have revealed the presence of iridoid and secoiridoid glycosides in all four species studied, and when considered together with an earlier literature report[6] suggest that such compounds may be chemotaxonomic markers for the genus.

Experimental

General

Reverse phase HPLC was conducted on an Agilent 1100 series instrument with variable wavelength UV detector set at 240 or at 254 nm or on a Shimadzu LC-20AT with a low temperature-evaporative light scattering detector (EL.S.D.-LT) fitted with a semi-preparative Waters® HPLC column (μBondapak C_{18} 7.8 × 300 mm) or a Phenomenex® column (Gemini 5 micron 10 × 250 mm). Positive or negative ion electrospray mass spectra (LRESMS) were determined using a Bruker Esquire HCT instrument or (HRESMS) on a MicroTof Q instrument, each with a standard ESI source. Optical rotation $[\alpha]_D$ measurements were taken on a Perkin-Elmer 241 MC or a JASCO P-1010 polarimeter. The ^{1}H and ^{13}C NMR data were measured on Bruker Avance 400, 500, or 750 MHz spectrometers at 298 K. Normal phase and reverse phase thin-layer chromatography were performed on Merck Art 5554 and 5559 aluminium backed plates precoated with silica gel Merk Kieselgel 60 F254, respectively. Normal phase flash column chromatography was undertaken on Scharlau silica gel 60, 0.04–0.06 mm (230–400 mesh ASTM), while reverse phase flash column chromatography was carried out on silica gel prepared using the method developed by Evans et al.[27] Acetylcholinesterase inhibition assay measurements were performed using a Bio-Rad Model 550 microplate reader at 415 nm.

Collection, Extraction, and Isolation

Stem bark of *F. blumei* G.Don (SC0106) was collected in Malang, East Java, Indonesia on 16 November 2006. Stem bark of *F. ceilanica* Thunb (SC0206) and *F. racemosa* JACK *ex* WALL (voucher sample SC0306) was collected on 16 November 2006 from Purwodadi Botanic Garden, East-Java, Indonesia. A methanolic extract of the stem bark of *F. auriculata* Jack, Mall (sample collected 26 February 2001; voucher FU0101) was a generous gift from Dr Achmad Fuad, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia. All plants were identified by staff of Purwodadi Botanic Garden, East Java, Indonesia; voucher samples are stored in the Faculty of Pharmacy, Airlangga University. Plant materials were chopped and air-dried at room temperature for 5 days and then powdered using a blender.

Powdered stem bark of *F. racemosa* (530 g) was macerated with hexanes (3 × 24 h), then with DCM, and finally by MeOH. The methanolic extract (18 g) was fractionated by vacuum liquid chromatography, with a gradient of $CHCl_3$, MeOH, and H_2O in increasing polarity, and afforded 9 fractions. Fraction 8, namely FRM8, was then partitioned by NP flash chromatography, using EtOAc, MeOH, and H_2O in order of increasing polarity. The first three fractions, namely FRM801, 802, and 803 eluting in EtOAc/MeOH (9:1), EtOAc/MeOH/H_2O (9:1:1), and EtOAc/MeOH/H_2O (8:2:1), respectively contained secologanoside (**2**) (134.6 mg). The fourth fraction, FRM804, was subjected to flash chromatography on SiO_2 using a solvent mixture of EtOAc/MeOH/H_2O (8:2:1), followed by EtOAc/MeOH/H_2O (5:5:1), and yielded 9 fractions. Fractions 7 and 8, namely FRM8047 and FRM8048, were then subjected to RP-HPLC using a gradient of 0–50% MeOH in H_2O for 30 min, with UV detection at 240 nm, and flowrate 1.5 mL min^{-1}. The component eluting at 20.9 min was fagraeoside (**1**) (3.5 mg).

Powdered stem bark of *F. blumei* (1 kg) was macerated in hexanes, followed by DCM and MeOH using the same procedure as *F. racemosa*. A portion of the MeOH extract (382 mg) was chromatographed on RP-silica gel using a solvent gradient from 0 to 100% MeOH in H_2O. A fraction eluting in 0–20% MeOH/H_2O contained di-*O*-methylcrenatin (**3**) (3.7 mg), potalioside B (**4**) (1.4 mg), and a mixture of adoxosidic acid (**5**) (3.4 mg) and adoxoside (**6**), which were further purified by RP-HPLC (0–50% MeOH in H_2O for 60 min, detection at 254 nm). The DCM extract (11.8 g) was chromatographed on kieselgel 60H using hexanes, DCM, and MeOH in order of increasing polarity, followed by column chromatography on Diaion HP-20, using a solvent gradient (20% H_2O in MeOH to 100% MeOH) yielding 5 fractions. Fraction 1 was then subjected to RP-HPLC (50% MeOH in H_2O for 50 min, detection at 254 nm) to afford (+)-pinoresinol (**7**) (3.8 mg) and salicifoliol (**8**) (1.2 mg).

Extraction of powdered stem bark of *F. auriculata* was undertaken using the same procedure employed for *F. racemosa* and *F. blumei*. The MeOH extract (368 mg) was subjected to RP-flash column chromatography with a solvent gradient from 0 to 100% MeOH in H_2O, and yielded 19 fractions. Fraction 4 was further purified by RP-HPLC with a gradient of 20 to 30% MeOH in water over 60 min to afford loganic acid (**9**) (1.16 mg). Fraction 5, eluting in 20% MeOH/H_2O contained potalioside B (**4**) (8.79 mg), while fraction 6 (50% MeOH/H_2O) afforded sweroside (**10**) (33.9 mg).

Stem bark of *F. ceilanica* (488 g) was extracted in the same way. A portion of the methanolic extract (3.9 g) was partitioned by RP-flash chromatography using a solvent gradient from 0 to 100% MeOH in H_2O. Repeated RP-flash chromatography was undertaken on a fraction eluting in 100% H_2O, followed by semi preparative RP-TLC with MeOH/H_2O (3/7) to obtain five fractions. Fractions 2 and 3 were further purified by RP-HPLC (15–50% MeOH in H_2O containing 0.1% TFA for 30 min, detection at 254 nm) to give taxifolin-6-*C*-glucoside (**11**) (22.9 mg) from fraction 2, and aromadendrin-6-*C*-glucoside (**12**) (14.1 mg), loganic acid (**9**) (5.8 mg), and secologanoside (**2**) (14.1 mg) from fraction 3.

Fagraeoside (1): Pale yellow amorphous solid (3.5 mg); $[\alpha]_D^{23}$ −38.6 (*c* 0.57, H_2O); UV (H_2O) λ_{max} (log ε) 241 (3.53) nm; ^{1}H and ^{13}C NMR (D_2O, 750 and 100 MHz), see Table 1; ^{1}H NMR (DMSO-d_6, 750 MHz) δ 7.82 (1H, br s, NH), 7.28 (1H, d, J = 2.4, H-3), 5.47 (1H, ddd, J = 17.2, 10.3, 10.0, H-8), 5.36 (1H, d, J = 1.7, H-1), 5.30 (1H, dd, J = 17.1, 2.0, H-10a), 5.22 (1H, dd, J = 10.3, 2.0, H-10b), 5.19 (1H, dd, J = 11.6, 3.0, H-7), 4.90 (1H, br d, J = 6.8, H-4″), 4.51 (1H, d, J = 8.0, H-1′), 3.68 (1H, m, H-6′a), 3.42 (1H, m, H-6′b), 3.14 (2H, m, H-3′ overlapping with H-5′), 3.03 (1H, m, H-4′), 2.98 (1H, m, H-2′), 2.85 (1H, m, H-5), 2.72 (1H, d, J = 16.0, H-3″a), 2.62 (1H, m, H-9), 2.17 (1H, dd, J = 16.0, 6.8, H-3″b), 2.00 (1H, m, H-6a), 1.20 (1H, m, H-6b); ^{13}C NMR (DMSO-d_6, 750 MHz, partial data from HMBC) δ 161.8 (C-11), 146.6 (C-3), 132.9 (C-8), 119.8 (C-10), 107.0 (C-4), 98.6 (C-1′), 95.4 (C-1), 77.1 (C-5′), 76.7 (C-3′), 72.8 (C-2′), 69.8 (C-4′), 62.6 (C-7), 61.0 (C-6′), 50.6 (C-4″), 42.0 (C-9), 34.4 (C-3″), 32.9 (C-6), 23.8 (C-5); HRESIMS calcd. for $C_{20}H_{26}N_2O_{11}$, 469.1458. Found: *m/z* 469.1468 $[M-H]^-$.

Secologanoside (2): Amorphous solid (134.6 mg); $[\alpha]_D^{20}$ −98.3 (*c* 0.12, H_2O); UV (H_2O) λ_{max} (log ε) 241 (3.53) nm; the ^{1}H and ^{13}C NMR data were comparable with published values;[9] LRESIMS *m/z* 413.2 $[M+Na]^+$.

Determination of Glucose Absolute Configuration

The glucose moieties were characterised according to the method of Hayes et al.[11] A sample of either fagraeoside

(0.74 mg) or secologanoside (5.63 mg) was heated for 2 h at 100°C with 10% HCl in MeOH (0.5 mL). After cooling, the solution was concentrated under N_2, then partitioned between water and $CHCl_3$ (0.5 mL), and the aqueous layer concentrated under N_2. The residue was dissolved in a mixture of trifluoroacetic anhydride (TFAA)/DCM (1/1, 0.2 mL) and then analysed by enantioselective chromatography (Chirasil-L-Val capillary column (25 m × 0.32 mm × 0.2 µm), FID detection, carrier gas: helium, injector: 200°C, colum flow 1.32 mL min^{-1}, split ratio: 56/0, and pressure 40 kPa. Temperature program: initial temperature 50°C for 6 min, raised at 4°C min^{-1} to a final temperature of 160°C for 5 min). For comparison, standards were prepared from D- and L-glucose. Under these conditions, retention times were L-Glc (26.22 and 29.88 min), D-Glc (26.31 and 30.07 min). For glycoside **1**, peaks were observed at 26.30 and 30.06 min, and for glycoside **2**, peaks were observed at 26.26 and 30.02 min corresponding to D-glucose. Co-injection of either glucose pentatrifluoroacetate product with the D-glucose reference standard gave in each case a single peak.

Evaluation of Anti-Inflammatory Activity and Cytotoxicity

The prostaglandin E_2 assay was undertaken using Swiss albino mouse embryo fibroblast cells 3T3 (American Type Culture Collection (ATCC), Manassas, VA) and a PGE_2 enzyme immuno-assay kit (Prostaglandin E_2 ELA kit-monoclonal, Cayman Chemical Co., Ann Arbor, MI, catalogue no. 5141010) using acetyl salicylic acid as a positive control. Cytotoxicity in 3T3 murine fibroblasts, and in P388D1 murine lymphoblasts (ATCC) was assayed using an ATPLite kit (PerkinElmer, Waltham, MA) with chlorambucil as a reference compound. Details of both assays have recently been published.[21]

Evaluation of Acetylcholinesterase Inhibition

Compounds were diluted in MeOH to a concentration of 1 mg mL^{-1}. Galanthamine HBr salt was diluted in MeOH to a concentration of 100 µM. A solution of acetylthiocholine iodide in Millipore water (15 mM, 30 µL) was placed in each well of a 96-well plate. Then in sequence, 5,5′-dithiobis-(2-nitrobenzoic acid) in Millipore water (3 mM, 150 µL) and a buffer solution of bovine serum albumin (0.1%) made up in TRIS-HCl buffer (50 mM, pH 8.0, 60 µL) were added, followed by the compound under assay (30 µL) or by galanthamine HBr salt (30 µL). A blank well contained methanol (30 µL) instead of the compound under assay. The plate was shaken carefully to homogenize the mixture and the absorbance of individual wells at 415 nm was measured 10 times at intervals of 30 s. Acetylcholinesterase solution (0.22 U mL^{-1}, 30 µL) was then added, the individual plates shaken and the absorbance measurements repeated a further 10 times. The experiments were done in quadruplicate in three independent experiments.

Accessory Publication

The Accessory Publication contains copies of representative NMR spectra, including the ^1H and ^{13}C spectra of fagraeoside (**1**) in D_2O, together with HMBC, NOESY, and ROESY data, and the ^1H and ^{13}C spectra of secologanoside (**2**) in D_2O. Also included are characterisation details and spectroscopic data of compounds **2**–**12**. The Accessory Publication is available on the Journal's website.

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