Flavonoid Glucosides from the Hairy Roots of *Catharanthus roseus*

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Four new flavonoid glucosides, 3′,4′-di-*O*-methylquercetin-7-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadec-13‴-ol-14‴-enyl]-β-D-glucopyranoside (**1**), 4′-*O*-methylkaempferol-3-*O*-[(4″→13‴)- 2‴,6‴,10‴,14‴-tetramethylhexadecan-13‴-olyl]-β-D-glucopyranoside (**2**), 3′,4′-di-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-methylenedodeca-3‴,10‴-dienyl]-β-D-glucopyranoside (**3**), and 4′-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-hydroxymethylenedodecanyl]-β-D-glucopyranoside (**4**), along with the three known compounds were isolated from the methanol extract of *Catharanthus roseus* hairy roots. Their structures were elucidated spectroscopically. The new flavonoid glucosides inhibited both MMP-9 activity and TNF-α production in THP-1 cells treated with lipopolysaccharide.

The Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don (Apocynaceae), is a widely used ornamental and medicinal plant.[1] *C. roseus* is a herbaceous shrub[2] that has been extensively studied because of its production of two valuable alkaloids, vincristine and vinblastine, which are used in the treatment of human neoplasms.[3] Ajmalicine, also produced by *C. roseus,* is used in the treatment of circulatory disorders and hypertension.[3] Biologically indole alkaloids produced by plants are believed to play a role as antimicrobial and antifeeding compounds.[4,5] Only a few phenolic compounds have been reported in this genus.[6,7] Recently, two flavonol trisaccharides of kaempferol and quercetin have been isolated and identified.[8] Several indole alkaloids have been reported from *C. roseus* cell suspension cultures.[9,10] However, the production of the most valuable compounds reported from this plant, vincristine and vinblastine, has not yet been achieved in these cultures.[3] The presence of anthocyanidins,[11] phenolics,[10,12] and terpenoid compounds[9,10] in cultures of *C. roseus* have also been published.

This paper deals with the isolation and structural elucidation of four new flavonoid glucosides, 3′,4′-di-*O*-methylquercetin-7-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadec-13‴-ol-14‴-enyl]-β-D-glucopyranoside (**1**), 4′-*O*-methylkaempferol-3-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadecan-13‴-olyl]-β-D-glucopyranoside (**2**), 3′,4′-di-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-methylenedodeca-3‴,10‴-dienyl]-β-D-glucopyranoside (**3**), and 4′-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-hydroxymethylenedodecanyl]-β-D-glucopyranoside (**4**), on the basis of ^{1}H and ^{13}C NMR, DEPT spectroscopic studies, including 2D-NMR COSY, HETCOR, HSQC, and chemical reactions from cultured roots. This is the first report of the isolation of flavonoid glucosides (**1**−**4**) along with three known compounds, β-sitosterol, 3-epibetulinic acid, and *n*-pentadecanyl octa-dec-19-en-oate, from hairy root cultures of *C. roseus*. In addition, the anti-inflammatory effects of the purified natural products are discussed.

Results and Discussion

Compound **1** was obtained as a pale yellow crystalline mass from EtOAc:MeOH (9:1 v/v) eluants (Figure 1). It tested positive for a flavonoid glucoside. The UV spectrum of **1** displayed characteristic bands for a flavonol derivative.[13,14] Its IR spectrum showed characteristic absorption bands for hydroxy (3439, 3370, 3280 cm^{-1}) and conjugated carbonyl (1674 cm^{-1}) groups. The positive ion FAB MS spectrum of **1** displayed a molecular ion peak at *m*/*z* 771 corresponding to a molecular formula of a flavone glucoside with an acyclic diterpene moiety. A molecular formula ($C_{43}H_{62}O_{12}$) was obtained from HR-FAB/MS.

The ^{1}H NMR spectrum of **1** indicated a flavonol glycoside moiety as it displayed two meta-coupled doublets at δ 7.15 (J = 3.0 Hz) and 7.12 (J = 3.0 Hz) for 5,7-oxygenated ring A, one meta-coupled doublet at δ 7.54 (J = 3.0 Hz), one ortho-coupled doublet at δ 7.32 (J = 8.5 Hz), and one doublet of doublets at δ 7.46 (J = 8.5, 3.0) assigned correspondingly to H-2′, H-5′, and H-6′, suggesting a 3′,4′-dioxygenated substitution pattern for ring B. The sugar unit in **1** was identified as β-glucopyranose by analysis of the coupling constant at the anomeric proton H-1″ appearing as a doublet at δ 5.05 (J = 7.5 Hz). Three one-proton doublets at δ 3.87 (J = 7.0 Hz), δ 3.51 (J = 9.0 Hz), and δ 3.48 (J = 9.0 Hz) were ascribed to the H-2″ and H_2-6″ sugar protons, respectively. The remaining sugar protons appeared as multiplets at δ 3.78 (H-3″), 4.32 (H-4″), and 4.49 (H-5″). Two broad three-proton signals at δ 3.84 and 3.81 were due to the methoxy protons. Three broad two-proton signals overlapping between δ 1.18−1.38 were assigned to H_2-3‴, H_2-7‴, and H_2-8‴. A one-proton doublet of doublets at δ 3.74 (J = 3.5, 6.5 Hz) and a one-proton broad multiplet at δ 6.77 were attributed to carbinol H-13‴ and vinylic H-15‴ protons, respectively. A three-proton doublet at δ 2.21 (J = 6.1 Hz) and a broad three-proton signal at δ 2.20 were associated with the H_3-16‴ and H_3-20‴ methyl protons located on the vinylic carbons. Four three-proton doublets at δ 0.80 (J = 6.5 Hz), 0.89 (J = 6.5 Hz), 0.86 (J = 7.0 Hz), and 0.82 (J = 6.5 Hz) were assigned correspondingly to the C-1‴, C-17‴, C-18‴, and C-19‴ secondary methyl protons. The remaining methylene and methine protons of the diterpenoid moiety appeared between δ 2.23−0.92. The ^{13}C NMR spectrum of **1** showed important signals for a C-4 flavone carbonyl carbon at δ 172.87; other flavone carbons between δ 169.32−103.62; an anomeric carbon at δ 106.54 (C-1″); sugar carbons between δ 78.28−61.24; vinylic carbons at δ 135.27 (C-14‴) and 122.61 (C-15‴); methyl carbons at δ 14.32 (C-1‴), 25.76 (C-16‴), 25.76 (C-17‴), 24.52 (C-18‴), 32.13 (C-19‴), and 25.81 (C-20‴); methoxy carbons at δ 61.16 and 56.39; and the remaining methine and methylene carbons between δ 56.05−22.90. The ^{1}H and ^{13}C NMR values of diterpene moiety in **1** were compared with those described for other acyclic diterpenoids.[15] The multiplicity of each carbon was determined by analysis of the DEPT spectrum. In the

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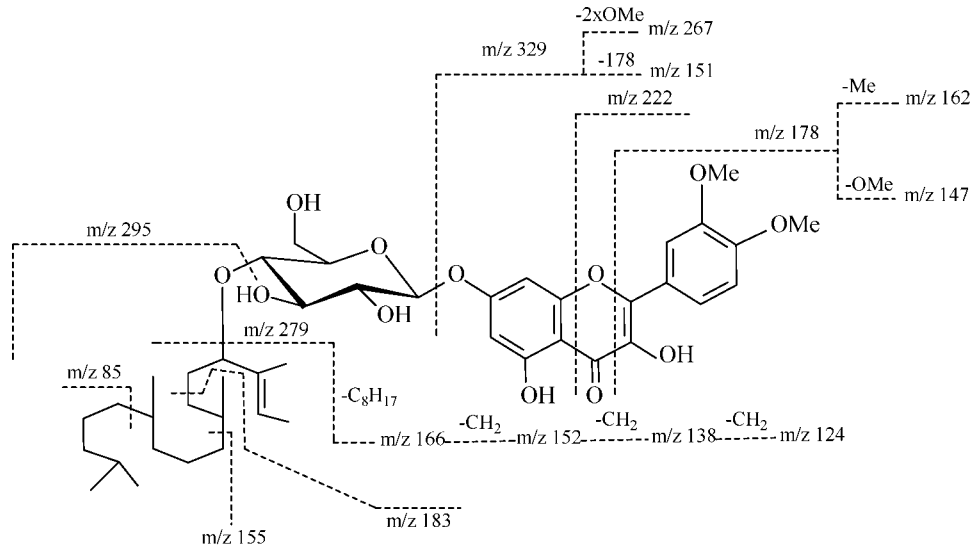

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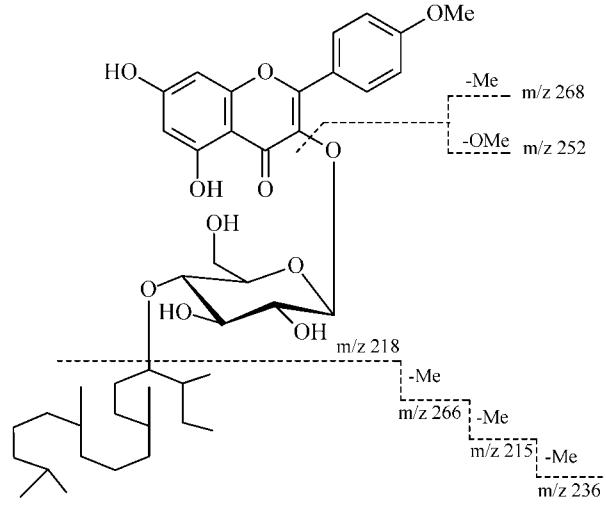

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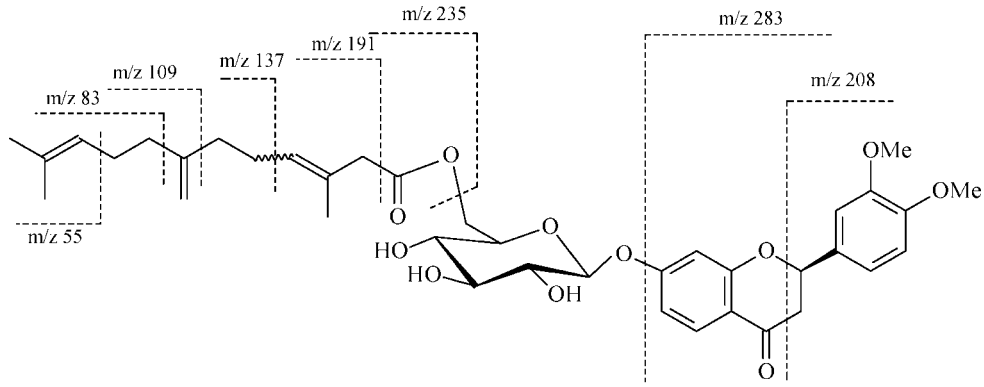
Figure 1. Fragmentation pattern of 3′,4′-di-*O*-methylquercetin-7-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadec-13‴-ol-14‴-enyl]-*β*-D-glucopyranoside (**1**) and 4′-*O*-methylkaempferol-3-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadecan-13‴-olyl]-*β*-D-glucopyranoside (**2**).

^{1}H−^{13}C HETCOR spectrum, C-4″ interacted with H-5″, H$_2$-6″, H-2″, and H-13‴; C-15‴ correlated with H$_3$-16‴, H$_3$-20‴, and H-13‴; and C-7 correlated with H-1″. In the HSQC spectrum, C-1″ correlated with H-1″; C-19‴ correlated with H-19‴. In the ^{1}H−^{1}H COSY spectrum H-2′ interacted with H-6′, H-5′ and OMe; H-1″ correlated with H-2″ and H-6; and H$_3$-20‴ correlated with H-13‴ and H$_2$-12‴. Acid hydrolysis of **1** yielded D-glucose (see Experimental Section). The chemical structure of **1** was established as 3′,4′-di-*O*-methylquercetin-7-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadec-13‴-ol-14‴-enyl]-*β*-D-glucopyranoside.

Compound **2** was obtained as a pale yellow crystalline mass from EtOAc:MeOH (9:1 v/v) eluants (Figure 1). It tested positive for a flavonoid glucoside. The UV spectrum of **2** in MeOH showed absorption maxima at 279 and 343 nm which was typical for a 3-substituted flavonol.[16] Its IR spectrum showed characteristic absorption bands for hydroxy (3414, 3385, 3260 cm^{-1}) and conjugated carbonyl (1685 cm^{-1}) groups. The positive ion FAB MS spectrum exhibited a molecular ion peak at *m/z* 743 corresponding to a flavone glucoside with an acyclic diterpene moiety. A complete molecular formula ($C_{42}H_{62}O_{11}$) was obtained from HR-FAB/MS.

The ^{1}H NMR spectrum of **2** displayed a broad two-proton signal at *δ* 7.05 assigned to H-6 and H-8. Two one-proton doublet of doublets at *δ* 6.84 (*J* = 3.0, 9.0 Hz) and *δ* 6.78 (*J* = 3.0, 9.0 Hz) were attributed to ortho-coupled H-2′ and H-6′, respectively. A two-proton doublet of doublets at *δ* 7.18, with couplings of 3.0 and 9.0 Hz was ascribed to ortho-, meta-coupled H-3′ and H-5′, suggesting an AA′ BB′-type coupling system in the B-ring. A one-proton doublet at *δ* 5.38 (*J* = 7.0 Hz) was attributed to the anomeric H-1″ of *β*-glucopyranoside. The sugar protons appeared between *δ* 4.71−3.28. A one-proton broad multiplet at *δ* 3.68 with a half-width of 15.5 Hz was due to the H-13‴ carbinol proton. A three-proton triplet at *δ* 0.84 (*J* = 6.9 Hz) and five three proton doublets at *δ* 5.38 (*J* = 7.0 Hz), 1.29 (*J* = 6.1 Hz), 1.23 (*J* = 6.6 Hz), 0.91 (*J* = 6.5 Hz), and 0.87 (*J* = 8.5 Hz) were assigned to the C-16‴ primary and C-1‴, C-17‴, C-18‴, C-19‴, C-20‴ secondary methyl protons, all attached to saturated carbons. The remaining methylene and methine protons resonated between *δ* 2.80−1.25. The ^{13}C NMR spectrum of **2** showed signals for a carbonyl carbon at *δ* 175.89 (C-4); aromatic carbons between *δ* 165.79−95.56; an anomeric carbon at *δ* 103.63; other glycosidic carbons between *δ* 71.0−63.06; a carbinol carbon at *δ* 71.93 (C-13‴); methyl carbons at *δ* 12.12



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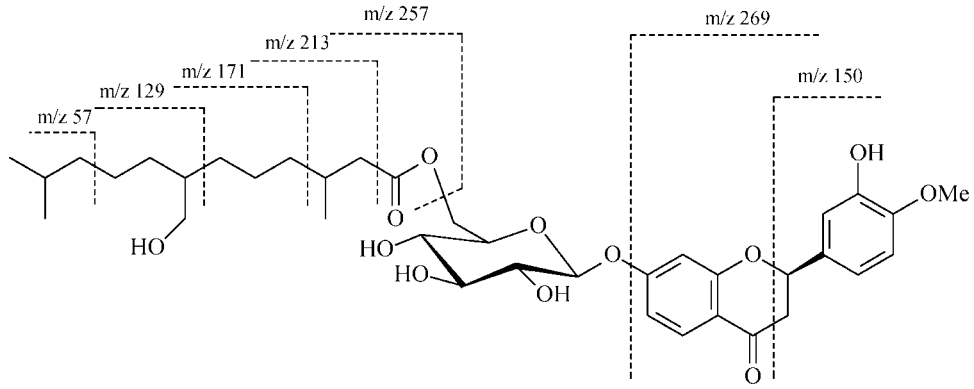

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Figure 2. Fragmentation pattern of 3′,4′-di-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-methylenedodeca-3‴,10‴-dienyl]-*β*-D-glucopyranoside (**3**) and 4′-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-hydroxymethylenedodecanyl]-*β*-D-glucopyranoside (**4**).

(C-1‴), 13.99 (C-16‴), 27.54 (C-17‴), 26.41 (C-18‴), 31.58 (C-19‴), and 24.37 (C-20‴); and a methoxy carbon at *δ* 56.17. The remaining methylene and methine carbons appeared between *δ* 59.97−19.79. The ^{1}H and ^{13}C NMR values of the diterpene moiety in **2** were compared with those described for other acyclic diterpenoids.[17] The multiplicity of each carbon was determined by analysis of the DEPT spectrum. The ^{1}H−^{1}H COSY spectrum of **2** showed the correlation of H-2′ with H-3′ and H-6′; H-1″ with H-2″, H-5″, and H_2-6″; H-4″ with H-13‴. The appearance of the C-3 signal at *δ* 135.92 in the ^{13}C NMR spectrum supported the attachment of the sugar moiety at this carbon.[18−20] In the ^{1}H−^{13}C HETCOR spectrum, C-3 correlated with H-1″, and C-13‴ correlated with H-4″. In the HSQC spectrum, C-1″ interacted with H-1″. Acid hydrolysis of **2** yielded D-glucose. The chemical structure of **2** was formulated as 4′-*O*-methylkaempferol-3-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadecan-13‴-olyl]-*β*-D-glucopyranoside.

Compound **3** was obtained as a pale yellow crystalline mass from EtOAc:MeOH (9:1 v/v) eluants (Figure 2). It tested positive for a flavonoid glucoside. The UV spectrum of **3** in MeOH showed absorption maxima at 281 and 330 nm which was typical of a flavanone derivative.[16] CD data indicated that the absolute configuration of C-2 in compound **3** is R.[21] Its IR spectrum showed characteristic absorption bands for hydroxy groups (3455, 3440 cm^{-1}), an ester group (1725 cm^{-1}), and an unsaturated carbonyl group (1690 cm^{-1}). Its molecular weight was established as *m/z* 680 corresponding to a molecular formula $C_{38}H_{48}O_{11}$ on the basis of MS and ^{13}C NMR spectra data. A complete molecular formula ($C_{38}H_{48}O_{11}$) was obtained from HR-FAB/MS.

The ^{1}H NMR spectrum of **3** showed three one-proton doublets at *δ* 7.39 (*J* = 7.5 Hz), 7.34 (*J* = 3.0 Hz), and 7.33 (*J* = 7.5 Hz) assigned to ortho-coupled H-5′, meta-coupled H-2′, and ortho-coupled H-5. Three one-proton multiplets at *δ* 7.16, 5.69, and 5.67 were ascribed to aromatic H-6, vinylic H-4‴, and H-10‴, respectively. A two-proton multiplet at *δ* 7.03 was attributed to aromatic H-6′ and H-8 suggesting ABX-type coupling systems of the A and B rings. A broad two-proton signal at *δ* 5.48 was attributed to the unsaturated methylene protons H_2-14″. A one-proton double doublet at *δ* 5.56 with coupling constants of 1.8 and 6.6 Hz was ascribed to H-2. A one-proton broad signal at *δ* 4.81 was assigned to the anomeric H-1″. The remaining sugar protons resonated between *δ* 3.78−3.30. A two-proton multiplet at *δ* 3.68 was due to H-2‴ methylene protons. Two one-proton doublets at *δ* 2.42 (*J* = 6.0 Hz) and 2.39 (*J* = 5.0 Hz) were attributed to the methylene H_2-3 protons characteristic for ring C. Three broad signals at *δ* 1.68, 1.67, and 1.52, each integrating for three protons, were ascribed to the C-12″, C-15″, and C-13″ methyl protons all located on vinylic carbons. Two broad three-proton signals at *δ* 3.56 and 3.34 were ascribed to the methoxy protons. The remaining methylene protons resonated between *δ* 1.94−1.71. The ^{13}C NMR spectrum of **3** exhibited the presence of a ketone carbonyl signal at *δ* 191.31 (C-4); aromatic carbons between *δ* 166.12−101.39; vinylic carbons between *δ* 139.55−122.39; an unsaturated C-14″ methylene carbon at *δ* 116.11; an anomeric carbon at *δ* 103.61 (C-1″); sugar carbons between at *δ* 74.16−60.53; methoxy carbons at *δ* 55.68 and 53.13; and methyl carbons at *δ* 13.41 (C-12‴), 27.07 (C-13‴), and 14.04 (C-15‴). The downfield shift of an oxymethylene carbon to *δ* 67.42 from the normal value near *δ* 61.00 supported[20] the location of the C_{15} acid at C-6″. The ^{1}H and ^{13}C NMR values of the sesquiterpene part in **3** were compared with the acyclic sesquiterpenoids.[17] The multiplicity of each carbon was determined by analyzing the DEPT spectrum. In the ^{1}H−^{1}H COSY spectrum of **3**, correlations were

observed as H_2-3 with H-2; H-6 with H-5 and H-8; H_2-6″ with H-4″; H-3″ with H_2-2‴; H_2-12‴ with H_3-15‴; and H_3-13‴ with H-4‴, H_2-2‴, and H_2-5‴. The $^1H-^{13}C$ HETCOR spectrum of **3** exhibited correlations of H-2 with C-2′ and C-6′; H-1″ with C-2″ and C-3″; H_2-6″ with C-4″ and C-1‴; H_3-12‴ with C-10‴ and C-15‴; and H_3-13‴ with C-4″ and C-2‴. In the HSQC spectrum of **3**, interactions were observed between C-13‴ and H-13‴. Acid hydrolysis of **3** yielded β-D-glucose as the glycone moiety. The structure of **3** was established as 3′,4′-di-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-methylenedodeca-3‴,10‴-dienyl]-β-D-glucopyranoside. However, the configuration of the C-3‴-C-4‴ double bond in the structure of **3** could not be confirmed.

Compound **4** was obtained as a pale yellow crystalline mass from EtOAc:MeOH (9:1 v/v) eluants (Figure 2). It tested positive for a flavonoid glucoside. The UV spectrum of **4** exhibited absorption maxima at 283 and 328 nm which was typical of a flavanone derivative.[16] CD experiment indicated that the absolute configurations of C-2 in compound **4** is R.[21] Its IR spectrum showed characteristic absorption bands for hydroxy (3441, 3390 cm^{-1}), ester (1725 cm^{-1}), and carbonyl (1690 cm^{-1}) groups. Its molecular weight was established at *m/z* 688 on the basis of mass and ^{13}C NMR spectra, which corresponded to the molecular formula of a flavanone glucoside attached with an acyclic sesquiterpenoid. A complete molecular formula, $C_{37}H_{52}O_{12}$, was obtained from HR-FAB/MS.

The ^1H NMR spectrum of **4** displayed four one-proton doublets at δ 7.33 (J = 8.5 Hz), 7.37 (J = 2.0 Hz), 7.09 (J = 3.0 Hz), and 6.97 (J = 7.5 Hz) assigned to ortho-coupled H-5, meta-coupled H-8 and H-2′, and ortho-coupled H-5′. Two one proton double doublets at δ 7.39 (J = 8.5, 2.0 Hz) and 7.06 (J = 3, 7.5 Hz) were ascribed to meta-, ortho-coupled H-6 and H-6′, respectively, suggesting ABX-type coupling systems of A- and B-rings. A one-proton double doublet at δ 5.32 (J = 1.5, 8.5 Hz) was ascribed to H-2. A one-proton doublet at δ 5.38 (J = 7.8 Hz) was attributed to the anomeric H-1″ proton. The remaining sugar protons appeared in the range δ 4.57−3.26. Shifting of the oxygenated methylene proton signal downfield to δ 3.64 (J = 6.0 Hz) and 3.61 (J = 6.0 Hz) suggested the attachment of the sesquiterpenoid moiety at C-6″. Two one-proton doublets at δ 3.26 (J = 9.0 Hz) and 3.25 (J = 9.0 Hz) were assigned to the hydroxy methylene H_2-14‴ protons. Two one-proton signals at δ 3.19 and 3.11 (d, J = 9.0 Hz) were ascribed to the methylene H_2-3 protons. A three broad proton signal at δ 3.30 was due to the methoxy protons. Three doublets at δ 0.88 (J = 7.5 Hz), 0.86 (J = 6.5 Hz), and 0.84 (6.5 Hz), each integrating for three protons, were assigned to the C-12‴, C-13‴, and C-15‴ secondary methyl protons. Two one-proton doublets at δ 2.95 (J = 8.0 Hz), and 2.93 (J = 8.0 Hz) were attributed C-2‴ methylene protons adjacent to the ester group. The remaining methylene and methine protons appeared between δ 2.78−1.28. The ^{13}C NMR spectrum of **4** exhibited signals for a carbonyl carbon at δ 185.67 (C-4); an ester carbonyl at δ 174.37 (C-1‴); flavanone carbons between δ 149.22−100.57; an anomeric carbon at δ 98.12 (C-1″); sugar carbons between δ 78.29−71.42; a oxymethylene carbon at δ 62.66 (C-14‴); methyl carbons at δ 22.73 (C-12‴), 25.01 (C-13‴), and 22.03 (C-15‴); and the remaining methylene and methine carbons between δ 44.82−26.34. The appearance of the C-6″ oxygenated methylene carbon downfield at δ 71.42 further supported the attachment of the sesquiterpenoid moiety at this carbon.[20] The ^1H and ^{13}C NMR values of the sesquiterpene moiety were compared with the similar type of molecules.[17] The multiplicity of each carbon was determined by DEPT experiments. The $^1H-^1H$ COSY spectrum of **4** showed the correlations of H-1″ with H-8, H-6, H-2″, and H-3″; H_2-6″ with H-4′ and H_2-2‴; H_2-14‴ with H-7‴, H_2-6‴, and H_2-8‴; H-2 with H_2-3, H-2′, and H-6′; and H_3-12‴ with H_3-15″. The $^1H-^{13}C$ HETCOR spectrum of **4** exhibited the correlations of H-6 with C-8; H-2 with C-3; C-4′ with H-5′, H-6′, and H-2′; and C-12‴ with H_3-15‴ and H_2-10‴. In the HSQC spectrum of **4**, interactions were observed of C-2‴ with H-2‴ and C-2′ with H-2′. Acid hydrolysis of **4** yielded β-glucose as the glycone moiety (TLC comparison). The chemical structure of **4** was elucidated as 4′-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-hydroxymethylenedodecanyl]-β-D-glucopyranoside. The configuration of the C-3‴ and C-7‴ stereogenic centers could not be defined.

β-Sitosterol,[22] 3-epi-betulinic acid,[23,24] and *n*-pentadecanyl octadec-19-en-oate[25] were identified by comparison with literature data. This is the first report of the isolation of 3-epi-betulinic acid and *n*-pentadecanyl octadec-19-en-oate from this plant.

To test the suppressive effect on the human monocytic cell line THP-1, **1**, **2**, **3**, and **4** were pretreated at concentrations between 6.5−25.9, 6.7−26.9, 7.3−29.4, and 7.3−29.0 μM, respectively, for 2 h. Then the cells were activated with lipopolysaccharide (LPS) and analyzed for matrix metalloproteinases (MMP-9) expression. These compounds were able to partially suppress MMP-9 in a dose-dependent manner (Figure 3A, C, E, and G).

To test the cytotoxicity on the THP-1 cell line, the purified compounds **1**, **2**, **3**, and **4** were pretreated at concentrations between 6.5−25.9 μM, 6.7−26.9, 7.3−29.4, and 7.3−29.0 μM, respectively, for 2 h, followed by LPS treatment. Cell viability was tested 24 h after LPS treatment. Cell viability of THP-1 cells was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, USA). Treatment of compounds **1**, **2**, **3**, and **4** in the presence of LPS did not affect cell viability of the THP-1 cell line up to concentrations of 25.9, 26.9, 29.4, and 29.0 μM, respectively (Figure 3B, D, F, and H). Treatment of these new flavonoid glucosides into cancer cells such as A549, CaSki, DLD-2, and MCF-7 cells revealed no cytotoxicity. This data indicates that compounds **1**−**4** purified from *C. roseus* suppressed MMP-9 induction in LPS-stimulated human monocytic cells without cytotoxicity. The compounds were also found to block cytokine expression of pro-inflammatory cytokine TNF-α (Figure 4).

Macrophages play an important role in the innate immunity in humans by destroying infection caused by bacteria, dead tissue cells, and small mineral particles through phagocytosis. Macrophages secrete extracellular signals, which play an important role in the regulation of inflammation. The activated macrophages produce proteolytic enzymes, pro-inflammatory cytokines/chemokines, cell adhesion molecules, nitric oxide (NO), and cylooxygenase-2 (COX-2).[26,27] These signals are involved in creating the pathogenesis of inflammatory disease especially the important proteolytic enzyme matrix metalloproteinases (MMPs).[27] These factors are involved in inflammatory diseases such as atherosclerosis, rheumatoid arthritis, tumor invasion, and metastasis by degrading many molecules in the extracellular matrix.[28] These new flavonoids (**1**−**4**) inhibited both MMP-9 activity and TNF-α production in THP-1 cells treated with LPS. The four new flavonoid glucoside compounds may be useful for the treatment of inflammatory diseases.

Although *C. roseus* was reported to contain indole alkaloids and phenolic compounds,[6,7,9,10] this is the first report of these new flavonoid glycosides (**1**−**4**) from the hairy roots of *C. roseus*. The compounds inhibited both MMP-9 activity and TNF-α production in THP-1 cells treated with LPS.

Experimental Section

General Experimental Procedures. Melting points were determined using Electrochemical Engineering (Electrothermal, Seoul, Korea) model IA9100 melting point apparatus. Optical rotation was measured with an instrument Ltd. (Seoul, Korea) model AA-10 polarimeter. UV spectra were measured with a TU-1800_{PC} UV−vis spectrophotometer. Circular dichroism (CD) was determined with a Jasco J-710 at Korea Basic Science Insititute, Ochang, Korea. IR spectra were recorded on a Thermo Mattson Infinity Gold FT-IR model 60-AR spectrophotometer at the Korea Institute of Science and Technology (KIST) Seoul, Korea. ^1H and ^{13}C NMR spectra were obtained at 500 and 125 MHz, respectively, using a Bruker Avance model DRX-500 spectrometer at

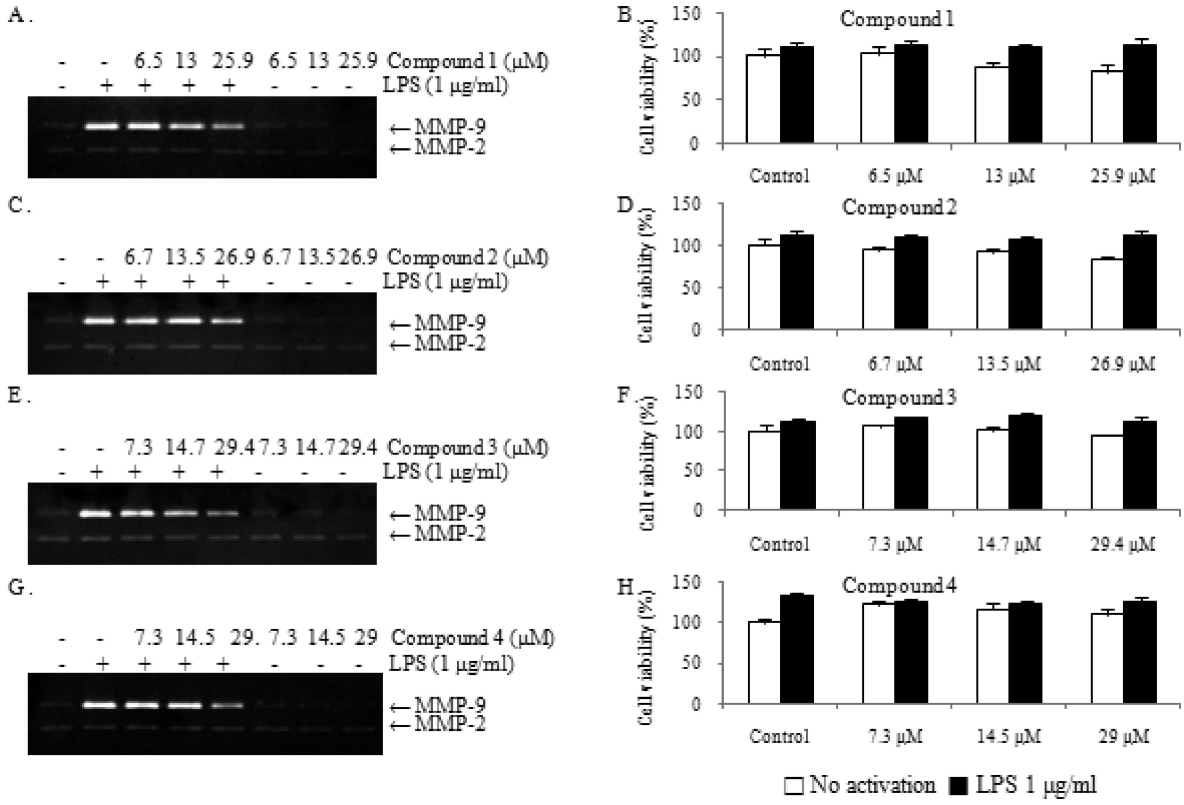

Figure 3. Inhibiting effect of compounds (**1**−**4**) on MMP-9 expression in THP-1 cells stimulated with LPS without affecting cell viability. (A, C, E, G) THP-1 cells were pretreated with the compounds at concentrations of 6.5, 13.0, and 25.9 μM for **1**; 6.7, 13.5, and 26.9 μM for **2**; 7.3, 14.7, and 29.4 μM for **3**; and 7.3, 14.5, and 29.0 μM for **4** for 2 h before stimulation with 1 μg/mL LPS. The culture supernatants were collected 24 h after activation and subjected to gelatin zymogram. (B, D, F, H) THP-1 cells were treated as compound in A, C, E, and G, and cell viability was tested with MTS assay as described in Experimental Section.

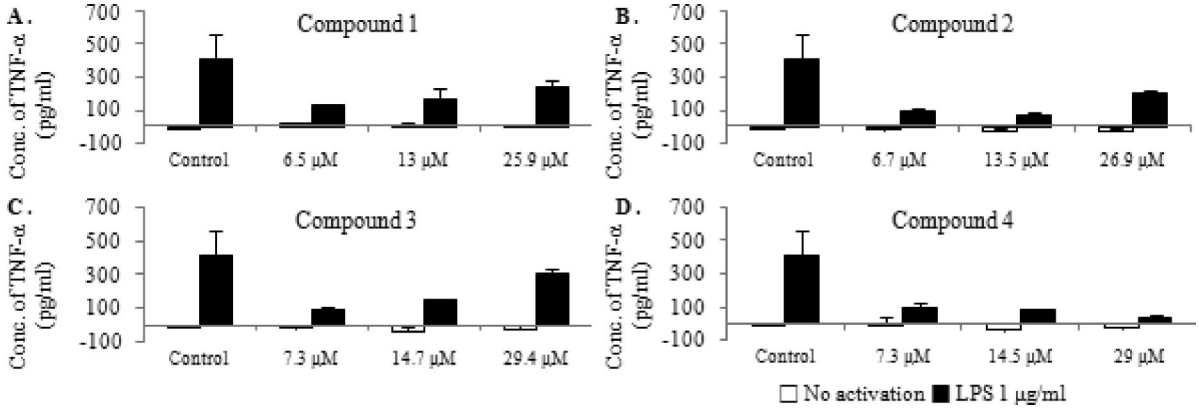

Figure 4. Blocking effect of compounds (**1**−**4**) on cytokines expression induced by LPS in monocytic cell lines. THP-1 cells were pretreated with compounds (**1**−**4**) for 2 h and activated with 1 μg/mL LPS. Culture supernatants were collected in 24 h and the TNF-α concentration were measured using sandwich ELISA. Measurements were done in duplicate.

Seoul National University (SNU), Seoul, Korea. NMR experiments included DEPT, COSY, HETCOR, and HSQC. NMR spectra were obtained in $CDCl_3$ and methanol-d_4 using TMS as an internal standard, with chemical shifts expressed in ppm (δ) and coupling constants (J) in Hz. EI MS and FAB MS were recorded on JEOL JMS-SX 102A and JEOL JMS-AX 505WA spectrophotometers, respectively, at Seoul National University. Thin layer chromatography was performed on precoated silica gel 60 F_{254} plates (Merck). Visualization of the TLC plates was performed using a 5% H_2SO_4 in C_2H_5OH spray reagent. Column chromatography was performed using silica gel (70−230 mesh) and LiChroprep RP-18 (40−63 μM; ODS silica gel) from Merck.

Chemicals. All chemicals were of analytical grade: *n*-hexane, EtOAc, MeOH, EtOH, H_2SO_4, and vanillin were purchased from Daejung Chemicals and Metals (Seoul, Korea). Precoated TLC plates (layer thickness 0.5 mm), silica gel for column chromatography (70−230 mesh ASTM) and LiChroprep RP-18 (40−63 μm) were from Merck (Darmstadt, Germany). Authentic standards of β-sitosterol and D-glucose were purchased from Sigma-Aldrich (St. Louis, MO).

Culture Conditions. The hairy root line used in this study was previously generated by infection of *C. roseus* cv. Little Bright Eye seedlings with *Agrobacterium rhizogenes* 15834.[29] The culture media consisted of a filter-sterilized solution of 3% sucrose, half-strength Gamborg's B5 salts, and full-strength Gamborg's vitamins with the pH adjusted to 5.7. The 50 mL cultures were grown in 250 mL Erlenmeyer flasks to late exponential phase in the dark at 26 °C and 100 rpm.

Plant Material. The powdered hairy roots of *C. roseus* (200 g) were immersed in MeOH (1.5 L) for 3 days at room temperature. The supernatant was concentrated under vacuum to yield an extract (22.5

Table 1. ^{1}H NMR Data (500 MHz, methanol-d_4) of Compounds **1** and **2**

position	**1** δ_H (J in Hz)	**2** δ_H (J in Hz)
2		
3		
4		
5		
6	7.15 d (3.0)	7.05 br s
7		
8	7.12 d (3.0)	7.05 br s
9		
10		
1′		
2′	7.54 d (3.0)	6.84 dd (3.0, 9.0)
3′		7.18 dd (3.0, 9.0)
4′		
5′	7.32 d (8.5)	7.18 dd (3.0, 9.0)
6′	7.49 dd (8.5, 3.0)	6.78 dd (3.0, 9.0)
1″	5.05 d (7.5)	5.38 d (7.0)
2″	3.87 d (7.0)	3.93 d (8.5)
3″	3.78 m	3.80 m
4″	4.32 m	4.35 m
5″	4.49 m	4.71 m
6″	3.51 d (9.0), 3.48 d (9.0)	3.24 br s, 3.28 br s
1‴	0.80 d (6.5)	0.86 d (6.5)
2‴	2.08 m	2.30 m
3‴	1.67 m, 1.25 br s	2.41 m, 2.17 m
4‴	2.23 m, 0.92 m	2.41 m, 2.51 m
5‴	1.98 m, 1.56 m	2.30 m, 2.03 m
6‴	1.99 m	2.44 m
7‴	1.93 m, 1.25 br s	2.17 m, 1.99 m
8‴	1.67 m, 1.25 br s	2.03 m, 1.95 m
9‴	1.58 m, 1.22 m	1.99 m, 1.85 m
10‴	2.70 m	2.59 m
11‴	1.91 m, 1.58 m	1.95 m, 1.25 m
12‴	1.30 m, 1.25 br s	1.85 m, 2.59 m
13‴	3.74 dd (3.5, 6.5)	3.68 m ($w_{1/2}$ = 15.5)
14‴		2.80 m
15‴	6.77 br m	1.54 m
16‴	2.21 d (6.1)	0.84 t (6.9)
17‴	0.89 d (6.5)	1.29 d (6.1)
18‴	0.86 d (7.0)	1.23 d (6.6)
19‴	0.82 d (6.5)	0.91 d (6.5)
20‴	2.20 br s	0.87 d (8.5)
OMe	3.84 br s, 3.81 br s	3.82 br s

Table 2. ^{13}C NMR Data (125 MHz, methanol-d_4) of Compounds **1** and **2**

position	**1** δ_C	**2** δ_C
2	144.08	144.05
3	138.50	135.92
4	172.87	175.89
5	167.53	159.47
6	95.45	99.12
7	169.32	165.79
8	109.38	97.56
9	156.57	154.26
10	103.62	100.55
1′	118.83	124.74
2′	114.75	127.46
3′	154.65	111.85
4′	154.51	134.65
5′	112.01	111.12
6′	108.34	131.25
1″	106.54	103.63
2″	78.14	70.81
3″	76.85	69.52
4″	77.97	71.60
5″	78.28	71.77
6″	61.24	63.06
1‴	14.32	12.12
2‴	52.12	53.61
3‴	17.08	19.79
4‴	22.90	21.23
5‴	23.29	22.09
6‴	56.05	46.93
7‴	29.57	24.16
8″	34.34	29.91
9″	49.19	39.05
10‴	52.06	56.47
11‴	29.91	43.05
12‴	51.41	56.96
13‴	76.97	71.93
14‴	135.27	59.97
15‴	122.61	34.19
16‴	25.76	13.99
17‴	25.56	27.54
18‴	24.52	26.41
19‴	32.13	31.58
20‴	25.81	24.37
OMe	61.16, 56.39	56.17

g). This material was suspended in H_2O and extracted with EtOAc and *n*-BuOH successively to produce EtOAc (11.2 g) and *n*-BuOH (7.4 g) extracts.

Separation and Isolation. The EtOAc extract (11.2 g) was separated on a silica gel column (70−230 mesh, 400 g, 4.5 × 90 cm) and was eluted with a gradient of *n*-hexane-EtOAc to yield 26 fractions (each fraction 250 mL): fractions 1−2 with *n*-hexane, fractions 3−4 with *n*-hexane-EtOAc (9:1), fractions 5−6 with *n*-hexane-EtOAc (8:2), fractions 7−8 with *n*-hexane-EtOAc (7:3), fractions 9−10 with *n*-hexane-EtOAc (1:1), fractions 11−12 with *n*-hexane-EtOAc (3:7), fractions 13−14 with EtOAc, fractions 15−16 with EtOAc-MeOH (9.5:0.5), fractions 17−18 with EtOAc-MeOH (9:1), fractions 19−20 with EtOAc-MeOH (7:3), fractions 21−22 with EtOAc-MeOH (1:1), fractions 23−24 with EtOAc-MeOH (3:7), and fractions 25−26 in MeOH. All fractions were examined by TLC. Fractions 1−4 were not further separated because of their low amounts. Further chromatography of the fractions 5−6 (800 mg) over silica gel (70−230 mesh, 1.5 × 25 cm) eluting with CH_2Cl_2-MeOH (99:1) afforded β-sitosterol (28 mg). The identity of β-sitosterol was confirmed through comparison with an authentic sample by TLC and spectroscopic data.[22] Fractions 7−8 (600 mg) were further chromatographed over silica gel (70−230 mesh, 1 × 20 cm) eluted with CH_2Cl_2-MeOH (100 mL, each of 99:1, 98.5:1.5, 98.2, 97.5:2.5, and 97:3) to obtain five fractions (frs.1 to frs. 5). After recrystallization of frs. 4−5 from $CHCl_3$, 3-epi-betulinic (120 mg) acid was obtained. The spectral data for 3-epi-betulinic acid were in agreement with reported values.[25] Fractions 11−12 with *n*-hexane-EtOAc (3:7) from the first column was further fractionated with $CHCl_3$-MeOH (99:1, 98:2, 97:3, 96:4, and 95:5) to afford five fractions (frs.1 to frs. 5). Fractions 4−5, after being mixed and rechromatographed over LiChroprep RP-18 (ODS silica gel; 40−63 μm; 50 g; each fraction 50 mL), eluting sequentially with MeOH containing 80, 60, 40, 20, 10, and 0% of H_2O, yielded *n*-pentadecanyl octadec-19-en-oate (23 mg). Fractions 17−18 with EtOAc-MeOH (9:1) from the first column were further fractionated with $CHCl_3$-MeOH (99:1, 97:3, 95:5, 93:7, and 9:1) to afford five fractions (frs. 1 to frs. 5). Fractions 3−5, after being mixed and rechromatographed over Lichroprep RP-18 (ODS silica gel; 40−63 μM; 50 g; each fraction 50 mL) and being eluted sequentially with MeOH containing 80, 60, 40, 20, 10, and 0% H_2O, yielded four compounds **1** (18 mg), **2** (19 mg), **3** (21 mg), and **4** (23 mg).

3′,4′-Di-*O*-methylquercetin-7-*O*-[(4″→13‴)-2‴,6‴,10‴,14‴-tetramethylhexadec-13‴-ol-14‴-enyl]-β-D-glucopyranoside (1). Yellow crystals (MeOH); mp 218−219°; R_f 0.71 ($CHCl_3$:MeOH; 9:1); $[\alpha]^{22}_D$ −52.1 (c 0.6, MeOH); UV (MeOH) λ_{max} 238, 273, 356 nm; IR (KBr) ν_{max} 3439, 3370, 3280, 2924, 2851, 1674, 1619, 1522, 1461, 1246, 1210, 1163, 1090 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; EI MS *m*/*z* (rel. int.) 441 [M − 329]$^+$ (2.2), 414 (17.5), 392 (13.6), 352 (27.9), 331 (2.2), 329 (6.5), 295 (5.8), 282 (3.7), 281 (12.7), 267 (11.0), 251 (7.0), 222 (16.9), 209 (9.7), 195 (15.4), 183 (9.9), 181 (16.2), 178 (8.9), 166 (18.7), 163 (8.1), 155 (14.2), 151 (15.3), 149 (35.5), 147 (10.4), 138 (23.4), 136 (22.1), 125 (37.7), 111 (57.5), 97 (89.1), 85 (47.1), 83 (84.3), 71 (67.8), 69 (68.1), 57 (100), 55 (66.4); FAB-MS *m*/*z* 771 [M + H]$^+$; HR-FAB/MS *m*/*z* 771.4319 (calcd for $C_{43}H_{63}O_{12}$, 771.4326).

4′-*O*-Methylkaempferol-3-*O*-[(4″→13‴)- 2‴,6‴,10‴,14‴-tetramethylhexadecan-13‴-olyl]-β-D-glucopyranoside (2). Yellow crystals (MeOH); mp 221−223°; R_f 0.68 ($CHCl_3$:MeOH; 9:1); $[\alpha]^{22}_D$ −42.1 (*c* 0.8, MeOH); UV (MeOH) λ_{max} 230, 279, 343 nm; IR (KBr) ν_{max} 3414, 3385, 3260, 2928, 2855, 1685, 1629, 1459, 1215, 1117, 1038,

Table 3. ^{1}H NMR Data (500 MHz, methanol-d_4) of Compounds **3** and **4**

position	**3** δ_H (J in Hz)	**4** δ_H (J in Hz)
2	5.56 dd (1.8, 6.6)	5.32 dd (1.5, 8.5)
3	2.42 d (6.0), 2.39 d (5.0)	3.19 d (15.5), 3.11 m
4		
5	7.33 d (7.5)	7.33 d (8.5)
6	7.16 m	7.39 dd (8.5, 2.0)
7		
8	7.03 m	7.37 d (2.0)
9		
10		
1′		
2′	7.34 d (3.0)	7.09 d (3.0)
3′		
4′		
5′	7.39 d (7.5)	6.97 d (7.5)
6′	7.03 m	7.06 dd (3, 7.5)
1″	4.81 br s	5.38 d (7.8)
2″	3.60 d (5.5)	3.82 m
3″	3.55 m	3.80 m
4″	3.58 m	3.26 m
5″	3.78 m	4.57 m
6″	3.31 d (6.5), 3.30 d (6.5)	3.64 d (6.0 m), 3.61 d (6.0)
1‴		
2‴	3.68 m	2.95 d (8.0), 2.93 d (8.0)
3‴		2.78 m
4‴	5.69 m	2.45 m 2.36 m
5‴	1.94 m	2.06 m, 2.02 m
6‴	1.93 m	1.60 m, 1.59 m
7‴		2.23 m
8‴	1.73 m	1.28 m
9‴	1.71 m	1.28 br s
10‴	5.67 m	1.28 br s
11‴		2.05 m
12‴	1.68 br s	0.88 d (7.5)
13‴	1.52 br s	0.86 d (6.5)
14‴	5.48 br s	3.26 d (9.0), 3.25 d (9.0)
15‴	1.67 br s	0.84 d (6.5)
OMe OMe	3.56 br s, 3.34 br s	3.30 br s
OMe	3.34 br s	

Table 4. ^{13}C NMR Data (125 MHz, methanol-d_4) of Compounds **3** and **4**

position	**3** δ_C	**4** δ_c
2	73.66	74.38
3	41.47	45.19
4	191.31	185.67
5	127.54	134.42
6	112.13	120.20
7	155.91	149.22
8	101.39	100.57
9	166.12	167.17
10	129.69	128.77
1′	129.52	132.81
2′	128.86	122.54
3′	143.10	141.73
4′	146.29	143.89
5′	118.75	118.73
6′	120.84	120.56
1″	103.61	98.13
2″	63.39	78.02
3″	60.57	68.97
4″	60.30	71.60
5″	74.16	78.29
6″	67.42	71.42
1‴	171.10	174.37
2‴	52.97	30.81
3‴	137.63	44.82
4‴	124.39	30.51
5‴	38.47	28.13
6‴	46.53	27.25
7‴	139.55	33.11
8‴	38.22	26.37
9‴	31.71	28.73
10‴	122.39	26.34
11‴	134.74	32.07
12‴	13.41	22.73
13‴	27.07	25.01
14‴	116.11	62.66
15‴	14.04	22.03
OMe	55.68	55.19
	53.13	

753 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; EI MS *m/z* 354 (8.2), 338 (22.9), 322 (52.1), 268 (8.9), 266 (9.4), 252 (6.1), 251 (3.3), 238 (4.5), 236 (3.4), 212 (11.1), 199 (100), 186 (37.8), 169 (10.5), 166 (6.4), 162 (2.2), 144 (9.9), 119 (7.7), 101 (6.5), 87 (3.0), 85 (3.8), 73 (6.7), 57 (7.4), 55 (8.8); FAB-MS *m/z* 743 [M + H]$^+$; HR-FAB/MS *m/z* 743.4365 (calcd for $C_{42}H_{63}O_{11}$, 743.4369).

3′,4′-Di-*O*-methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-methylenedodeca-3‴,10‴-dienyl]-β-D-glucopyranoside (3). Yellow solid (MeOH); mp 228−229°; R_f 0.67 ($CHCl_3$:MeOH; 9:1); $[\alpha]^{22}_D$ −32.1 (*c* 0.3, MeOH); UV (MeOH) λ_{max} 231, 281, 330 nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 360.8 (−0.13), 269.0 (+3.50); IR (KBr) ν_{max} 3455, 3440, 2926, 2845, 1725, 1690, 1629, 1456, 1383, 1310, 1260 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 3 and 4; EI MS *m/z* (rel. int.) 680 [M]$^+$ ($C_{38}H_{48}O_{11}$), 283 (5.1), 235 (33.3), 219 (22.9), 208 (21.4), 192 (23.7), 191 (13.4), 180 (24.2), 137 (16.5), 123 (30.9), 109 (24.3), 83 (33.0), 69 (39.0) 55 (47.7); FAB-MS *m/z* 681 [M + H]$^+$; HR-FAB/MS *m/z* 681.3267 (calcd for $C_{38}H_{48}O_{11}$, 681.3273).

4′-*O*-Methylbutin-7-*O*-[(6″→1‴)-3‴,11‴-dimethyl-7‴-hydroxymethylenedodecanyl]-β-D-glucopyranoside (4). Yellow crystals (MeOH); mp 212−214°; R_f 0.71 ($CHCl_3$:MeOH; 9:1); $[\alpha]^{22}_D$ −64.1 (*c* 0.8, MeOH); UV (MeOH) λ_{max} 228, 283, 328 nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 327.8 (−0.76), 270.6 (+14.39); IR (KBr) ν_{max} 3441, 3390,, 2924, 2853, 1725, 1690, 1647, 1461, 1390, 1180, 1075 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 3 and 4; EI MS *m/z* (rel. int.) 688 [M]$^+$ ($C_{37}H_{52}O_{12}$), 415 (2.5), 269 (9.6), 257 (41.2), 241 (11.1), 203 (2.4), 199 (10.5), 171 (17.1), 166 (20.5), 150 (45.7), 129 (48.4), 85 (49.2), 71 (74.1) 57 (100); FAB-MS *m/z* 689 [M + H]$^+$; HR-FAB/MS *m/z* 689.3533 (calcd for $C_{37}H_{52}O_{12}$, 689.3539).

Acid Hydrolysis of 1−4. Compounds **1**−**4** (4 mg each) were refluxed with 1 M HCl:dioxane (1:1, v/v) in (2 mL) a water bath for 4 h. The reaction mixture was evaporated and partitioned with $CHCl_3$ and water four times. Each extract was concentrated. The $CHCl_3$ extract contained the aglycone portion while the water extract contained D-glucose (cochromatographed on TLC with an authentic sample). For the determination of the absolute configuration of glucose in the compounds, compound **1** (32 mg), **2** (35 mg), **3** (38 mg), and **4** (36 mg) were hydrolyzed in the same procedure and subjected to silica gel chromatography with a gradient of $CHCl_3$−MeOH−H_2O (7:3:0.5 to 6:4:2). The specific rotations of glucose in compound **1**, **2**, **3**, and **4** were $[\alpha]^{24}_D$ +21.9 (*c* 0.32, H_2O), +20.0 (*c* 0.34, H_2O), +21.1 (*c* 0.38, H_2O), +19.0 (*c* 0.36, H_2O), respectively.

Cell Culture and Cytotoxic Effect of Compounds. A human macrophage cell line THP-1 was obtained from the American type Culture Collection. THP-1 cells were incubated in RPMI-1640 medium containing 10% fetal bovine serum, penicillin-streptomycin (10,000 unit/mL penicillin and 10,000 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 incubator. To test the cytotoxic effects of compounds on THP-1 cells, we washed the cells three times with the medium containing 0.1% fetal bovine serum and then seeded on sterilized 96-well tissue culture plate (5×10^4 cell/well). The cells were pretreated with the compounds at concentrations of 6.5, 13.0, and 25.9 μM for **1**; 6.7, 13.5, and 26.9 μM for **2**; 7.3, 14.7, and 29.4 μM for **3**; and 7.3, 14.5, and 29.0 μM for **4** for 2 h. Next, cells were stimulated with 1 μg/mL lipopolysaccharide; LPS (Sigma, St. Louis, MO) in the continuous presence of compounds **1**−**4** for 24 h. Cell viability was evaluated in each well by the addition of MTS. The MTS assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product. After exposure, the MTS/PMS reagent was added and cell cultures were incubated at 37 °C for 2 h. At the end of the incubation period, absorbance was recorded at 492 nm.[30]

Gelatin Zymogram. The MMP-9 activity in the culture supernatant was determined by substrate gel electrophoresis. Zymograph using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin was performed according to the reported method.[31] The samples were mixed with 4% SDS, 20% glycerol, 0.01% bromophenol, and 0.125 M Tris-HCl mixture and eletrophoresed on

10% polyacrylamide gel at room temperature. After electrophoresis, the gels were sequentially treated with 2.5% Triton X-100 for 40 min to remove SDS, distilled H_2O for 40 min to remove Triton X-100, and digestion buffer (50 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3) for 14 h at 37 °C. The gels were finally stained with 0.1% Coomassie Brilliant Blue R250 and destained for visualization of the bands.

Enzyme-Linked Immunosorbent Assay (ELISA) for TNF. Tumor necrosis factor (TNF-α) levels in the culture supernatant were measured using a sandwich ELISA (R&D Systems, USA) as described previously.[32] The detection limit of ELISA was <10 pg/mL.

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