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Securiosides A and B, Novel Acylated Triterpene Bisdesmosides with Selective Cytotoxic Activity Against M-CSF-Stimulated Macrophages

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Abstract—We report the discovery of securiosides A (**1**) and B (**2**), novel acylated triterpene bisdesmosides, isolated from the roots of *Securidaca inappendiculata*. Securiosides A and B showed potent selective cytotoxic activity against M-CSF-stimulated macrophages and were suggested to have potential as new agents for the treatment of inflammatory diseases such as RA and atherosclerosis.

Introduction

Macrophages regulate inflammatory reactions from the initiation to tissue repair process through secretion of various mediators, such as cytokines and reactive oxygen species.[1] The state of macrophage accumulation in inflammatory tissues may influence pathological outcome. For instance, the excessive accumulation of macrophages may cause tissue destruction. The number and growth state of macrophages are implicated in several pathological processes including rheumatoid arthritis (RA) and atherosclerosis. Mulherin and co-workers revealed that synovial macrophage numbers closely correlated with articular destruction in RA.[2] It was also suggested that atherosclerotic processes involve proliferation of macrophage-derived foam cells.[3–5] Therefore, the compounds having growth inhibitory activity or selective cytotoxic activity against macrophages are candidates for new drugs to control inflammatory diseases such as RA and atherosclerosis. As our initial approach to develop cytotoxic agents against macrophages, we have evaluated the inhibitory activity for macrophage growth of about 60 Chinese crude drugs which are used for the treatment of inflammation. Among them, the inhibitory activity of the H_2O extract of *Securidaca inappendiculata* roots was very potent against macrophages and was less effective to other cell types, namely bone marrow cells and lymphocytes. Based on these findings, we carried out further bioassay-guided fractionation of the H_2O extract of *S. inappendiculata* roots, resulting in the isolation of two novel bisdesmosidic triterpene saponins, named securiosides A (**1**) and B (**2**), as the active constituents responsible for the macrophages cytostasis. This communication reports the isolation and structural determination of securiosides A and B and their selective cytotoxic activity against macrophages.

Screening Procedures

The plant materials (59 species) used for screening study were from the same source listed elsewhere, of which we had screened the inhibitory substance for calprotectin-induced apoptosis.[6] The inhibitory activity of an H_2O extract of each plant material against murine peritoneal macrophages was examined. Macrophage growth assay was performed as follows. Briefly, peritoneal cells were

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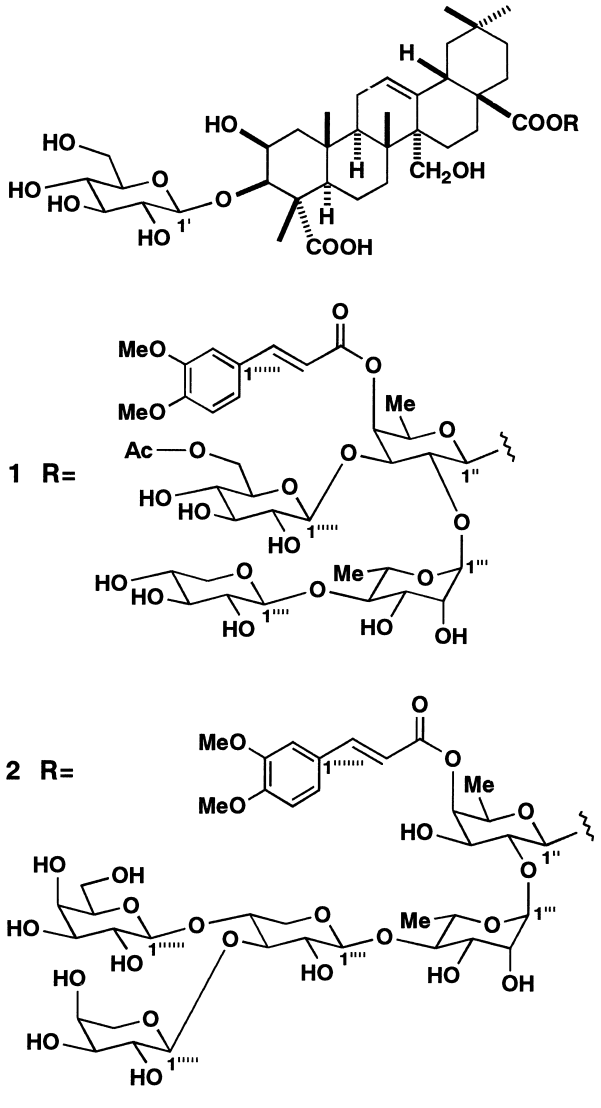
obtained 3–5 days after ip injection of 30 mg of starch into each male C3H/He mouse (Japan SLC, Shizuoka, Japan). These cells were suspended in RPMI 1640 medium (Nissui-Seiyaku, Tokyo, Japan) supplemented with 5% heated-inactivated FCS (Summit, Ft. Collins, CO, USA), penicillin (100 U/mL) and kanamycin (60 mg/mL), and added into wells of 96-well microplates (Corning, Corning, NY) at 2×10^4 cells/well. The adherent macrophages were added with test samples and simultaneously with L-cell-conditioned medium that was used as crude M-CSF.[7] The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air with 0.2 mL of medium without changing the medium for 6 days. The growth of macrophages was evaluated by the incorporation of ^{3}H-thymidine as previously described.[8] The H_2O extract of *Cimicifuga foetida* rhizomes (Ranunculaceae), *Arctium lappa* fruits (Compositae), *Crinum asiaticum* var. *sinicum* stems (Amaryllidaceae), and *Securidaca inappendiculata* roots (Polygalaceae) were found to show the macrophage growth-inhibitory activity. In this paper, we studied the properties of the substances included in *S. inappendiculata* roots since its activity was more potent and selective to macrophages.



Isolation

The roots of *S. inappendiculata* (210 g) were extracted with hot H_2O (3 L, 3 h). After removal of the fine debris in the extract by centrifugation, it was freeze-dried to give a crude H_2O extract (12 g). The H_2O extract showed a selective cytotoxicity against M-CSF-stimulated macrophages with an IC_{50} value of 2.5 μg/mL, and was successively fractionated with Et_2O and then with *n*-BuOH. The cytotoxic activity was observed only in the *n*-BuOH-soluble phase. The *n*-BuOH phase was further separated by subjecting it to Si gel column chromatography into a $CHCl_3$/MeOH (3:1) eluate fraction and an MeOH eluate fraction. The activity was concentrated in the MeOH eluate fraction, which was purified by column chromato-graphy on Si gel eluting with $CHCl_3$/MeOH/H_2O (20:10:1) and ODS Si gel with MeCN/H_2O (2:3) to result in the isolation of securiosides A (**1**) and B (**2**), as the active constituents responsible for the selective macrophages cytostasis.

Structural Elucidation

Securioside A (**1**) was obtained as an amorphous solid, $[\alpha]_D$ −24.0° (MeOH).[9] Its molecular formula, $C_{72}O_{106}O_{33}$, was deduced from the positive- and negative-ion FABMS (*m*/*z* 1521 $[M+Na]^+$; *m*/*z* 1497 $[M-H]^-$), ^{13}C NMR spectrum combined with DEPT data, and from the result of elemental analysis. The ^{1}H NMR spectrum of **1** in C_5D_5N contained five three-proton singlet signals at δ 1.96, 1.56, 1.14, 0.94, and 0.77, and five anomeric proton signals at δ 6.47 (br s), 6.19 (d, $J=8.2$ Hz), 5.07 (d, $J=7.7$ Hz), 5.06 (d, $J=7.8$ Hz), and 5.05 (d, $J=6.9$ Hz), suggesting **1** to be a triterpene pentaglycoside. In addition, the presence of an acetyl group and an (*E*)-3,4-dimethoxycinnamoyl group was shown by the IR, ^{1}H NMR, and ^{13}C NMR spectra.[9] Alkaline methanolysis of **1** with 3% NaOMe in MeOH gave a deacy derivative (**1a**, $C_{59}H_{94}O_{29}$) and methyl 3,4-dimethoxycinnamate. Subsequent acid hydrolysis of **1a** with 1 M HCl (dioxane: H_2O, 1:1) produced a triterpene aglycone (**1b**), identified as 2β,3β-dihydroxy-27-norolean-13(14)-ene-23,28-dioic acid,[10] and D-glucose, D-fucose, D-xylose, and L-rhamnose as the carbohydrate compounds. The monosaccharides, including their absolute configurations, were identified by direct HPLC analysis of the hydrolysate, which was performed on an aminopropyl-bonded Si gel column using MeCN/H_2O (3:1) as solvent system, with detection being carried out by using optical rotation (OR) detector. Comparison of the NMR data of **1a** with those of the aglycone (**1b**) were indicative of **1b** being an artifactual sapogenin produced under acid conditions. The genuine sapogenin was presumed to be 2β,3β,27-trihydroxy-olean-12-ene-23,28-dioic acid (**1c**). This was confirmed by alkaline hydrolysis **1a** with 5% KOH aq at 95 °C for 2 h, which cleaved the ester linkage of the glycoside moiety at C-28, followed by enzymatic hydrolysis using naringinase (pH 4.2, AcOK/AcOH buffer, rt, 30 h), resulting in the production of **1c**. In order to determine the sugar sequences and their linkage positions to the aglycone of **1**, spectral analysis was carried out with the deacyl derivative (**1a**). The ^{1}H–^{1}H COSY and

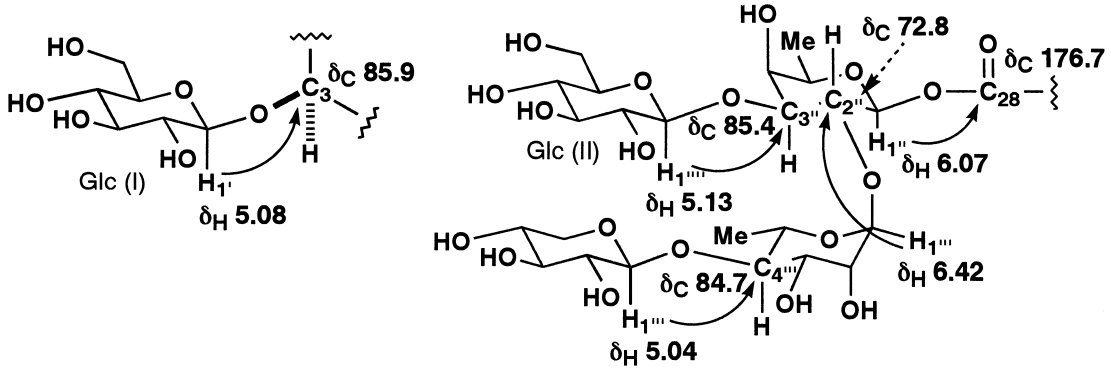


Figure 1. HMBC correlations of the sugar moieties of **1a** in pyridine-d_5.

TOCSY experiments allowed the sequential assignments of the signals from H-1 to H_2-5, H_2-6, or Me-6 of each monosaccharide. Then, the HMQC spectrum was used to associate the protons with the relevant carbon resonances. Comparison of the carbon shifts thus assigned with those of reference methyl glycosides,[11] taking into account the known effects of *O*-glycosylation and the results of acid hydrolysis, indicated that **1a** contained two terminal β-D-glucopyranosyl units, a terminal β-D-xylopyranosyl unit, a C-4 substituted α-L-rhamnopyranosyl unit, and a C-2 and C-3 disubstituted β-D-fucopyranosyl unit. The β-orientations of the anomeric center of the glucosyl, xylosyl, and fucosyl moieties were supported by the relatively large *J* values of their anomeric protons ($J = 6.9$–8.2 Hz). For the rhamnosyl moiety, the large $^1J_{C,H}$ value (173 Hz) and three-bond coupled strong HMBC correlations from the anomeric proton to C-3 and C-5 (the dihedral angle between H-1 and C-3, and between H-1 and C-5 about 180°) indicated that the anomeric proton was equatorial thus possessing an α-pyranoid anomeric form.[12] In the HMBC spectrum, correlation peaks were observed from δ 5.08 (glucose (I) H-1) to δ 85.9 (aglycone C-3), δ 5.04 (xylose H-1) to δ 84.7 (rhamnose C-4), δ 5.13 (glucose (II) H-1) to δ 85.4 (fucose C-3), δ 6.42 (rhamnose H-1) to δ 72.8 (fucose C-2), and from δ 6.07 (fucose H-1) to δ 176.7 (aglycone C-28). Thus, it was revealed that one glucose was attached at the aglycone C-3 and that the branched tetraglycoside, xylosyl-(1→4)-rhamnosyl-(1→2)-[glucosyl-(1→3)]-fucosyl, was linked to the aglycone C-28. Finally, attention focused on the ester linkage positions of acetic acid and (*E*)-3,4-dimethoxycinnamic acid in **1**. When the ^{1}H and ^{13}C NMR spectra of **1** were compared with those of **1a**, downfield shifts by *O*-acylation were detected at H-4/C-4 of the fucosyl moiety and H_2-6/C-6 of glucosyl (II) residue. Furthermore, in the HMBC spectrum, the carbonyl carbon signal at δ 171.0 showed long-range correlations with the methyl singlet signal at δ 2.07 and the glucose (II) H-6b signal at δ 4.75 (dd, $J = 11.5$, 4.4 Hz). Another carbonyl carbon signal at δ 166.8 was correlated with the olefin proton signal at δ 7.87 (d, $J = 15.8$ Hz) and the fucose H-4 signal at δ 6.03 (br d, $J = 3.2$ Hz). All of these data were consistent with structure 3-*O*-β-D-glucopyranosyl-2β,3β,27-trihydroxyolean-12-ene-23,28-dioic acid 28-*O*-β-D-xylopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[6-*O*-acetyl-β-D-glucopyranosyl-(1→3)]-4-*O*-(*E*)-3,4-dimethoxycinnamoyl-β-D-fucopyranosyl ester, which was assigned to **1**.

Securioside B (**2**), $C_{75}H_{112}O_{36}$, was obtained as an amorphous solid.[13] The ^{1}H NMR spectrum of **2** exhibited signals for six anomeric protons at δ 6.35 (br s), 6.18 (d, $J = 8.1$ Hz), 5.74 (d, $J = 5.7$ Hz), 5.06 (d, $J = 7.6$ Hz), 4.95 (d, $J = 7.8$ Hz), and 4.91 (d, $J = 7.7$ Hz), as well as signals for five methyl groups arising from the aglycone moiety at δ 1.97, 1.54, 1.14, 0.92, and 0.79. The presence of an (*E*)-3,4-dimethoxycinnamoyl group in **2** as in **1** was readily confirmed by the ^{1}H and ^{13}C NMR spectra. Alkaline hydrolysis of **2** with 3% NaOMe in MeOH furnished a deacyl derivative (**2a**) and methyl (*E*)-3,4-dimethoxycinnamate. Acid hydrolysis of **2a** with 1 M HCl (dioxane–H_2O) gave **1b**, and D-glucose, D-galactose, D-fucose, D-xylose, L-rhamnose, and L-arabinose. On the other hand, alkaline hydrolysis of **2a** with 5% KOH aq at 95 °C for 2 h yielded **1c**. These data implied that **2** was structurally related to **1** with the difference of the acylated glycoside moiety attached at C-28 of the aglycone. The ^{13}C NMR assignments of the sugar parts of **2a**, which were established by the same NMR analysis procedures as for **1a**, revealed that **2a** was composed of a terminal β-D-glucopyranosyl unit, a terminal β-D-galactopyranosyl unit, a terminal α-L-arabinopyranosyl unit, a C-3 and C-4 disubstituted β-D-xylopyranosyl unit, a C-4 substituted α-L-rhamnopyranosyl unit, and a C-2 and C-3 disubstituted β-D-fucopyranosyl unit. The terminal glucosyl group was ascertained to be linked to C-3 of the aglycone by an HMBC correlation from δ 5.05 (glucose H-1) to δ 85.9 (aglycone C-3). The other HMBC correlations from δ 5.71 (arabinose H-1) to δ 81.1 (xylose C-3), δ 4.90 (galactose H-1) to δ 72.4 (xylose C-4), δ 4.93 (xylose H-1) to δ 85.0 (rhamnose C-4), δ 6.37 (rhamnose H-1) to δ 74.3 (fucose C-2), and from δ 6.06 (fucose H-1) to δ 176.7 (aglycone C-28) established the pentaglycoside structure attached at C-28 of the aglycone as arabinosyl-(1→3)-[galactosyl-(1→4)]-xylosyl-(1→4)-rhamnosyl-(1→2)-fucosyl. The ester linkage at the fucose C-4 position in **2** was formed from 3,4-dimethoxycinnamic acid as was evident from an HMBC correlation between the signals of the fucose H-4 proton at δ 5.76 (br d, $J = 3.7$ Hz) and the carbonyl carbon at δ 167.7. Accordingly, the structure of **2** was formulated as 3-*O*-β-D-glucopyranosyl-2β,3β,27-trihydroxyolean-

12-ene-23,28-dioic acid 28-*O*-α-L-arabinopyranosyl-(1→3)-*O*-[β-D-galactopyranosyl-(1→4)]-β-D-xylopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-4-*O*-(*E*)-3,4-dimethoxy-cinnamoyl-β-D-fucopyranosyl ester.

Securiosides A (**1**) and B (**2**) are novel acylated triterpene bisdesmosides, and, as far as we know, their acylated branched oligoglycoside structures are reported for the first time in triterpene saponins.

Biological Activities of 1 and 2

Securiosides A (**1**) and B (**2**) exhibited not only growth-inhibitory activity but also potent cytotoxic activity against M-CSF-stimulated macrophages from CH3/He male mice with an IC_{50} value of less than 0.25 μM, while the corresponding deacyl derivatives (**1a**, **2a**), soyasaponins, glycyrrhizin, and some cinnamic acid derivatives were not cytotoxic themselves. This indicates that the aromatic acyl group attached at the fucosyl residue of the glycoside moiety is essential for the appearance of the activity. It is presumed that M-CSF-stimulated macrophages specifically recognize the acylated glycoside moiety of the saponins and take in the whole saponin molecules, leading to macrophage death. It is also noteworthy that **1** and **2** showed more than 10-fold higher cytotoxic activity against M-CSF-stimulated macrophages than against non-stimulated macrophages, GM-CSF-stimulated macrophages, bone-marrow cells, and lymphocytes. The selective macrophage death by **1** and **2** appeared not to be induced by changes in their membrane architecture, but to be mediated through induction of apoptosis, because they did not hemolyze sheep erythrocytes even at the sample concentration of 10 μg/mL and induced change in macrophage morphology and DNA fragmentation. Further investigation into the detailed mechanism on the activity of **1** and **2**, and in vivo evaluation for the animal models of RA and atherosclerosis are now under way.

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9. Securiosides A (**1**): $[\alpha]_D$ −24.0° (*c* 0.10, MeOH); FABMS (positive) *m/z* 1521 $[M+Na]^+$; FABMS (negative) *m/z* 1497 $[M-H]^-$; anal. C 55.11%, H 7.59%, calcd for $C_{72}H_{106}O_{33}\cdot 4H_2O$, C 55.02%, H 7.20%; UV (MeOH) λ_{max} 321.5 (log ε 4.21), 298.5 (log ε 4.13), 231.5 (log ε 4.09) nm; IR (film) ν_{max} 3375, 1710, 1600, 1510, 1452 cm^{-1}; ^1H NMR (C_5D_5N) δ 7.87 (1H, d, *J*=15.8 Hz, H-7′′′′′′), 7.06 (1H, br s, H-2′′′′′′), 7.05 (1H, br d, *J*=8.1 Hz, H-6′′′′′′), 6.91 (1H, d, *J*=8.1 Hz, H-5′′′′′′), 6.50 (1H, d, *J*=15.8 Hz, H-8′′′′′′), 6.47 (1H, br s, H-1′′′), 6.19 (1H, d, *J*=8.2 Hz, H-1′′), 6.03 (1H, br d, *J*=3.2 Hz, H-4′′), 5.84 (1H, br s, H-12), 5.07 (1H, d, *J*=7.7 Hz, H-1′), 5.06 (1H, d, *J*=7.8 Hz, H-1′′′′), 5.05 (1H, d, *J*=6.9 Hz, H-1′′′′′), 5.04 (1H, m, H-6′′′′a), 4.75 (1H, dd, *J*=11.5, 4.4 Hz, H-6′′′′b), 3.80 and 3.75 (each 3H, s, OMe×2), 2.07 (3H, s, Ac), 1.96 (3H, s, Me-24), 1.79 (3H, d, *J*=6.1 Hz, Me-6′′′), 1.56 (3H, s, Me-25), 1.40 (3H, d, *J*=6.3 Hz, Me-6′′), 1.14 (3H, s, Me-26), 0.90 (3H, s, Me-30), 0.77 (3H, s, Me-29); ^{13}C NMR (C_5D_5N) δ 44.2, 70.3, 85.9, 52.8, 52.5, 21.3, 33.4, 41.1, 49.2, 37.0, 23.5, 128.0, 138.8, 47.9, 24.4, 23.9, 46.9, 42.0, 45.2, 30.7, 33.7, 32.2, 180.7, 14.2, 17.4, 18.7, 64.4, 176.6, 33.0, 23.9 (C-1–C-30), 105.3, 75.1, 78.3, 71.5, 78.3, 62.6 (C-1′–C-6′), 94.5, 72.6, 83.0, 73.9, 70.8, 17.0 (C-1′′–C-6′′), 101.4, 71.8, 72.3, 84.6, 68.5, 18.7 (C-1′′′–C-6′′′), 107.3, 76.0, 78.7, 70.9, 67.4 (C-1′′′′–C-5′′′′), 105.4, 75.2, 78.2, 70.9, 74.9, 63.9 (C-1′′′′′–C-6′′′′), 127.7, 110.9, 150.1, 152.0, 111.9, 123.5, 145.5, 116.2, 166.8 (C-1′′′′′′–C-9′′′′′′), 55.9 and 55.7 (OMe×2), 171.0 and 20.9 (Ac).
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13. Securiosides B (**2**): $[\alpha]_D$ −17.0° (*c* 0.10, MeOH); FABMS (positive) *m/z* 1611 $[M+Na]^+$, FABMS (negative) *m/z* 1587 $[M-H]^-$; anal. C 52.09%, H 7.31%, calcd for $C_{75}H_{112}O_{36}\cdot 8H_2O$, C 51.96%, H 7.44%; UV (MeOH) λ_{max} 321.5 (log ε 4.21), 299.5 (log ε 4.13), 233.0 (log ε 4.09) nm; IR (film) ν_{max} 3370, 1714, 1598, 1514, 1454 cm^{-1}; ^1H NMR (C_5D_5N) δ 7.96 (1H, d, *J*=15.9 Hz, H-7′′′′′′′), 7.11 (1H, br s, H-2′′′′′′), 7.09 (1H, br d, *J*=8.2 Hz, H-6′′′′′′′), 6.93 (1H, d, *J*=8.2 Hz, H-5′′′′′′′), 6.56 (1H, d, *J*=15.9 Hz, H-8′′′′′′′), 6.35 (1H, br s, H-1′′′), 6.18 (1H, d, *J*=8.1 Hz, H-1′′), 5.84 (1H, br s, H-12), 5.76 (1H, br d, *J*=3.7 Hz, H-4′′), 5.74 (1H, d, *J*=5.7 Hz, H-1′′′′′), 5.06 (1H, d, *J*=7.6 Hz, H-1′), 4.95 (1H, d, *J*=7.8 Hz, H-1′′′′), 4.91 (1H, d, *J*=7.7 Hz, H-1′′′′′′), 3.81 and 3.74 (each 3H, s, OMe×2), 1.97 (3H, s, Me-24), 1.75 (3H, d, *J*=6.3 Hz, Me-6′′′), 1.54 (3H, s, Me-25), 1.36 (3H, d, *J*=6.3 Hz, Me-6′′), 1.14 (3H, s, Me-26), 0.92 (3H, s, Me-30), 0.79 (3H, s, Me-29); ^{13}C NMR (C_5D_5N) δ 44.1, 70.3, 86.0, 52.8, 52.4, 21.4, 33.5, 41.1, 49.3, 36.9, 23.5, 127.7, 138.9, 48.0, 24.4, 24.0, 47.0, 42.0, 45.2, 30.7, 33.8, 32.4, 180.8, 14.2, 17.4, 18.7, 64.4, 176.7, 33.0, 23.9 (C-1–C-30), 105.5, 75.2, 78.3, 71.4, 78.3, 62.7 (C-1′–C-6′), 94.6, 74.3, 74.1, 74.7, 70.9, 16.6 (C-1′′–C-6′′), 101.9, 71.7, 72.5, 84.9, 68.4, 17.4 (C-1′′′–C-6′′′), 106.6, 75.0, 81.0, 72.6, 65.3 (C-1′′′′–C-5′′′′), 103.0, 71.6, 73.5, 66.6, 63.5 (C-1′′′′′–C-5′′′′), 103.6, 71.6, 76.4, 70.0, 77.0, 62.2 (C-1′′′′′′–C-6′′′′′), 127.8, 111.4, 150.1, 152.1, 112.0, 123.5, 145.8, 116.0, 167.7 (C-1′′′′′′′–C-9′′′′′′′), 56.0 and 55.8 (OMe×2).