Regioselective synthesis of di-*C*-glycosylflavones possessing anti-inflammation activities†

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Received 16th April 2010, Accepted 28th June 2010
DOI: 10.1039/c0ob00011f

Three methods are utilized to synthesize a variety of 6,8-di-*C*-glycosylflavones bearing identical or distinct glycosyl moieties. Some *C*-glycosylation compounds are found to have better anti-inflammation activities than the parent flavones. Among them, 6,8-di-*C*-glucosylapigenin (known as vicenin-2) shows inhibition of TNF-α expression and NO production with IC_{50} values of 6.8 and 5.2 μM, respectively.

Introduction

Dendrobium huoshanense[1] (Orchidaceae) is a valuable herbal plant used in traditional Chinese medicine.[1–3] The polysaccharide constituent of stem mucilage is found to exhibit specific functions in activating murine splenocytes to produce several cytokines, including IFN-γ, IL-10, IL-6, and IL-1α, as well as hematopoietic growth factors GM-CSF and G-CSF.[4] The bioactive small molecules in *D. huoshanense* are reported to include the bibenzyl,[5] phenanthrene,[6] fluorene,[7] coumarin,[8] sesquiterpene,[9] flavanone,[10] and alkaloid[11] structural types. We recently also isolated four 6,8-di-*C*-glycosylflavones with a core structure of apigenin bearing pentoside (arabinoside or xyloside) and rhamnosyl–hexoside (glucoside or galactoside) substituents.[12]

The constituents of *C*-glycosylflavones are rich in Rutaceous, Compositous and Fabaceous plants.[13] The naturally-occurring *C*-glycosides are generally linked at C-6 and/or C-8 on the A-ring of flavonoid nucleus. Monosaccharides of D-Glc, D-Gal, D-Ara, L-Ara, D-Xyl and L-Rha are commonly found in natural glycosylapigenins.[14] It has been difficult to isolate di-*C*-glycosyl flavonoids from nature sources;[12,14d,14f] thus, organic synthesis is an alternative and effective method to obtain sufficient quantities of these bioactive compounds. In this study, a series of 6,8-di-*C*-glycosyl flavonoids bearing identical or distinct glycosyl substituents were synthesized and their biological activities were examined.

Results and discussion

1. Synthesis

At the first sight, direct di-*C*-glycosylation of naringenin (**1**) looks attractive to attain di-*C*-glycosylflavones having identical glycosyl substituent, in particular, (±)-naringenin is commercially available. However, Sato's[15] and our current studies showed that the $Sc(OTf)_3$-promoted glycosylation of (±)-naringenin with D-glucose gave a low yield (<20%) of the desired 6,8-di-*C*-β-D-glucosylnaringenin (**2aa**)[16] along with a significant amount (~15%) of mono-*C*-glycosylation product and other unidentified side products (Scheme 1). Purification of 6,8-di-*C*-glucosylnaringenin or the peracetylation derivative **2aaAc** was only realized by repeated chromatography to remove undesired side products. Oxidation of the peracetylated flavanone **2aaAc** with DDQ, followed by saponification, yielded 6,8-di-*C*-β-D-glucosylapigenin (**3aa**, vicenin-2).[15,17] Attempts to direct *C*-glycosylation on the A-ring of apigenin failed, presumably because the electron-withdrawing effect in the conjugated system of flavone disfavored the electrophilic aromatic substitution reaction.

In another approach, Sato and coworkers have carried out the $Sc(OTf)_3$-promoted glycosylation of phloroacetophenone (**4**) with D-glucose.[18] Accordingly, the reaction gave 43% and 38% yields of mono- and di-*C*-glycosyl phloroacetophenones under optimized conditions. In our hands, the $Sc(OTf)_3$-promoted glycosylation of phloroacetophenone with D-xylose, followed by benzylation of the phenolic groups, afforded pure 6,8-di-*C*-xylosyl phloroacetophenone (**5bbBn**) in 16% isolated yield (Scheme 2). Condensation of **5bbBn** with substituted benzaldehydes in the presence of KOH gave diglycosylchalcones **7bb1–3** in 60–70% yields. Without prior benzylation, the phenoxide ions would be generated in the presence of KOH base, and thus prevent the desired aldol reaction.[19] By acid catalysis, diglycosylchalcones **7bb1–3** underwent intramolecular Michael reaction smoothly to give 6,8-di-*C*-glycosylflavanones **8bb1–3** after debenzylation. To avoid undesired oxidation of the phenolic moieties, flavanones **8bb1–3** were protected as the peracetylates prior to oxidation with Me_2SO/I_2.[19a,20] Reacetylation was performed to facilitate isolation of the products because some acetyl groups were cleaved by HI (generated *in situ*) under the reaction conditions. By subsequent saponification, the target compounds of 6,8-di-*C*-glycosylflavones **9bb1–3** having different substituents on the B-ring were obtained in reasonable yields.

This synthetic approach can be applied to build libraries of 6,8-di-*C*-glycosylflavanones (*e.g.* **8bb1–3**) and 6,8-di-*C*-glycosylflavones (*e.g.* **9bb1–3**) containing different substituents on the B-ring because the precursors of di-*C*-glycosylchalcone are easily prepared by using a variety of substituted benzaldehydes (*e.g.* **6a–c**). In the case of the di-*C*-glycosylchalcone bearing two different glycosyl moieties at 3′- and 5′-positions, both 2′- and

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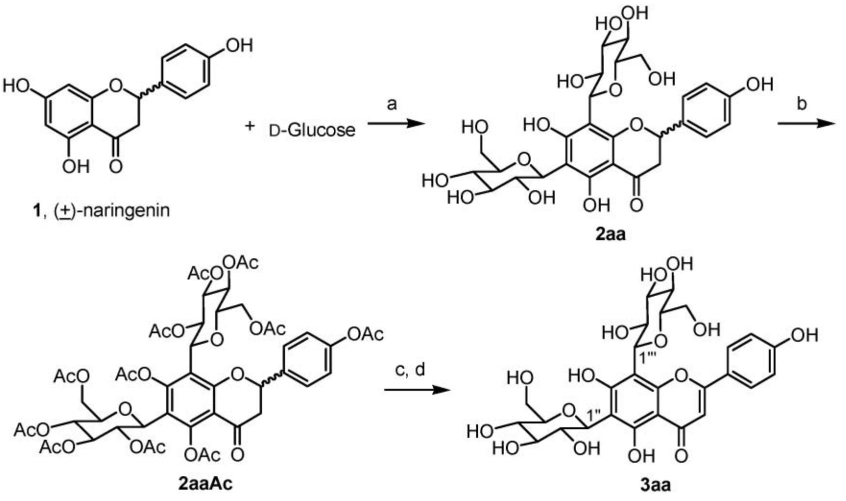
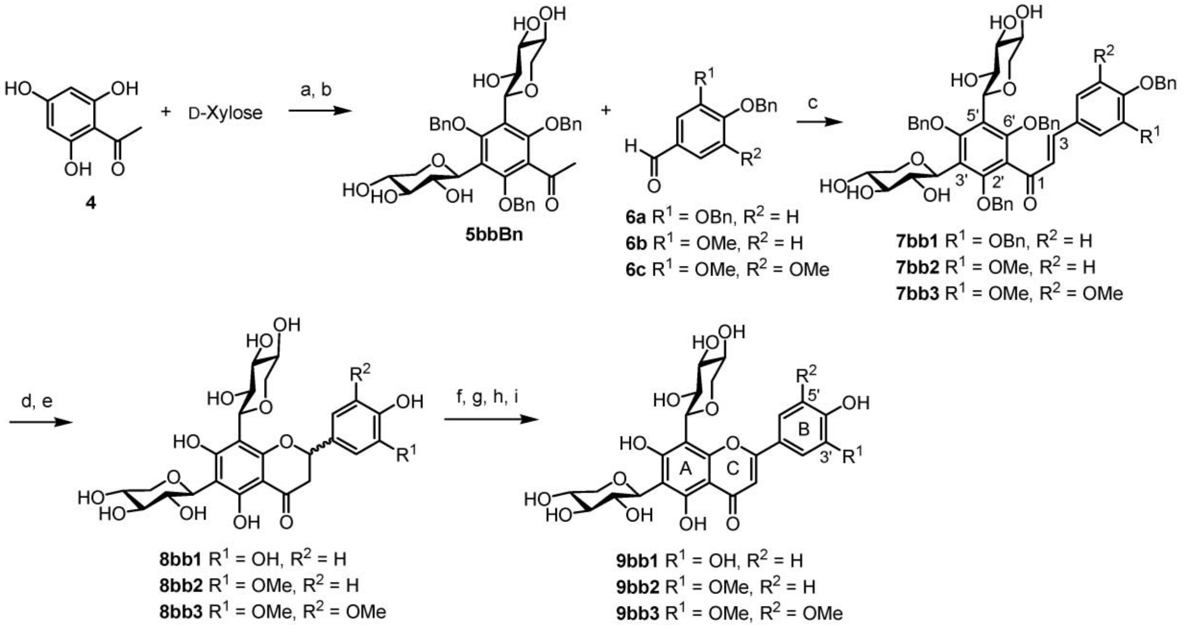
† Electronic supplementary information (ESI) available: Experimental details and NMR spectra. See DOI: 10.1039/c0ob00011f



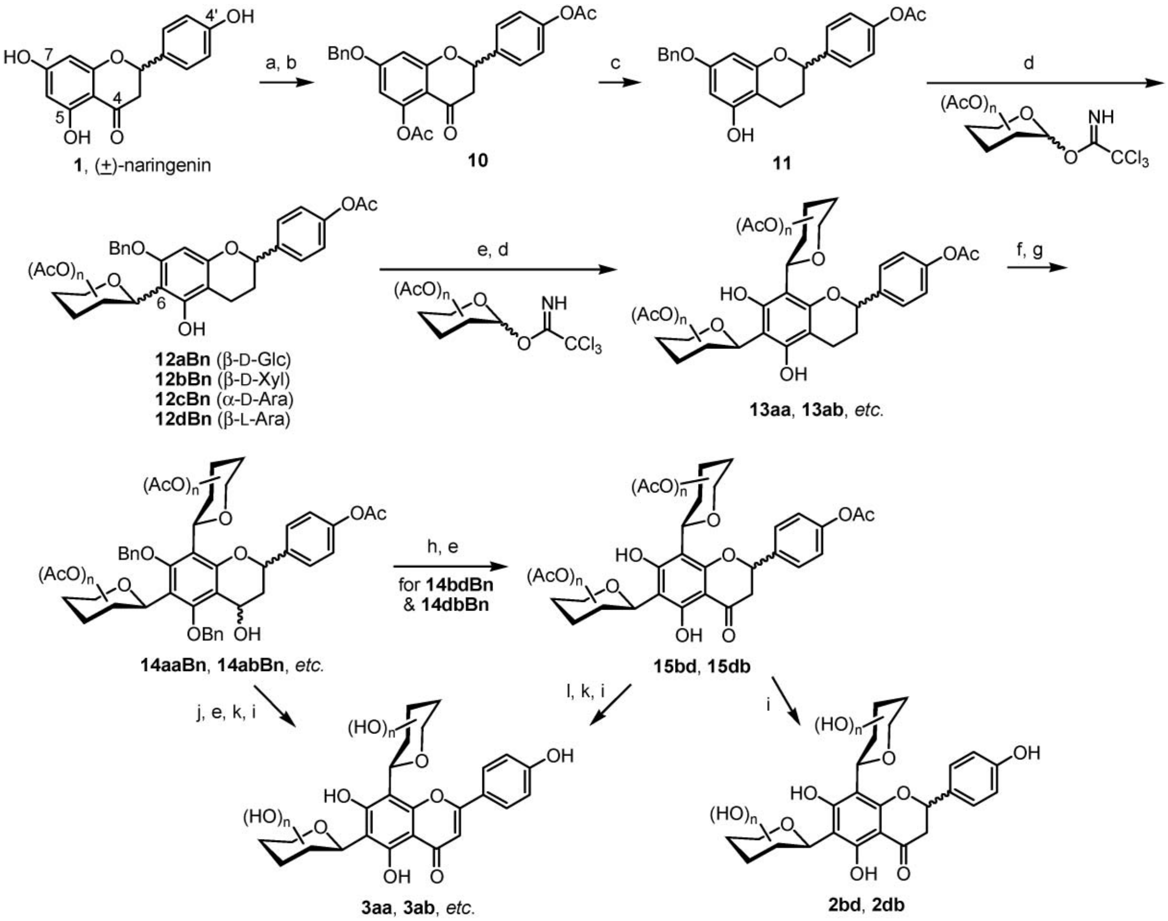
Scheme 1 Synthesis of 6,8-di-*C*-glucosylapigenin by the $Sc(OTf)_3$-promoted di-*C*-glycosylation of (±)-naringenin with D-glucose. *Reagents and conditions*: (a) $Sc(OTf)_3$, EtOH, H_2O, reflux, 16 h; (b) Ac_2O, DMAP, pyridine, 0 to 25 °C, 24 h; (c) DDQ, PhCl, 130 °C, 24 h; (d) NaOMe, MeOH, 25 °C, 1 h.



Scheme 2 Stepwise synthesis of di-*C*-glycosylflavones *via* diglycosylation of phloroacetophenone with D-xylose. *Reagents and conditions*: (a) $Sc(OTf)_3$, EtOH, H_2O, reflux 14 h; (b) BnBr, K_2CO_3, DMF, 25 °C, 12 h; 16% from **4**; (c) KOH, MeOH, 45 °C; 60–70%; (d) HCl, MeOH, reflux, 25 min; (e) H_2, Pd/C, MeOH, EtOAc, 25 °C, 3 h; (f) Ac_2O, pyridine, 25 °C, 12 h; (g) cat. I_2, DMSO, 130 °C, 3 h; (h) Ac_2O, pyridine, 25 °C, 4 h; (i) MeONa, MeOH, 25 °C, 3 h.

6′-alkoxy groups can act as the Michael donors in the acid-catalyzed cyclization, giving two regioisomers of di-*C*-glycosylflavanone. To circumvent this problem, a regioselective synthesis of 6,8-di-*C*-glycosyl flavonoids was thus explored by tandem *C*-glycosylations of flavans to introduce individual glycosyl residues at the designated 6- or 8-positions (Scheme 3).

Comparing the three phenol groups in naringenin, the 5-OH is the least reactive due to its intramolecular bonding with the C-4 carbonyl group, whereas the 7-OH is activated by conjugation with the carbonyl group at the *para* position. Thus, alkylation of (±)-naringenin (**1**) with benzyl bromide (1.3 equiv.) in the presence of K_2CO_3 (1 equiv.) occurred selectively at the most acidic 7-OH group. Flavanone **10** was obtained in 97% overall yield by acetylation of the remaining 4′- and 5-OH groups. Furthermore, flavanone **10** was transformed into flavan **11** in order to avoid difficulty in *C*-glycosylations caused by the electron-withdrawing effect of the C-4 carbonyl group. Flavan **11** was obtained in 90% yield by reduction of **10** with $NaBH_4$ (2.0 equiv.).[21] Deacetylation at C-5 along with removal of the C-4 carbonyl is rationalized.[21] Accordingly, the acetyl group of 5-OAc is readily transferred to the 4-OH group that resulted from an initial reduction of the C-4 carbonyl in flavanone **10**. Such transesterification thus facilitates the elimination of an HOAc molecule to form an *o*-quinonemethide intermediate, which is reduced further by $NaBH_4$ to give the observed flavan product **11**.

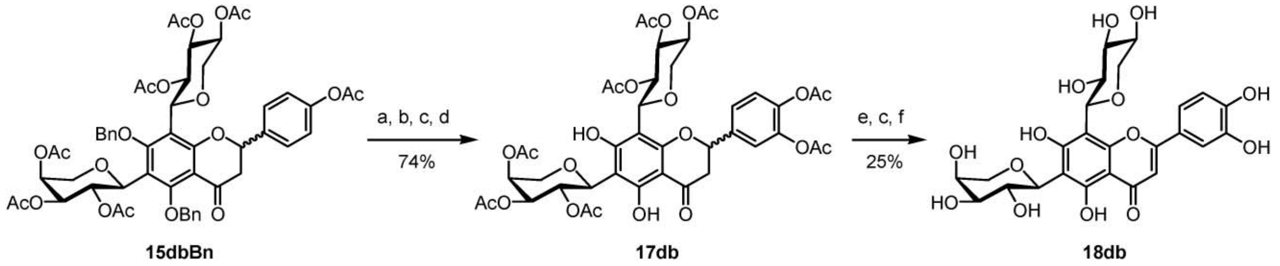


Scheme 3 Synthesis of di-*C*-glycosylapigenins *via* regioselective tandem glycosylations of flavans. *Reagents and conditions*: (a) BnBr, K_2CO_3 (1 equiv.), DMF, 25 °C, 12 h; 75%; (b) Ac_2O, pyridine, DMAP, 25 °C, 4 h; 95% from **1**; (c) $NaBH_4$, THF–H_2O, 0 °C, 45 min; 90%; (d) cat. TMSOTf, CH_2Cl_2, −15 °C (30 min) to 25 °C (3 h); 55–79%; (e) H_2, Pd/C, EtOAc, MeOH, 25 °C, 1 h; 83–92%; (f) BnBr, K_2CO_3, DMF, 50 °C, 5 h; (g) CAN, CH_3CN, H_2O, 25 °C, 2 h; (h) PDC, CH_2Cl_2, reflux, 4 h; (i) NaOMe, MeOH, 25 °C, 12 h; 79–91%; (j) DDQ, PhCl, 140 °C, 24 h; (k) Ac_2O, pyridine, DMAP, 25 °C, 4 h; (l) cat. I_2, Me_2SO, 140 °C, 4 h.

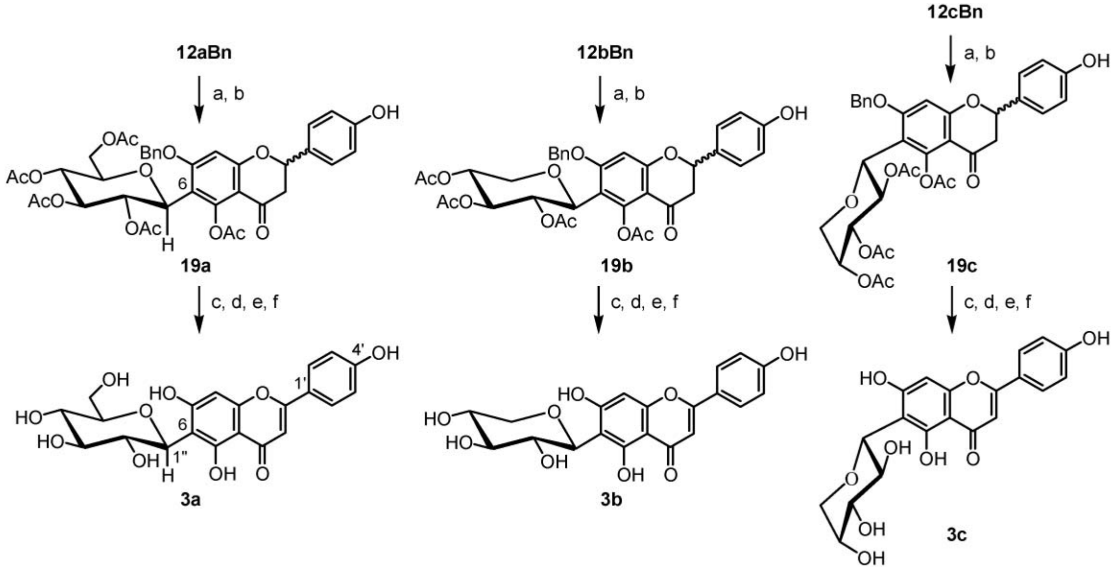
In this synthetic route, D-glucose (series a), D-xylose (series b), D-arabinose (series c) and L-arabinose (series d) were prior elaborated to the corresponding peracetylglycosyl trichloroacetimidates to act as the glycosyl donors. The first *C*-glycosylation of **11** occurred selectively at the C-6 position, giving **12aBn–12dBn**, by using trimethylsilyl trifluoromethanesulfonate (TMSOTf) as the reaction promoter. The regioselective *C*-glycosylation could be attributable to a Fries-type *O*→*C* glycoside rearrangement of the initially formed *O*-glycosylation compound.[22] However, the possibility of direct Friedel–Crafts *C*-glycosylation at the most activated position (C-6) of the aromatic A-ring is not excluded.[22] After removal of the benzyl group in **12aBn–12dBn** by hydrogenolysis, the second *C*-glycosylation with another glycosyl donor, either the same with or different from that in the first glycosylation, proceeded smoothly at C-8 to give a series of 6,8-di-*C*-glycosylflavans (**13aa**, **13ab** and other analogs).

The acetates of 6-*C*-glycosylflavans **12aBn**, **12bBn** and **12cBn** could be oxidized by excess cerium(IV) ammonium nitrate (CAN, see Scheme 5);[23] however, attempts to oxidize 6,8-di-*C*-glycosylflavans (*e.g.* **13aa**) and their peracetylation derivatives failed. Finally, the bis-benzylation derivatives were found to be smoothly oxidized by CAN to give 6,8-di-*C*-glycosylflavanols (*e.g.* **14aaBn**) in good yields. Di-*C*-glycosylflavanols **14bdBn** and **14bdBn** were further oxidized by pyridinium dichromate (PDC) to give the corresponding di-*C*-glycosylflavanones, which were subjected to hydrogenation and saponification to give 6,8-di-*C*-glycosylnaringenins **2bd** and **2db**. On the other hand, di-*C*-glycosylflavanols (*e.g.* **14aaBn**) were directly oxidized by excess amounts of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to afford the corresponding di-*C*-glycosylflavones. After debenzylation, the product was converted to the peracetate derivative for isolation and structural characterization. The final saponification culminated in the desired 6,8-di-*C*-glucosylapigenins (**3aa**). Di-*C*-glycosylflavanones (*e.g.* **15aa**) were also oxidized in I_2/Me_2SO, followed by removal of the protecting groups, to give 6,8-di-*C*-glycosylapigenins (*e.g.* **3aa**). A series of 6,8-di-*C*-glycosylapigenin compounds bearing the same or distinct glycosyl moieties at the designated positions (C-6 and C-8) were thus synthesized by similar procedures (Scheme 3).

Chemical elaboration of the B-ring in 6,8-di-*C*-glycosylflavanone was also feasible by mimicking the enzymatic oxidation of phenol in the biosynthesis of flavonoids.[23c] For example, flavanone **15dbBn** was selectively hydrolyzed by K_2CO_3 (1 equiv.) in CH_2Cl_2–MeOH under mild conditions to expose the



Scheme 4 Elaboration of the B-ring in 6,8-di-*C*-glycosylflavone. *Reagents and conditions*: (a) K_2CO_3 (1 equiv.), CH_2Cl_2–MeOH, 25 °C, 1 h; (b) IBX (2 equiv.), DMSO, 25 °C, 5 h; then Na_2SO_3, 25 °C, 4 h; (c) Ac_2O, pyridine, DMAP, 25 °C, 6 h; (d) H_2, Pd/C, THF–MeOH, 25 °C, 3 h; (e) cat. I_2, DMSO, 140 °C, 4 h; (f) NaOMe, MeOH, 25 °C, 2 h.



Scheme 5 Synthesis of 6-*C*-glycosylflavones that support the structural determination of 6,8-di-*C*-glycosylflavones. *Reagents and conditions*: (a) AcCl, Et_3N, DMAP, CH_2Cl_2, 25 °C, 10 h; (b) CAN, MeCN–AcOH–H_2O, 50 °C, 10 h; (c) cat. I_2, DMSO, 140 °C, 1 h; (d) H_2, Pd/C, 25 °C, 1 h; (e) Ac_2O, pyridine, 25 °C, 10 h; (f) MeONa, MeOH, 25 °C, 5 h.

phenolic group on the B-ring (Scheme 4). The phenolic moiety was then oxidized with 2-iodoxybenzoic acid (IBX),[24] followed by reductive workup of the *o*-quinone intermediate with sodium dithionite ($Na_2S_2O_4$), to give a catechol product. After acetylation and debenzylation, flavanone **17db** was further oxidized with I_2/Me_2SO and isolated as a peracetylated product, which was subjected to saponification to afford 6,8-di-*C*-glycosylluteolin **18db**.

2. Structure elucidation

As shown in the above-delineated schemes, various 6,8-di-*C*-glycosylflavones bearing substituents of D-glucoside, D-xyloside, D-arabinoside and L-arabinoside were synthesized. The substitutions at C-6 and C-8 were confirmed by the disappearance of the signals for H-6 and H-8 in the ^1H NMR spectra. However, structural determination of the sterically demanding molecule of 6,8-di-*C*-glycosylflavone is not trivial at all by NMR analysis because it often exists as a mixture of rotamers. Moreover, *C*-glycosylation of (±)-naringenin would produce 6,8-di-*C*-glycosylnaringenins (*e.g.* **2bd**) and 6,8-di-*C*-glycosylflavans (*e.g.* **13aa**) as mixtures of diastereomers, of which structure elucidation was further complicated by the existence of rotamers. Three 6-*C*-glycosylapigenins **3a**, **3b** and **3c** (Scheme 5) were thus synthesized and fully characterized to provide supporting evidence for the structural assignments of the 6,8-di-*C*-glycosylapigenins.

All the glycosyl substituents in **3a–c** were found to exist in the pyranoside forms with the aglycone in an equatorial position, *i.e.* glucoside and xyloside in the β-configuration, and arabinoside in the α-configuration. The "anomeric" proton (H-1″) in the axial position in compound **3a** occurred at δ_H 4.89 as a doublet with a large coupling constant ($J = 10$ Hz). The structure of **3a** was unambiguously assigned as 6-*C*-(β-D-glucopyranosyl)apigenin because the synthetic sample exhibited the physical and spectral properties ([α], IR, HRMS, ^1H and ^{13}C NMR) consistent with those reported for a natural product, isovitexin.[25] Compound **3b** contained a β-xylopyranoside, rather than furanoside, because the H-1″ at δ_H 4.79 (d, $J = 9.9$ Hz) showed a $^3J_{H,C}$ correlation with the C-5″ at δ_C 70.6 in the HMBC spectrum. The α-arabinopyranoside in **3c** was also inferred from the HMBC correlation of C-5″ (at δ_C 69.8) with the axial H-1″ (at δ_H 4.79, d, $J = 9.8$ Hz).

The ^1H and ^{13}C resonances of the sugar moieties in di-*C*-glycosylflavones were assigned according to their ^1H, ^{13}C, DEPT, COSY, HSQC and HMBC spectra. The α- or β-configurations of sugar residues could be deduced by the coupling constants of the "anomeric" protons H-1″ and H-1‴, if their resonances were

Table 1 Anti-inflammation activities of water-, ethanol-, and methanol-soluble extracts of *Dendrobium huoshanense*

Pretreated extract	Induced TNF-α (%)^{a}	Nitric oxide production (%)^{a}	Cell growth (%)^{b}
DH-H_2O	129.3^{c}/139.2^{d}	ND^{e}/ND^{e}	115^{f}/120^{g}
DH-MeOH	92.7^{c}/66.8^{d}	42.9^{h}	102^{f}/100^{g}
DH-EtOH	88.2^{c}/44.8^{d}	32.2^{h}	94^{f}/94^{g}

[a] The value detected in cells by treatment only with LPS (100 ng mL^{-1}) was defined as 100%. [b] The value detected in cells with no treatment was defined as 100%. [c] The extract (100 µg mL^{-1}) was added prior to treatment with LPS. [d] The extract (250 µg mL^{-1}) was added prior to treatment with LPS. [e] Not determined. [f] The extract (100 µg mL^{-1}) was added to cells for 6 h. [g] The extract (250 µg mL^{-1}) was added to cells for 6 h. [h] The extract (50 µg mL^{-1}) was added prior to treatment with LPS.

distinguishable from other protons. For example, compound **3db**, identical to a natural product isolated from *Viola yedoensis*,[14f] showed the H-1″ and H-1‴ at δ_H 5.03 (d, J = 10 Hz) and 4.78 (d, J = 9.6 Hz), respectively, consistent with the α-configuration of L-arabinoside and the β-configuration of D-xyloside. From time to time, the assignments were also assisted by NMR analysis of the peracetylation derivatives of di-*C*-glycosylflavones. For example, peracetylation of **3aa** (vicenin-2) gave a derivative **3aaAc**,[15] which displayed two axial protons H-1″ and H-1‴ at δ_H 4.78 and 4.55 with large coupling constant (J = 10 Hz) corresponding to the 6β- and 8β-oriented glucosides. Though H-1″ and H-1‴ in **9bb1** were covered by the signals of methanol (small amount often found in CD_3OD solvent), the "anomeric" protons were diagnostic in the peracetylation derivative **9bb1Ac** to deduce the 6β- and 8β-configurations of xylopyranosides. Compound **9bb1Ac** also displayed the correlations of C-1″ (at δ_C 74.2) with H-5″ (at δ_H 4.40) as well as C-1‴ (at δ_C 72.8) with H-5‴ (at δ_H 4.15) to support the assignment of **9bb1** as 6,8-di-*C*-β-D-xylopyranosylluteolin.

3. Biological activities

Flavonoids possess several biological activities such as anti-cancer, antibacterial, anti-inflammatory, immunomodulatory and antioxidants.[14g,26] *D. huoshanense* was claimed to have anti-inflammation activities in traditional Chinese medicinal practice. When various fractions from *D. huoshanense* were tested for anti-inflammation activities in our preliminary studies, we also found that ethanol- or methanol-soluble fractions (DH-EtOH and DH-MeOH, respectively), but not water-soluble fractions (DH-H_2O), can inhibit expression of TNF-α and other inflammatory cytokines in lipopolysaccharide (LPS)-activated RAW264.7 cells (Table 1). In addition to cytokine expression, we also monitored the expression of nitric oxide (NO), which is involved in inflammation and immunoregulation.[27] NO production appeared to decrease upon DH-EtOH and DH-MeOH treatment in LPS-activated cells. The fraction was later identified to contain 6,8-di-*C*-glycosyl flavonoids.[12]

To further dissect the structure-and-activity relationship, mono-glycosyl and di-glycosyl flavonoids were synthesized as mentioned above. The synthesized analogues with various sugars were further tested for their anti-inflammation activities by monitoring TNF-α expression as well as NO production rate. It was found that TNF-α expression in Raw264.7 cells decreased to ~75% on treatment with 50 µM of 6,8-di-*C*-β-D-xylopyranosylluteolin (**9bb1**). Changing the 3′-OH group to methoxy (**9bb2**) or addition of extra methoxy groups (**9bb3**) on the B-ring did not improve the anti-inflammation activities. In contrast, the corresponding peracetates (**9bb1Ac**, **9bb2Ac** and **9bb3Ac**) greatly suppressed TNF-α expression to 27–50%, indicating that acetylation may help the availability of the compounds to the cells.

Table 2 Anti-inflammation activities of apigenin and (di)glycosylapigenins

Compound	C-6	C-8	IC_{50}/µM^{a}	
			TNF-α	NO
Apigenin	H	H	18.5 ± 3.5	19 ± 6.8
3a	D-Glc	H	9.7 ± 3.0	5.2 ± 1.3
3b	D-Xyl	H	35 ± 7.0	11 ± 4.3
3c	D-Ara	H	32 ± 5.6	9.5 ± 0.4
3aa	D-Glc	D-Glc	6.8 ± 2.5	3.9 ± 0.9
3ab	D-Glc	D-Xyl	32 ± 4.2	8.1 ± 0.3
3ac	D-Glc	D-Ara	27.5 ± 2.1	9.9 ± 1.6
3bb	D-Xyl	D-Xyl	19.5 ± 6.3	6.9 ± 1.9
3bc	D-Xyl	D-Ara	24.5 ± 0.7	6.7 ± 0.5
3bd	D-Xyl	L-Ara	>100	>100
3cb	D-Ara	D-Xyl	27 ± 1.4	8.8 ± 0.8
3cc	D-Ara	D-Ara	27 ± 5.6	13 ± 2.1
3db	L-Ara	D-Xyl	>100	>100

[a] Concentration of the indicated compound required for 50% inhibition of TNF-α expression or NO production.

As shown in Table 2, compound **3a**, having a glucose moiety on the apigenin scaffold, but not **3b** or **3c** with xylose or arabinose moieties, increased anti-inflammation activities, as the IC_{50} for TNF-α expression decreased from 18.5 µM (for apigenin) to 9.7 µM. Diglycosylapigenin **3aa** with an extra glucose on the C-8 position further enhanced the potency up to an IC_{50} value of 6.8 µM. Correlating with inhibition of TNF-α expression, both **3a** and **3aa** also inhibited iNOS expression (data not shown) and then NO production, with IC_{50} values of 5.2 and 3.9 µM, respectively.

Conclusion

Three methods were applied to the synthesis of di-*C*-glycosylflavones. The first method (Scheme 1) used a Lewis acid, $Sc(OTf)_3$, to promote the glycosylations of (±)-naringenin with unmodified monosaccharides to give the di-*C*-glycosylation products accompanied by mono-*C*-glycosylation and unidentified compounds. This synthetic procedure is straightforward; however, it is tedious to obtain pure di-*C*-glycosylapigenins by repeated chromatography.

The second method (Scheme 2) for the synthesis of di-*C*-glycosylflavones starts with the $Sc(OTf)_3$-promoted diglycosylation of phloroacetophenone. The subsequent aldol condensations with substituted benzaldehydes gave diglycosylchalcones, which underwent intramolecular Michael reactions to afford a series of 6,8-di-*C*-glycosylflavanones and 6,8-di-*C*-glycosylflavones with various substituents on the B-ring. This method is limited to the synthesis of those compounds bearing the same glycosyl moieties because the intramolecular Michael reactions would lack regiochemical control in cases where the di-*C*-glycosylchalcone bears two different glycosyl moieties.

Finally, the problems encountered in the first and second methods were circumvented by regioselective tandem glycosylations of flavans (Scheme 3). Flavan is more electron-rich than flavanone and flavones, rendering facile *C*-glycosylation. To an appropriate flavan, the first glycosylation was introduced to the C-6 position, and the second glycosylation with the same or distinct glycosyl donor occurred at the C-8 position. The prepared 6,8-di-*C*-glycosylflavans were then elaborated to a series of 6,8-di-*C*-glycosylflavones by modification at the B- and C-rings.

The prepared 6,8-di-*C*-glycosylflavones were found to exhibit anti-inflammation activities by inhibiting TNF-α expression and NO production. Introduction of glucosyl moieties as in **3aa** improved the anti-inflammation activities compared with the parent compound of apigenin. Our study also indicates that acetylation may help the availability of the compounds (*e.g.* **9bb1Ac**) to suppress TNF-α expression.

Experimental

General

All the reagents and solvents were reagent grade and were used without further purification unless otherwise specified. All solvents were anhydrous grade unless indicated otherwise. All non-aqueous reactions were carried out in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel using aqueous *p*-anisaldehyde as visualizing agent. Silica gel (0.040–0.063 mm particle sizes) and LiChroprep RP-18 (0.040–0.063 mm particle sizes) were used for column chromatography. Flash chromatography was performed on silica gel of 60–200 μm particle size. Molecular sieves were activated under high vacuum at 220 °C over 6 h.

Melting points were recorded on a Yanaco or Electrothermal MEL-TEMP® 1101D apparatus in open capillaries and are not corrected. Infrared (IR) spectra were recorded on Nicolet Magna 550-II or Thermo Nicolet 380 FT-IR spectrometers. Nuclear magnetic resonance (NMR) spectra were obtained on Varian Unity Plus-400 (400 MHz) or Bruker AVANCE (400 and 600 MHz) spectrometers. Chemical shifts (δ) are given in parts per million (ppm) relative to δ_H 7.26/δ_C 77.0 (central line of t) for $CHCl_3/CDCl_3$, δ_H 4.80 for H_2O/D_2O, δ_H 3.31/δ_C 48.2 for CD_3OD-d_4, or δ_H 2.49/δ_C 39.5 for DMSO-d_6. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (double of doublets) and br (broad). Coupling constants (*J*) are given in Hz. Distortionless enhancement polarization transfer (DEPT) spectra were taken to determine the types of carbon signals. The ESI-MS experiments were conducted on a Bruker Daltonics BioTOF III high-resolution mass spectrometer. Optical rotations were measured on digital polarimeter of Japan JASCO Co. DIP-1000. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}.

Representative synthetic procedures

3,5-Di-(*C*-β-D-xylopyranosyl)acetophenone (5bb). A mixture of phloroacetophenone (**4**, 372 mg, 2 mmol), D-xylose (900 mg, 6 mmol), and $Sc(OTf)_3$ (197 mg, 0.4 mmol) in EtOH (6 mL)/H_2O (3 mL) was heated at reflux for 14 h. The reaction mixture was cooled, and concentrated under reduced pressure. The residue was subject to column chromatography on silica gel (acetone–EtOAc–H_2O–HOAc, 15 : 30 : 2 : 1 to 30 : 30 : 5 : 1) to give a crude sample of 3,5-di-*C*-β-D-xylopyranosylphloroacetophenone (**5bb**).

For analytical purposes, the crude sample of **5bb** was treated with Ac_2O (5 mL) in pyridine (5 mL) for 12 h at room temperature. The reaction mixture was partitioned between 1 M HCl and EtOAc. The organic phase was washed with 1 M HCl and brine, dried over anhydrous $MgSO_4$, filtered, and then concentrated by rotary evaporation. The residue was purified by column chromatography on silica gel (EtOAc–hexane, 1 : 1 to 1.5 : 1) to give **5bbAc** (320 mg, 20%).

To a stirred solution of compound **5bbAc** (188 mg, 0.23 mmol) in dry methanol (6 mL) was added dropwise sodium methoxide (60 mg, 1.1 mmol) in dry methanol (3 mL). The resulting solution was stirred at room temperature for 1 h. Dowex 50W×8 (H^+) was added to the stirred reaction mixture until the solution became neutral. The resulting mixture was filtered and washed with methanol. The filtrate was evaporated *in vacuo*, and washed by ether and hexane to give compound **5bb** (91 mg, 92%). $C_{18}H_{24}O_{12}$; colorless solid, mp 154–155.5 °C; TLC (Me_2CO–EtOAc–H_2O–HOAc, 30 : 30 : 5 : 1) R_f 0.25; ^{1}H NMR (CD_3OD, 400 MHz) δ 4.81 (2 H, d, J = 10 Hz, α-anomeric H), 4.08 (2 H, dd, J = 11.6, 5.6 Hz), 3.70–3.60 (4 H, m), 3.44 (2 H, t, J = 9 Hz), 3.37–3.30 (2 H, m), 2.64 (3 H, s); ^{13}C NMR (CD_3OD, 100 MHz) δ 205.5, 163.4 (2 ×), 162.6, 106.5, 104.3 (2 ×), 79.4 (2 ×), 77.7 (2 ×), 74.2 (2 ×), 71.8 (2 ×), 71.4 (2 ×), 33.4; HRMS (ESI) calcd for $C_{18}H_{23}O_{12}$: 431.1190, found: m/z 431.1195 $[M - H]^-$.

2,4,6,3′,4′-Pentabenzyloxy-3,5-di-*C*-(β-D-xylopyranosyl)chalcone (7bb1). To a solution of crude **5bb** (112 mg, 0.26 mmol) in dry DMF (3 mL) were added $PhCH_2Br$ (200 mg, 1.17 mmol) and K_2CO_3 (161 mg, 1.17 mmol). The mixture was stirred at room temperature for 12 h, and complete consumption of **5bb** was shown by TLC analysis. The mixture was filtered, concentrated by evaporation under reduced pressure, and purified by column chromatography on silica gel ($CHCl_3$–MeOH, 8 : 1) to give **5bbBn** (160 mg, 88%).

Potassium hydroxide (200 mg, 3.6 mmol) was added to a solution of **5bbBn** (280 mg, 0.4 mmol) and 3,4-dibenzyloxybenzaldehyde (380 mg, 1.2 mmol) in MeOH (5 mL). The solution was stirred at 45 °C for 24 h. After confirming the disappearance of **5bbBn** by TLC analysis, 1 M HCl was added to neutralize the reaction mixture. The volatiles were removed by rotary evaporation under reduced pressure, and the residue was partitioned between water and EtOAc. The organic phase was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation. The residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 13 : 1) to give **7bb1** (245 mg, 61%). $C_{60}H_{58}O_{14}$; yellow prisms, mp 121.8–123.8 °C; TLC ($CHCl_3$–MeOH, 10 : 1) R_f 0.13; $[\alpha]^{25}_D$ −38.08 (*c* 2.1, EtOAc); IR ν_{max} (neat) 3400, 2921, 1576, 1508, 1454, 1268, 1134, 1085 cm^{-1}; ^{1}H NMR (a mixture of rotamers, $CDCl_3$, 400 MHz) δ 7.44–7.02 (27 H, m), 6.96 (1 H, d, J = 8.4 Hz), 6.79 (2 H, dd, J = 12.2, 3.8 Hz), 5.09 (2 H, s), 5.04 (2 H, s), 4.95 (2 H, t, J = 12.4 Hz), 4.78–4.66 (4 H, m), 4.55–4.49 (2 H, m), 4.22 (1 H, t, J = 9.2 Hz), 4.08 (1 H, t, J = 8.8 Hz), 3.87 (2 H, d, J = 6 Hz), 3.36–3.32 (1 H, m), 3.25–3.16 (3 H, m), 3.14–3.06 (2 H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 193.9, 160.9, 157.6, 157.0, 151.3, 148.5, 146.2, 136.5, 136.3, 136.1, 128.4–127.0 (5 ×), 125.5, 124.0, 123.3, 122.2, 113.8, 79.8 (2×), 78.9 (2×), 78.4 (2×), 75.9 (2×), 75.7 (2×), 71.2, 71.1, 70.7

(2 ×), 69.9; HRMS (ESI) calcd for $C_{60}H_{59}O_{14}$: 1003.3905, found: *m*/*z* 1003.3917 $[M + H]^+$.

5,7,3′,4′-Tetrahydroxy-6,8-di-*C*-(β-D-xylopyranosyl)flavanone (8bb1). A solution of **7bb1** (153 mg, 0.152 mmol) in concentrated HCl (1.5 mL) and MeOH (3 mL) was heated at reflux for 25 min. After the consumption of **7bb1** as shown by TLC analysis, the reaction mixture was concentrated by rotary evaporation under reduced pressure. The residue dissolved in MeOH–EtOAc (1 : 1, 6 mL) was vigorously stirred with 10% Pd–C (40 mg) under an H_2 atmosphere at room temperature for 2 h. The mixture was filtered through a Celite pad, and rinsed with MeOH. The filtrate was concentrated, and purified by column chromatography on silica gel (acetone–EtOAc–H_2O–HOAc, 15 : 30 : 2 : 1) to give **8bb1** (62 mg, 74%). $C_{25}H_{28}O_{14}$; TLC (Me_2CO–EtOAc–H_2O–HOAc, 30 : 30 : 5 : 1) R_f 0.25; ^1H NMR (CD_3OD, 400 MHz) δ 6.95 (1 H, d, J = 8.8 Hz), 6.84–6.77 (2 H, m), 5.35–5.30 (1 H, m), 4.80–4.70 (2 H, m), 4.03–3.93 (4 H, m), 3.69–3.56 (2 H, m), 3.50–3.22 (4 H, m), 3.11–3.02 (1 H, m), 2.83–2.71 (1 H, m); HRMS (ESI) calcd for $C_{25}H_{28}O_{14}Na$: 575.1371, found: *m*/*z* 575.1379 $[M + Na]^+$.

6,8-Di-*C*-(β-D-xylopyranosyl)-5,7,3′,4′-tetrahydroxyflavone (9bb1). A solution of **8bb1** (200 mg, 0.36 mmol) in Ac_2O (6 mL) and pyridine (6 mL) was stirred 12 h at room temperature. The mixture was partitioned between 1 M HCl and EtOAc. The organic phase was washed with 1 M HCl and brine, dried over anhydrous $MgSO_4$, filtered, and then concentrated by rotary evaporation. The residue was purified by column chromatography on silica gel (EtOAc–hexane, 1 : 2 to 1 : 1) to give **8bb1Ac** (260 mg) as a diastereomeric mixture.

A solution of flavanone **8bb1Ac** (56 mg, 0.057 mmol) and I_2 (4.4 mg, 0.017 mmol) in DMSO (4 mL) was stirred at 130 °C for 3 h. The mixture was cooled, quenched by addition of $Na_2S_2O_{3(aq)}$, and partitioned with water and EtOAc. The organic phase was washed with water and brine, dried over anhydrous $MgSO_4$, and filtered. After the volatiles were removed by rotary evaporation under reduced pressure, the residue dissolved in pyridine (3 mL) and Ac_2O (3 mL) was stirred 3 h at room temperature. The mixture was partitioned between 1 M $HCl_{(aq)}$ and EtOAc. The organic phase was washed with 1 M HCl and brine, dried over anhydrous $MgSO_4$, filtered, and then concentrated by rotary evaporation. The residue was purified by column chromatography on silica gel (EtOAc–hexane, 1 : 1 to 1.5 : 1) to give **9bb1Ac** (48 mg, 87%).

To a stirred solution of **9bb1Ac** (9 mg, 0.009 mmol) in dry methanol (3 mL) was added dropwise a solution of sodium methoxide (10 mg, 0.18 mmol) in dry methanol (1 mL). The mixture was stirred at room temperature for 3 h, and neutralized by addition of Dowex 50W×8 (H^+). The mixture was filtered and washed with methanol. The filtrate was evaporated *in vacuo*, washed by ether and hexane to give **9bb1** (4.5 mg, 90%). $C_{25}H_{26}O_{14}$; colorless solid, mp 203–205 °C; TLC (acetone–EtOAc–H_2O–HOAc, 30 : 30 : 5 : 1) R_f 0.24; IR v_{max} (KBr) 3399, 2922, 2856, 1645, 1625, 1578, 1440, 1351, 1299, 1221, 1087, 1056 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.42 (1 H, d, J = 7.8 Hz), 7.41 (1 H, s), 6.92 (1 H, d, J = 7.8 Hz), 6.57 (1 H, s), 4.85 (2 H, covered by the signal of methanol), 4.14–3.98 (4 H, m), 3.81 (1 H, br s), 3.71–3.65 (2 H, m), 3.54–3.42 (3 H, m); ^{13}C NMR (CD_3OD, 100 MHz) δ 182.7, 165.1, 161.6, 159.9, 155.4, 149.7, 145.6, 122.5, 119.5, 115.5, 113.3, 107.6, 104.2, 102.8 (2 ×), 78.8, 78.6, 75.4, 72.1, 70.7 (2 ×), 70.6 (2 ×), 70.2 (2 ×); HRMS (ESI) calcd for $C_{25}H_{25}O_{14}$: 549.1244, found: *m*/*z* 549.1247 $[M - H]^-$.

4′-Acetoxy-7-benzyloxy-5-hydroxyflavan (11). A suspension mixture of (±)-naringenin (**1**,7.5 g, 27.6 mmol) and K_2CO_3 (3.82 g, 27.6 mmol) in anhydrous DMF (100 mL) was stirred at room temperature for 10 min, and then benzyl bromide (4.3 mL, 35.8 mmol) was added dropwise. The mixture was warmed to room temperature, and stirred for 12 h at room temperature. The reaction was quenched by addition of saturated aqueous NH_4Cl, and the mixture was concentrated *in vacuo*. The residual solid was washed with water and evaporated to dryness under reduced pressure. The crude product (~10.9 g) was used in the next step without further purification.

A solution of the above-prepared crude product and Ac_2O (20 mL) in pyridine (30 mL) was treated with 4-dimethylaminopyridine (DMAP, 60 mg, 0.5 mmol). The solution was stirred for 4 h at room temperature; the mixture was diluted with EtOAc and washed with 1 M HCl. After neutralization with saturated aqueous $NaHCO_3$, the organic layer was dried over anhydrous $MgSO_4$, filtrated and concentrated. The residue was purified by flash column chromatography (EtOAc–hexane, 1 : 4 to 1 : 2.5) to afford **10** (11.7 g, 95% for two steps).

To a solution of **10** (8.58 g, 19.32 mmol) in THF (70 mL) and H_2O (35 mmol) was slowly added $NaBH_4$ (1.47 g, 38.64 mmol) at 0 °C. The mixture solution was stirred for 45 min at 0 °C, and then quenched by addition of saturated aqueous NH_4Cl. The organic layer was separated and the aqueous layer was extracted with EtOAc (5 ×). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography (20% to 30% EtOAc in hexane) to afford **11** (6.75 g, 90%). $C_{24}H_{22}O_5$; colorless solid; mp 165.5–167 °C; TLC (EtOAc–hexane, 3 : 7) R_f 0.3; IR (film) 3360, 2928, 1732, 1655, 1129 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 7.41–7.28 (7 H, m), 7.08 (2 H, d, J = 8.5 Hz), 6.17 (1 H, d, J = 2.4 Hz), 6.06 (1 H, d, J = 2.4 Hz), 4.98–4.96 (3 H, m), 4.81 (1 H, s, OH), 2.72–2.63 (2 H, m), 2.29 (3 H, s), 2.21–2.18 (1 H, m), 2.03–1.97 (1 H, m); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.5, 158.4, 156.6, 154.5, 150.2, 139.0, 136.9, 128.5 (2 ×), 128.4, 127.9, 127.4 (2 ×), 127.2 (2 ×), 121.6 (2 ×), 102.0, 95.5, 95.3, 70.0, 29.4, 21.1, 18.9; HRMS calcd for $C_{24}H_{23}O_5$: 391.1545, found: *m*/*z* 391.1549 $[M + H]^+$.

4′-Acetoxy-6-*C*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-7-benzyloxy-5-hydroxyflavan (12aBn). To a solution of flavan **11** (1.95 g, 5 mmol) and 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl trichloroacetimidate (2.71 g, 5.5 mmol) in anhydrous CH_2Cl_2 (40 mL) at −15 °C was added dropwise trimethylsilyl triflate (110 μL, 0.5 mmol). The mixture solution was stirred for 30 min, and then warmed to room temperature within 3 h. The reaction was quenched by addition of saturated aqueous $NaHCO_3$ (15 mL), and the mixture was extracted with EtOAc (5×). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by flash column chromatography (30% EtOAc in hexane) to afford **12aBn** as a white foam (2.81 g, 79%), which contained an inseparable mixture of diastereomers (existing as rotamers) as shown by the ^1H and ^{13}C NMR spectra. TLC (EtOAc–hexane, 1 : 2) R_f 0.35; IR (film) 3512, 2942, 1752, 1623, 1321, 1229 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 7.51 (1 H, br s), 7.37–7.29 (7 H, m), 7.06–7.04 (2 H, m), 6.06 (0.5 H, s), 6.04 (0.5 H, s), 5.39–5.37 (1 H, m), 5.32–5.24 (2 H, m), 5.20–5.18 (1 H, m), 4.94–4.85 (3 H, m), 4.28–4.25 (1 H, m), 4.10–4.08 (1 H, m), 3.81 (1 H, d, *J* = 9.8 Hz), 2.78–2.75 (1 H, m), 2.63–2.61 (1 H, m), 2.24 (3 H, s), 2.13–2.97 (11 H, m), 1.77 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.6, 170.3, 169.4, 169.0, 168.9, 156.88/156.87, 155.26/155.18, 155.13/155.08, 150.26/150.23, 139.0, 136.8, 128.7 (2 ×), 128.0, 127.29/127.24 (2 ×), 127.1/127.0 (2 ×), 121.6 (2 ×), 104.2, 101.6/101.5, 92.9/92.8, 77.5/77.3, 76.1/76.0, 74.1, 73.94/73.90, 70.49/70.46, 70.34/70.31, 67.9, 61.49/61.45, 29.5/29.3, 21.1, 20.79, 20.6, 20.5, 20.4/20.3, 19.2/19.0; HRMS calcd for $C_{38}H_{40}NaO_{14}$: 743.2316, found: *m*/*z* 743.2325 [M + Na]$^+$.

4′-Acetoxy-6,8-di-*C*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-5,7-dihydroxyflavan (13aa). Compound **12aBn** (720 mg, 1 mmol) was subjected to hydrogenolysis on Pd/C (10%, 50 mg) in CH_3OH (10 mL)/EtOAc (10 mL) for 1 h at room temperature under an atmosphere of hydrogen. The mixture was filtered through Celite; the filtrate was concentrated to yield a crude product, which was chromatographed on a short silica gel column (EtOAc–hexane (1 : 1)) to afford **12a** (580 mg, 92%) containing an inseparable mixture of diastereomers (existing as rotamers) as shown by the ^{1}H and ^{13}C NMR spectra.

To a solution of monoglycosylflavan **12a** (300 mg, 0.47 mmol) and 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl trichloroacetimidate (296 mg, 0.6 mmol) in anhydrous CH_2Cl_2 (25 mL) at −15 °C was added dropwise trimethylsilyl triflate (6 μL, 0.024 mmol). The mixture solution was stirred for 30 min, and then warmed to room temperature over a period of 3 h. The reaction was quenched by addition of saturated aqueous $NaHCO_3$ (15 mL), and the mixture was extracted with EtOAc (5×). The combined organic extracts were dried over $MgSO_4$ and evaporated *in vacuo*. The crude product was purified by flash column chromatography (EtOAc–hexane (2 : 5)) to give the di-*C*-glycosylflavan **13aa** (347 mg, 77%), which contained a diastereomeric mixture (existing as rotamers) as shown by the ^{1}H NMR spectrum. $C_{45}H_{52}O_{23}$; colorless foam; TLC (EtOAc–hexane, 1 : 1) R_f 0.40; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.43 (1 H, d, *J* = 8.2 Hz), 7.39–7.36 (3 H, m), 7.13–7.10 (2 H, m), 5.44–5.18 (6 H, m), 5.15–4.76 (2.5 H, m), 4.32–4.28 (2 H, m), 4.14–3.98 (2.5 H, m), 3.91–3.81 (2 H, m), 2.82–2.74 (1 H, m), 2.65–2.56 (1 H, m), 2.31–1.81 (29 H, 9 × OAc; C_3–H_a and H_b); HRMS calcd for $C_{45}H_{52}NaO_{23}$ (M^+ + Na): 983.2797, found: *m*/*z* 983.2800.

4′-Acetoxy-6,8-di-*C*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-5,7-dibenzyloxy-4-hydroxyflavan (14aaBn). A mixture of **13aa** (347 mg, 0.36 mmol), benzyl bromide (170 mg, 1.08 mmol) and K_2CO_3 (174 mg, 1.26 mmol) in anhydrous DMF (6 mL) was heated at 50 °C for 5 h. The mixture was cooled to room temperature, diluted with EtOAc (10 mL), and filtered. The filtrate was concentrated by rotary evaporation under reduced pressure, and the residue was partitioned with EtOAc (20 mL) and water (8 mL). The organic layer was separated, washed with water (8 mL), dried over $MgSO_4$, filtered and concentrated by rotary evaporation under reduced pressure.

The crude product of **13aaBn** was dissolved in CH_3CN–water (5 : 1, 24 mL) and stirred with cerium(IV) ammonium nitrate (CAN, 6 mmol) for 2 h at room temperature. The mixture was partitioned between EtOAc (50 mL) and water (20 mL). The aqueous layer was extracted with EtOAc (3×). The combined organic extracts were dried over $MgSO_4$, concentrated, and purified by flash column chromatography (EtOAc–hexane (45 : 55)) to give **14aaBn** (296 mg, 71% for two steps), which contained a diastereomeric mixture (existing as rotamers) as shown by the ^{1}H and ^{13}C NMR spectra. $C_{59}H_{64}O_{24}$; pale yellow foam; TLC (EtOAc–hexane, 1 : 1) R_f 0.30; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.63–7.35 (12 H, m), 7.18–7.15 (2 H, m), 6.09–5.86 (1.5 H, m), 5.46–5.18 (3.5 H, m), 5.15–5.00 (4.8 H, m), 4.98–4.71 (5.2 H, m), 4.27–3.81 (4.5 H, m), 3.75–3.56 (1 H, m), 3.36–3.28 (0.5 H, m), 2.32–1.67 (29 H, 9 × OAc; C_3–H_a and H_b); HRMS calcd for $C_{59}H_{64}NaO_{24}$: 1179.3685, found: *m*/*z* 1179.3692 [M + Na]$^+$.

4′-Acetoxy-6-*C*-(tri-*O*-acetyl-β-D-xylopyranosyl)-8-*C*-(tri-*O*-acetyl-α-L-arabinopyranosyl)-5,7-di-hydroxyflavanone (15bd). A solution of **14bdBn** (773 mg, 0.763 mmol) and PDC (1.15 g, 3.052 mmol) in CH_2Cl_2 (30 mL) was heated at reflux for 4 h. The mixture was concentrated by rotary evaporation, filtered through a short silica pad, and rinsed with EtOAc. The filtrate was concentrated; the residue was rinsed with Et_2O–hexane to afford **15bdBn** (760 mg, 98%) as an inseparable mixture of diastereomers (existing as rotamers).

A solution of **15bdBn** (223 mg, 0.22 mmol) in EtOAc–CH_3OH (1 : 1, 10 mL) was subjected to hydrogenolysis by vigorously stirring with 10% Pd/C (50 mg) under an atmosphere of H_2 at room temperature for 2 h. The mixture was filtered through a pad of Celite, rinsed with CH_3OH, and concentrated under reduced pressure. The residue was rinsed with Et_2O–*n*-pentane to give **15bd** (172 mg, 99%) as an inseparable mixture of diastereomers (existing as rotamers). $C_{39}H_{42}O_{20}$; white prisms, mp 174–176 °C; TLC (EtOAc–hexane, 3 : 2) R_f 0.35; IR ν_{max} (neat) 3308, 2924, 1749, 1632, 1370, 1220 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.76 (1 H, br, OH), 8.76 (1 H, br, OH), 7.68 (0.7 H, d, *J* = 8 Hz), 7.40 (1.3 H, d, *J* = 8 Hz), 7.18 (2 H, d, *J* = 8 Hz), 6.00–5.89 (1 H, m), 5.64 (1 H, d, *J* = 11.6 Hz), 5.43–5.24 (3 H, m), 5.14–4.86 (4 H, m), 4.30–4.23 (1 H, m), 4.14–3.97 (1 H, m), 3.81–3.71 (1 H, m), 3.51–3.41 (1 H, m), 3.17–2.72 (2 H, m), 2.33 (3 H, s), 2.27 (3 H, s), 2.05 (3 H, s), 2.03 (3 H, s), 1.99 (3 H, s), 1.88 (3 H, s), 1.85 (3 H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 196.2/194.9, 170.1, 169.8, 169.6, 169.3, 168.8, 168.5, 168.1, 163.2/162.5, 160.9, 158.8, 150.4, 135.4/135.2, 127.9/126.3 (2 ×), 121.9/121.5 (2 ×), 104.1, 103.0, 101.9/100.9, 78.7, 74.1, 73.4, 72.6/72.2, 71.4, 71.0, 69.9/69.0, 68.6/68.3, 67.9, 67.4/67.1, 66.9/66.3, 43.5/42.4, 20.8 (2 ×), 20.4 (3 ×), 20.3, 20.0; HRMS (ESI) calcd for $C_{39}H_{41}O_{20}$: 829.2191, found: *m*/*z* 829.2195 [M − H]$^-$.

6-*C*-β-D-xylopyranosyl-8-*C*-α-L-arabinopyranosyl-4,5,7-trihydroxyflavanone (2bd). To a solution of **15bd** (60.6 mg, 0.073 mmol) in CH_3OH (5 mL) was added sodium methoxide (50 mg, 0.93 mmol) at room temperature. The mixture was stirred for 2 h, neutralized with Dowex 50 W × 8 (H^+), filtered and evaporated *in vacuo*. The residue was washed with CH_2Cl_2 and Et_2O to give **2bd** (35 mg, 89%) as an inseparable diastereomeric mixture (existing as rotamers). $C_{25}H_{28}O_{13}$; yellow prisms, mp 192–193.5 °C; IR ν_{max} (neat) 3364, 2913, 1625, 1518, 1455, 1342, 1206, 1088 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.35 (2 H, d, *J* = 8.4 Hz), 6.80 (2 H, d, *J* = 8.4 Hz), 5.35 (1 H, d, *J* = 12.8 Hz), 4.72 (1 H, d, *J* = 10.4 Hz), 4.68 (1 H, d, *J* = 9.2 Hz), 4.20 (1 H, br), 4.04 (1 H, t, *J* = 9.2 Hz), 3.95–3.90 (3 H, m), 3.63–3.60 (2 H, m), 3.54 (1 H, dd, *J* = 9.2, 2.4 Hz), 3.37 (1 H, t, *J* = 9.2 Hz), 3.30–3.24 (1 H, m), 3.00 (1 H, dd, *J* = 17.2, 12.8 Hz), 2.72 (1 H, dd, *J* = 17.2, 1.6 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 198.6, 165.6, 163.9, 161.7, 158.8, 130.9, 129.1/128.9 (2 ×), 116.3 (2 ×), 106.4, 105.2, 103.1, 80.7, 80.4, 76.9, 75.8, 75.3, 72.2, 71.7, 71.6, 71.5, 71.1, 70.4, 44.4; HRMS (ESI) calcd for $C_{25}H_{27}O_{13}$: 535.1452, found: *m/z* 535.1453 $[M - H]^-$.

6,8-Di-*C*-(β-D-glucopyranosyl)-5,7,4′-trihydroxyflavone (vicenin-2) (3aa)[15,17]. A solution of **14aaBn** (231 mg, 0.2 mmol) and DDQ (227 mg, 1 mmol) in chlorobenzene (10 mL) was stirred for 24 h at 140 °C. The reaction mixture was evaporated *in vacuo* and the residue was partitioned between EtOAc (20 mL) and saturated aqueous $NaHCO_3$ (20 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated to give a crude diglycosylapigenin derivative (210 mg), which was subjected to hydrogenolysis by stirring with Pd/C (30 mg) in CH_3OH (10 mL)/EtOAc (10 mL) for 1 h at room temperature under an atmosphere of hydrogen. The mixture was filtered through Celite, and the filtrate was concentrated to yield the crude debenzylated product (181 mg) as a pale yellow solid.

To a solution of this debenzylated crude product in pyridine (5 mL) was added Ac_2O (5 mL). The mixture was stirred for 10 h, and then concentrated under reduced pressure. The residue was partitioned between EtOAc (30 mL) and water (20 mL). The organic layer was washed with 1 M aqueous HCl (10 mL) and water (10 mL). After neutralization with saturated aqueous $NaHCO_3$, the organic layer was dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (60% EtOAc in hexane) to afford **3aaAc** (142 mg, 66% overall yield).

A solution of compound **3aaAc** (142 mg, 0.13 mmol) in dry MeOH (5 mL) was treated with 30 wt% methanolic solution of NaOMe (0.5 mL). The mixture was warmed to room temperature and continuous stirred for 12 h, the mixture was then neutralized with Amberlite IR-120 (H^+), filtered, and rinsed with methanol. The filtrate was concentrated by rotary evaporation under reduced pressure, and the residue was washed with Et_2O to afford **3aa** (66 mg, 85%). $C_{27}H_{31}O_{15}$; pale-yellow powder, mp > 250 °C; $[\alpha]_D^{20}$ = +53.2 (*c* = 0.5, MeOH) [lit.[15] $[\alpha]_D^{21}$ = +56.9 (*c* = 0.745, MeOH)]; IR (film) 3351, 2923, 2872, 1657, 1623, 1501 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6, at 90 °C) δ 13.6 (1 H, br s), 13.5 (1 H, br s), 9.20 (1 H, br s), 7.95 (2 H, d, *J* = 8.6 Hz), 6.94 (2 H, d, *J* = 8.6 Hz), 6.69 (1 H, s), 4.89 (1 H, br d, *J* > 7.3 Hz, H-1‴$_{ax}$, 8β-Glc), 4.80 (1 H, d, *J* = 9.7 Hz, H-1″$_{ax}$, 6β-Glc), 3.77 (1 H, d, *J* = 11.8 Hz), 3.74 (1 H, d, *J* = 11.8 Hz), 3.72–3.68 (2 H, m), 3.64–3.58 (2 H, m), 3.44 (1 H, d, *J* = 9.2 Hz), 3.40–3.32 (5 H, m); ^{13}C NMR (150 MHz, DMSO-d_6, at 50 °C) δ 181.6, 163.5, 160.6, 160.1, 157.9, 154.5, 128.3 (2 ×), 120.8, 115.2 (2 ×), 106.8, 104.6, 103.3, 101.9, 81.2, 80.3, 78.2, 77.2, 73.4, 72.7, 71.3, 70.3/70.0, 69.1, 68.5, 60.7, 59.2; HRMS calcd for $C_{27}H_{31}O_{15}$: 595.1663, found: *m/z* 595.1668 $[M + H]^+$.

3′,4′-Di-acetoxy-6-*C*-(tri-*O*-acetyl-α-L-arabinopyranosyl)-8-*C*-(tri-*O*-acetyl-α-D-xylopyranosyl)-5,7-di-hydroxyflavanone (17db). Under an atmosphere of argon, a solution of **15dbBn** (63 mg, 0.062 mmol) in CH_2Cl_2–CH_3OH (1 : 2, 6 ml) was stirred with K_2CO_3 (8.6 mg, 0.062 mmol) at room temperature for 1 h. The mixture was neutralized with Dowex 50 W × 8 (H^+), filtered and concentrated under reduced pressure. The residue was treated with 2-iodoxybenzoic acid (IBX, 35 mg, 0.124 mmol) in DMSO (2 mL) at room temperature for 5 h. As the reaction progressed, the color changed from a translucent yellow solution to an opaque brown solution. Sodium dithionite ($Na_2S_2O_4$, 51 mg, 0.29 mmol) was added, and the mixture was stirred for an additional 4 h. The mixture was quenched by addition of H_2O (20 mL), and then partitioned between 1 M $HCl_{(aq)}$ and EtOAc (3 × 10 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure.

The crude product having a benzenediol moiety was treated with Ac_2O (3 mL) in pyridine (5 mL) and DMAP (10 mg, 0.08 mmol) at room temperature for 6 h. The mixture was quenched with CH_3OH, concentrated under reduced pressure, and partitioned between 1 M $HCl_{(aq)}$ and EtOAc. After neutralization with saturated aqueous $NaHCO_3$, the organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated. The residual solid was subjected to hydrogenolysis under an atmosphere of H_2 by vigorous stirring with 10% Pd/C (20 mg) in THF–CH_3OH (2 : 1, 30 mL) at room temperature for 3 h. The mixture was filtered through a pad of Celite, rinsed with CH_3OH and concentrated. The residue was purified by column chromatography on silica gel (EtOAc–hexane, 2 : 3 to 1 : 1) to afford **17db** as a mixture of diastereomers (41 mg, 74% overall yield).

$C_{41}H_{44}O_{22}$; white prisms, mp 166–168 °C; TLC (EtOAc–hexane, 3 : 2) R_f 0.26; IR ν_{max} (neat) 3246, 2916, 1731, 1637, 1374, 1218 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.56 (1 H, br, OH), 8.70 (1 H, br, OH), 7.40–7.26 (3 H, m), 5.75 (1 H, br), 5.67 (1 H, d, *J* = 12 Hz), 5.41 (2 H, br), 5.31–5.22 (2 H, m), 5.14 (1 H, d, *J* = 9.2 Hz), 4.97 (1 H, t, *J* = 10 Hz), 4.89 (1 H, d, *J* = 10.4 Hz), 4.21 (1 H, dd, *J* = 10.4, 5.2 Hz), 4.10 (1 H, d, *J* = 12 Hz), 3.81 (1 H, d, *J* = 13.2 Hz), 3.36 (1 H, t, *J* = 9.6 Hz), 2.95 (1 H, t, *J* = 12.8 Hz), 2.83 (1 H, d, *J* = 15.6 Hz), 2.29 (6 H, s, 2 × OAc), 2.23 (3 H, s, 1 × OAc), 2.07–1.93 (12 H, m, 4 × OAc), 1.83–1.78 (3 H, m, 1 × OAc); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 195.8, 170.0, 169.8, 169.6, 169.4, 168.6, 167.9, 167.8, 167.7, 163.6, 162.1, 160.9, 142.2, 141.7, 137.2, 123.8, 123.2, 120.4, 103.0, 102.0 (2 ×), 78.2/77.6, 74.1, 73.7, 73.3, 72.1/71.6, 71.3, 69.8/69.0, 68.7/68.6, 68.3, 67.5, 67.2, 43.1/42.6, 21.1, 21.0, 20.8, 20.8, 20.7, 20.6, 20.5, 20.4; HRMS (ESI) calcd for $C_{41}H_{43}O_{22}$: 887.2246, found: *m/z* 887.2266 $[M - H]^-$.

6-*C*-α-L-Arabinopyranosyl-8-*C*-α-D-xylopyranosyl)-3′,4′,5,7-tetrahydroxyflavone (18db). A solution of **17db** (47 mg, 0.053 mmol) was heated with iodine (4 mg, 0.016 mmol) in DMSO (2 mL) at 140 °C for 4 h. The mixture was quenched by addition of aqueous $Na_2S_2O_3$, and extracted with EtOAc (3 × 10 mL). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was treated with added Ac_2O (3 mL) in pyridine (3 mL) and DMAP (10 mg, 0.08 mmol) at room temperature for 16 h. The mixture was quenched with CH_3OH, concentrated, and partitioned between 1 M $HCl_{(aq)}$ and EtOAc. After neutralization with saturated aqueous $NaHCO_3$, the organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography on silica gel (EtOAc–hexane, 3 : 2) to afford **18dbAc** (13 mg, 25% overall yield).

Compound **18dbAc** (10 mg, 0.01 mmol) was stirred with sodium methoxide (5 mg, 0.09 mmol) in CH_3OH (4 mL) at room temperature for 2 h. The mixture was neutralized with Dowex 50 W × 8 (H^+), filtered and concentrated under reduced pressure. The residue was rinsed with CH_2Cl_2 and Et_2O to give **18db** (5 mg, 90%).

$C_{25}H_{26}O_{14}$; yellow prisms, mp > 250 °C (decomposed); $[\alpha]_D^{25}$ −41 (c 0.33, H_2O); IR ν_{max} (neat) 3360, 2925, 1626, 1580, 1518, 1443, 1368, 1207, 1086 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.44 (1 H, s), 7.41 (1 H, s), 6.92 (1 H, d, J = 8 Hz), 6.57 (1 H, s), 4.94 (1 H, d, J = 10 Hz), 4.80 (1 H, covered by the signal of methanol), 4.57 (1 H, br), 4.10–4.02 (4 H, m), 3.98 (1 H, br), 3.75 (1 H, d, J = 12.8 Hz), 3.65–3.60 (1 H, m), 3.49 (1 H, t, J = 9.2 Hz), 3.40 (1 H, t, J = 10.8 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 184.2, 168.6, 166.8, 164.6, 157.1, 151.0, 147.0, 123.9, 120.7, 116.7, 114.6, 111.7, 105.3, 104.1 (2 ×), 80.3, 76.5 (2 ×), 75.4, 72.9, 72.0 (3 ×), 71.3, 70.5; HRMS (ESI) calcd for $C_{25}H_{25}O_{14}$: 549.1244, found: m/z 549.1251 $[M - H]^-$.

5,4′-Diacetoxy-6-*C*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-7-benzyloxyflavanone (19a). Compound **12aBn** (1.44 g, 2 mmol) was treated with AcCl (0.35 mL, 5 mmol), Et_3N (1.4 mL, 10 mmol) and DMAP (25 mg, 0.2 mmol) in CH_2Cl_2 (30 mL) at 0 °C. The mixture was stirred for 10 h at room temperature, and concentrated by rotary evaporation. The residue was partitioned between EtOAc (80 mL) and water (30 mL). The organic layer was separated, and washed with 1 M aqueous HCl (30 mL) and water (30 mL). After neutralization with saturated aqueous $NaHCO_3$, the organic layer was dried over $MgSO_4$, filtered, and concentrated to give a crude acetylation product **12aBnAc** (~ 1.60 g).

The crude product **12aBnAc** (~ 1.60 g) was treated with CAN (8.16 g, 14.9 mmol) in a mixed solvents of CH_3CN (20 mL), AcOH (10 mL) and water (10 mL). The mixture was stirred for 45 min at 50 °C, quenched by addition of saturated aqueous $NaHCO_3$, and extracted with EtOAc (5 ×). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography (EtOAc–hexane (3 : 7)) to afford flavanone **19a** (807 mg, 52% for two steps) as an inseparable diastereomeric mixture (existing as rotamers). $C_{40}H_{40}O_{16}$; white foam; TLC (EtOAc–hexane, 1 : 2) R_f 0.32; ^1H NMR (600 MHz, $CDCl_3$) δ 7.54 (1 H, d, J = 7.5 Hz), 7.44–7.35 (6 H, m), 7.13–7.11 (2 H, m), 6.47 (0.6 H, d, J = 5.5 Hz), 6.42 (0.4 H, d, J = 3.8 Hz), 5.93–5.89 (0.6 H, m), 5.65–5.61 (0.4 H, m), 5.47–5.31 (1.3 H, m), 5.27–5.13 (2.4 H, m), 5.07–4.99 (1.7 H, m), 4.69–4.64 (0.6 H, m), 4.41–4.37 (0.3 H, m), 4.22–4.19 (0.7 H, m), 4.04 (0.7 H, d, J = 12.4 Hz), 3.95–3.92 (0.3 H, m), 3.72–3.69 (1 H, m), 3.02–2.90 (1 H, m), 2.68–2.64 (1 H, m), 2.46–1.80 (18 H, 6 × OAc); HRMS calcd for $C_{40}H_{40}NaO_{16}$: 799.2214, found: m/z 799.2217 $[M + Na]^+$.

5,7,4′-Trihydroxy-6-*C*-(β-D-glucopyranosyl)flavone (3a, Isovitexin)[25]. A mixture of flavanone **19a** (807 mg, 1.04 mmol) and iodine (26 mg, 0.1 mmol) in DMSO (20 mL) was stirred for 1 h at 140 °C. The mixture was poured into water (20 mL) and extracted with EtOAc (5×). The combined organic layers were washed with 10% $Na_2S_2O_3$ aqueous solution, water and brine. The organic layer was dried over $MgSO_4$ and concentrated. The residue was dissolved in EtOAc (15 mL)/CH_3OH (15 mL), and subjected to hydrogenolysis on 10% Pd/C (80 mg) for 1 h at room temperature under an atmosphere of hydrogen. The mixture was filtered through a pad of Celite; the filtrate was concentrated to afford a crude product as pale-yellow solids. The crude product was treated with Ac_2O (10 mL) in pyridine (10 mL) for 10 h at room temperature. The mixture was concentrated *in vacuo*; the residue was partitioned between EtOAc (30 mL) and water (20 mL). The organic layer was washed with 1 M aqueous HCl (10 mL) and water (10 mL). After neutralization with saturated aqueous $NaHCO_3$, the organic layer was dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (EtOAc–hexane (1 : 1)) to afford flavone **3aAc** (536 mg, 71% for three steps).

A solution of **3aAc** (73 mg, 0.1 mmol) in dry MeOH (5 mL) was stirred with 30 wt% methanolic solution of NaOMe (0.2 mL) at room temperature for 5 h. The mixture was neutralized with Amberlite IR-120 (H^+), filtered, and rinsed with methanol. The filtrate was concentrated by rotary evaporation under reduced pressure, and the residual yellow solids were washed with Et_2O to afford **3a** (38 mg, 87%).

3aAc. $C_{35}H_{34}O_{17}$; colorless foam; TLC (EtOAc–hexane, 1 : 1) R_f 0.4; IR (film) 2938, 1721, 1600, 1214, 1135 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$, as mixture of rotamers) δ 7.82 (2 H, d, J = 8.2 Hz), 7.29 (1 H, s), 7.22 (2 H, d, J = 8.2 Hz), 6.57 (1 H, s), 5.68 (0.7 H, t, J = 9.5 Hz), 5.61 (0.3 H, t, J = 9.5 Hz), 5.29 (1 H, t, J = 9.3 Hz), 5.14 (1 H, t, J = 9.7 Hz), 4.85–4.81 (1 H, m), 4.39 (1 H, br d, J = 13.0 Hz), 3.96 (1 H, br d, J = 12.3 Hz), 3.79 (1 H, br d, J = 9.4 Hz), 2.46 (3 H, s), 2.45 (3 H, s), 2.30 (3 H, s), 2.15–1.91 (9 H, m), 1.79 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$, as mixture of rotamers) δ 176.0, 170.4, 170.2, 169.9, 169.6, 168.9, 168.6, 167.8, 161.7, 157.2, 153.4, 153.3, 148.7, 128.3, 127.6 (2 ×), 122.4 (2 ×), 119.0, 114.5, 111.8, 108.7, 76.5, 74.3, 72.3, 69.5, 68.1, 61.9, 21.3, 21.2, 21.1, 20.7, 20.67, 20.63, 20.4; HRMS calcd for $C_{35}H_{35}O_{17}$: 727.1874, found: m/z 727.1877 $[M + H]^+$.

3a. $C_{21}H_{20}NaO_{10}$; yellow powder; mp 220–222 °C; $[\alpha]_D^{20}$ = +28.9 (c = 1.0, MeOH) [lit.[24a] $[\alpha]_D^{27}$ = +27.5 (c = 1.0, MeOH)]; IR (film) 3412, 2928, 1662, 1254, 1163 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) δ 7.77 (2 H, d, J = 8.7 Hz), 6.89 (2 H, d, J = 8.7 Hz), 6.53 (1 H, s), 6.45 (1 H, s), 4.89 (1 H, d, J = 10.0 Hz, $H_{anomeric}$, 6-β-configuration), 4.20–4.17 (1 H, m), 3.89 (1 H, dd, J = 12.2, 2.2 Hz), 3.75 (1 H, dd, J = 12.2, 5.5 Hz), 3.50–3.48 (2 H, m), 3.44–3.41 (1 H, m); ^{13}C NMR (150 MHz, CD_3OD) δ 183.0, 165.2, 163.9, 161.8, 161.1, 157.7, 128.5 (2 ×), 122.0, 116.1 (2 ×), 108.2, 104.2, 102.8, 94.3, 81.7, 79.2, 74.3, 71.6, 70.9, 62.0; HRMS calcd for $C_{21}H_{20}NaO_{10}$: 455.0954, found: m/z 455.0958 $[M + Na]^+$.

Cell culture

Mice macrophage cell line Raw264.7 was obtained from ATCC and were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and penicillin/streptomycin (100 units/ml) in a 37 °C humidified chamber with 5% CO_2.

Measurement of cell viability

The effects of different compounds on cell proliferation were analyzed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). This assay uses luciferase-catalyzed reaction to quantify ATP in the cells, which is used as an indicator of metabolically active cells. Briefly, Raw264.7 cells were incubated in 96-well plates at 4 × 10^4 cells per well for 24 h, and were subsequently treated with 100, 250, 500 and 1000 μg mL^{-1} of compounds, respectively, for another 6 h. The cells were then lysed by the addition of 20 μL of the reagent. The mixture was diluted 1 : 1 with fresh DMEM, and luminescence in each well was measured 10 min after reagent addition using an EnVision 2101 multilabel reader (Perkin Elmer).

RNA isolation and RT-PCR

Raw264.7 cells were cultured in 6-well plates at 3 × 10^6 cells per well for 24 h and treated with interested compounds for 30 min followed by addition of 100 ng mL^{-1} LPS for another 6 h. The cells were collected and stored at –80 °C until use. Total RNA was extracted using Trizol (Invitrogen) and processed for RT-PCR as described previously.[4]

Determination of protein level of TNF-α

Raw264.7 cells were plated in 96-well plates at 4 × 10^4 cells per well in duplicates for 24 h. The cells were pre-treated with compounds in various concentrations for 30 min, and then treated with 100 ng mL^{-1} LPS for 6 h. The supernatants were collected by centrifugation at 1000 rpm for 5 min at indicated time intervals, and processed for TNF-α determination using an ELISA kit (R&D) based on the manufacturer's instructions. The optical density was determined using a microplate reader at 450 nm. The concentration of cytokine released was determined using the standard curve. IC_{50} values were calculated using Graphpad Prism (Graphpad Software Inc., San Diego, CA).

Measurement of nitric oxide concentration

To measure the concentration of NO, a standard procedure using Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine dihydrochloride in 2.5% H_3PO_4) was used. Briefly, macrophage RAW264.7 cells were plated in 96-well plates in 100 μL RPMI medium without phenol-red for 24 h, and treated with compounds for 30 min followed by a two-day treatment of 100 ng mL^{-1} LPS. At the indicated time, cell medium was collected and mixed with Griess reagent in 1 : 1 ratio in wells of a 96-well plate. Optical density at 550 nm was measured with a microplate reader (Spectra Max, Molecular Devices) after 10 min incubation at room temperature. The concentration of nitrite in the samples was calculated from a sodium nitrite standard curve. IC_{50} values were calculated using Graphpad Prism version software 4.0.

Acknowledgements

We are grateful for the financial support of Yuen-Foong-Yu Biotech Co., Taiwan, and Academia Sinica.

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