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# Isolation of Constituents and Anti-complement Activity from *Acer okamotoanum*

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A novel acylated sterol glucoside (**1**) along with four known compounds, β-amyrin acetate (**2**), 3β,24-dihydroxytaraxer-14-ene (**3**), cleomiscosin A (**4**), and cleomiscosin C (**5**), were isolated from the leaf and twig of *Acer okamotoanum* Nakai (Aceraceae). The structure of the new compound was determined to be β-sitosterol glucoside-3'-*O*-hexacosanoicate based on chemical and spectroscopic analyses. In addition, the novel compound was found to exhibit a significant inhibitory effect ($IC_{50}$ value of 0.2 μM) on the complement system activated by the classical pathway.

**Key words:** *Acer okamotoanum*, Aceraceae, Acylated sterol glucoside, β-Sitosterol glucoside-3'-*O*-hexacosanoicate, Anti-complement activity

## INTRODUCTION

The complement system plays a significant role in host defense. The complement system can be activated by one of several cascade mechanisms, including the classical pathway (CP), alternative pathway (AP), or the MBL/MASP (mannan binding lectin/MBL-associated serine protease) pathway. Thirty odd complement fragments comprise the complement system, including proteolytic pro-enzymes, non-enzymatic components that form functional complexes, co-factors, regulators, and receptors (Ember and Hugli, 1997). The proteolytic cascade allows for considerable amplification since each proteinase molecule activated at one step can, in turn, generate multiple copies of an activated enzyme later in the cascade, which then cleaves non-enzymatic compounds, such as C3, C4, and C5. The larger fragments derived from C3, C4, and C5 (i.e. C3b, C4b, and C5b) are involved in biologic effector functions, such as opsonization, phagocytosis, and immunomodulation. However, the smaller molecules, C3a, C4a, and C5a, called anaphylatoxins, induce the release of mediators from mast cells and lymphocytes, which can cause a variety of inflammatory diseases and can be fatal if occurring after organ transplantation (Abbas *et al.*, 1997). Therefore, modulation of complement activity should prove beneficial in the treatment of inflammatory diseases.

The genus *Acer* is comprised of 15 species in Korea, which are primarily maple trees growing in mountainous regions. The leaf, branch, and root of some species in this genus have been used in folk medicine for the treatment of arthralgia and fractures (Kim *et al.*, 1998). The plant *Acer okamotoanum* is an endemic species in Korea. Previous phytochemical studies on this plant resulted in the isolation of some flavonol glycosides and phenolic compounds, along with their anti-HIV-1 integrase activities (Kim *et al.*, 1998a). In our search to find anti-complement active compounds originating from the natural plants, we investigated the constituents of the leaf and twig of *A. okamotoanum*, from which a novel acylated sterol glucoside (**1**) and four known compounds were isolated. This paper describes the isolation, structure elucidation, and anti-complement activity of these compounds.

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## MATERIALS AND METHODS

### General experimental procedures

Melting points were measured using an Electrothermal melting point apparatus. Optical rotation was determined on a JASCO P-1020 polarimeter. IR spectra were obtained on a JASCO FT/IR-5300 spectrometer with KBr discs. FAB-MS and HR-FAB-MS were obtained using a JEOL JMS-DX 300 spectrometer. UV spectra were obtained using a Beckman Du-650 UV-VIS recording spectrophotometer. $^1$H- (500 and 300 MHz) and $^{13}$C-NMR (125 and 75 MHz) were recorded on a Bruker FT-NMR spectrometer using TMS as an internal standard. HPLC was performed using a Shimazu liquid chromatograph model Class-vp version 6.12, equipped with an SPD-10A UV-vis detector (Shimadzu), an RI-8010 detector (Tosoh, Japan), and a Shodex OR-2 detector (Showa-Denko, Japan). HPLC was performed using a Mightysil column (250 mm × 10 mm, RP-$C_{18}$, 5 μm, Kanto, Japan) and a Spherisorb S5 ODS2 column (250 mm × 10 mm, RP-$C_{18}$, 5 μm, Waters, Milford, MA). All solvents for HPLC were filtrated through a 0.45 μm membrane filter (Waters).

### Plant material

The leaf and twig of *Acer okamotoanum* Nakai were collected in August 2001 at Ullung Island in Gyungsangbukdo and were identified by one of the authors (K. Bae). The voucher specimen (CNU 1289) has been deposited at the herbarium in the College of Pharmacy, Chungnam National University.

### Extraction and isolation

The leaf and twig of *A. okamotoanum* (2.7 kg) were extracted three times with methanol (MeOH) under reflux for 24 h, filtrated, and concentrated to give a MeOH extract (390.0 g). The MeOH extract was suspended in $H_2O$ and partitioned with hexane, ethyl acetate (EtOAc), and butanol (BuOH). The resulting fractions were concentrated *in vacuo* to give a hexane-soluble fraction (60.0 g), an EtOAc-soluble fraction (65.0 g), and a BuOH-soluble fraction (85.0 g).

The hexane-soluble fraction (60.0 g) was chromatographed on a silica gel column eluted with a stepwise gradient of hexane-EtOAc (80:1→1:1) to yield six fractions ($H_1$-$H_6$:1.0 g, 2.5 g, 2.2 g, 0.8 g, 2.1 g, 5.3 g, respectively). Compounds **2** (28.0 mg) and **3** (51.2 mg) were crystallized, with hexane, from fractions $H_2$ and $H_4$ respectively. Compound **1** (22.0 mg) was obtained from fraction $H_6$ by repeated silica gel column chromatography eluted with $CHCl_3$-MeOH (20:1).

The EtOAc-soluble fraction (65.0 g) was chromatographed on a silica gel column eluted with a stepwise gradient of $CHCl_3$-MeOH (40:1→1:1) to yield five fractions ($E_1$-$E_5$: 0.4 g, 1.2 g, 8.5 g, 10.0 g, 1.2 g, respectively). Fraction $E_1$ was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (20:1) to obtain three subfractions ($E_{11}$-$E_{13}$: 80.0 mg, 140.0 mg, 165.0 mg). Subfraction $E_{11}$ was further purified using preparative HPLC (YMC-Pack Pro C-18; 150 × 20 mm i.d.; S-4 μm, 8 nm; UV detector 254 nm) with 25% MeOH in $H_2O$ as the eluted solvent to yield compound **4** (6.5 mg, $t_R$ 22.0 min). Subfraction $E_{12}$ was purified by the same method to yield compound **5** (21.0 mg, $t_R$ 23.5 min).

### Compound (1)

White amorphous powder; $[\alpha]_D^{25}$ -20.0° (*c* 0.20, MeOH); IR (KBr) $\nu_{max}$ cm$^{-1}$: 3450, 2930, 1720, 1640, 1485, 1385, 1080, 1020; HR-FAB-MS *m/z* 978.5119 $[M+Na]^+$ (calcd for $C_{61}H_{110}O_7Na$: 978.5117); $^1$H-NMR (500 MHz, $CD_3OD$) d: 5.37 (1H, br s, H-6), 4.42 (1H, d, *J* = 7.6 Hz, H-1'), 3.65-3.68 (2H, m, H-6'), 3.66 (1H, m, H-3'), 3.54 (1H, m, H-3), 3.46 (1H, m, H-5'), 3.41 (1H, m, H-2'), 3.33 (1H, m, H-4'), 2.35 (2H, t, $CH_2$ of fatty acid), 1.27 (46H, br s, aliphatic chain H), 1.01 (3H, s, $CH_3$-19), 0.93 (3H, d, *J* = 3.0 Hz, $CH_3$-21), 0.89 (3H, d, 26"-$CH_3$), 0.85 (3H, t, *J* = 6.5 Hz, $CH_3$-29), 0.84 (6H, d, *J* = 7.0 Hz, $CH_3$-26 and 27), 0.69 (3H, s, $CH_3$-18); $^{13}$C-NMR (125 MHz, $CD_3OD$) δ: 174.9 (s, C-1"), 140.7 (s, C-5), 122.5 (d, C-6), 101.6 (d, C-1'), 80.0 (d, C-3), 76.4 (d, 5'), 74.3 (d, C-2'), 73.9 (d, C-3'), 70.6 (d, C-4'), 63.7 (t, C-6'), 57.1 (d, C-14), 56.5 (d, C-17), 50.6 (d, C-9), 46.2 (d, C-24), 42.7 (s, C-13), 42.6 (t, C-12), 40.2 (t, C-4), 37.6 (t, C-1), 37.1 (s, C-10), 36.5 (d, C-20), 34.6 (t, C-22), 34.3 (t, C-2"), 32.3 (d, C-7), 32.2 (t, C-8), 30.1 (t, C-2), 30.1 (t, C-4"), 30.0 (t, C-5"~20"), 29.9 (t, C-21"), 29.7 (d, C-25), 29.6 (t, C-22"), 29.5 (t, C-23"), 28.7 (t, C-16), 27.6 (t, C-24"), 26.5 (t, C-23), 25.0 (t, C-3"), 24.7 (t, C-15), 23.5 (t, C-28), 23.1 (t, C-25"), 21.5 (t, C-11), 19.8 (q, C-26), 19.4 (q, C-19), 19.4 (q, C-27), 19.1 (q, C-19), 14.5 (q, C-26"), 12.4 (q, C-29), 12.3 (q, C-18).

### Alkaline hydrolysis of 1

Compound **1** (10.0 mg) was dissolved in 9.0 mL of methanol, treated with 1.0 mL of 1N NaOH, and mixed for 1 h. Following incubation at room temperature overnight, 10.0 mL of water was added to the reaction mixture to give a white powder. The powder was then crystallized in $CHCl_3$-MeOH (2:1) to yield compound **1a** (5.3 mg). The reaction filtrate was neutralized with 1N HCl (pH < 1) and extracted with dichloromethane (4 × 5.0 mL). The extract was then evaporated and crystallized with EtOAc to give compound **1b** (1.5 mg).

### Compound (1a)

White powder; mp 288-290°C; IR (KBr) $\nu_{max}$ cm$^{-1}$: 3420, 1640, 1450, 1380, 1110, 1080, 1020; FAB-MS *m/z*: 599 $[M+Na]^+$; $^1$H- and $^{13}$C-NMR data see reference (Chang *et*

*al.*, 1981).

### Compound (1b)

White powder; mp 69-71°C; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3400, 2920, 1700, 1450, 730, 720; FAB-MS *m/z*: 397 $[M+H]^+$; $^1H$- and $^{13}C$-NMR data see reference (Trag *et al.*, 2005).

### Enzymatic Hydrolysis of 1a

Naringinase (100.0 mg, from *Penicillium decumbens*) was added to a suspension of compound **1a** (5.0 mg) in 50 mM acetate buffer (pH 5.5). The mixture was stirred at 37°C for 5 h. The reaction mixture was then extracted with EtOAc (3 × 10.0 L), and the organic layer was evaporated to dryness. The residue was recrystallized from EtOAc to give compound **1c** (2.4 mg), a white crystal. The water layer was passed through a Sep-Pak C18 cartridge (Waters, Milford, MA). The water layer was then analyzed by HPLC under the following conditions: solvent MeCN-$H_2O$ (3:1); flow rate 0.5 mL/min; detection RI (Refractive Index Detector) and OR (Optical Rotation Detector). The D-glucose present in the water layer was identified by comparing its retention time and polarity to those of an authentic sample: 17.5 min (D-glucose, positive polarity).

### Compound (1c)

White needle; mp 127-128°C; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3400, 2920, 1680, 1450, 1055, 840, 800; EI-MS *m/z*: 414 $[M]^+$; $^1H$- and $^{13}C$-NMR data see reference (Grag and Nes, 1984).

### Anti-complement assay

A diluted solution of normal human serum (complement serum, 80 μL) was mixed with a gelatin veronal buffer ($GVB^{2+}$, 80 μL) with or without sample. Each sample was dissolved in DMSO, which was used as a negative control. The mixture was pre-incubated at 37°C for 30 min, followed by the addition of sensitized erythrocytes (sheep red blood cells, 40 μL). After incubation under the same conditions, the mixture was centrifuged (4°C, 1500 rpm) and the optical density of the supernatant (100 μL) was measured at 450 nm (Yamada *et al.*, 1985). Tiliroside was used as a positive control (Jung *et al.*, 1998). The anti-complement activity was determined as the mean of the triplicate measurements and was expressed as the 50% inhibitory concentration ($IC_{50}$) values from the complement-dependent hemolysis of the control (Oh *et al.*, 2000).

## RESULTS AND DISCUSSION

In our search to obtain anti-complement active compounds from natural plants, an acylated sterol glucoside (**1**) and two triterpenoids (**2**, **3**) were isolated, by repeated column chromatography, from the hexane-soluble fraction from the MeOH extract of the leaf and twig of *A. okamotoanum*. Two coumarins (**4**, **5**) were isolated from the EtOAc-soluble fraction. By comparing the physicochemical and spectral data of compounds **2-5** with the previously reported values (Fig. 1), compounds **2-5** were identified as β-amyrin acetate (Shashi *et al.*, 1994), 3β,24-dihydroxytaraxer-14-ene (Ogihara *et al.*, 1987), cleomiscosin A (Ray *et al.*, 1985), and cleomiscosin C (Ray *et al.*, 1985), respectively. Each of these known compounds (**2-5**) was isolated from this plant for the first time.

Compound **1** was obtained as a white amorphous powder and was determined to be a steroid based on its positive reaction in the Liebermann-Burchard test. Absorption bands at 3450 (OH), 2930 (CH), 1720 (C=O),

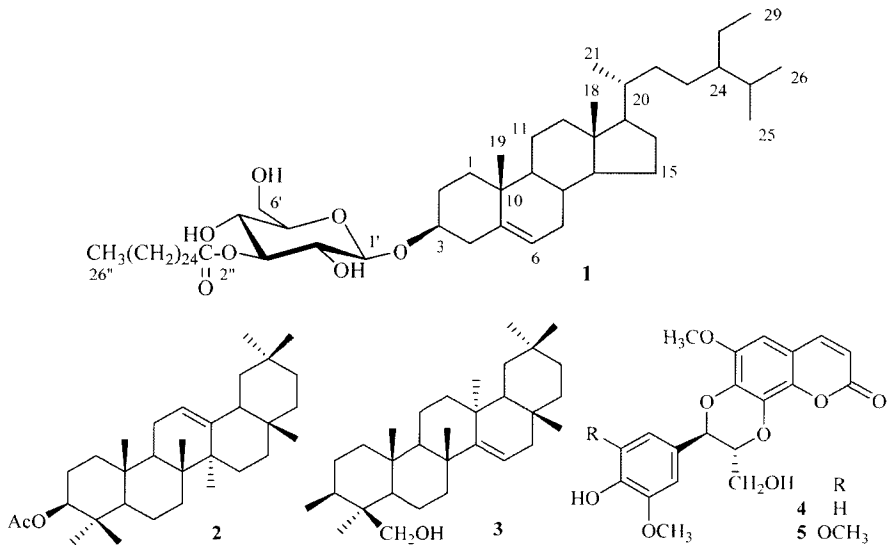


**Fig. 1.** Structures of compounds **1-5** from *Acer okamotoanum*

1640 (C=C), and 1080 $cm^{-1}$ (glycosidic C-O) were observed in the IR spectrum. HR-FAB-MS gave a molecular ion at *m/z* 978.5119 $[M+Na]^+$, providing the formula $C_{61}H_{110}O_7$ (calcd for $C_{61}H_{110}O_7Na$: 978.5117). The $^1$H-NMR spectrum showed two singlet methyl groups at δ 1.01 and 0.69, four doublet methyl groups at δ 0.93, 0.89, 0.84 × 2, a triplet methyl group at δ 0.85, an anomeric proton at δ 4.42 (1H, *J* = 7.6 Hz), an olefinic proton δ 5.37 (1H, br s), aliphatic chain protons at δ 1.27 (46H, br s), and a pair of methylene protons at δ 2.35 (2H, t). The $^{13}$C-NMR spectrum showed the presence of a carbonyl carbon at δ 174.9, olefinic carbons at δ 140.7 and 122.5, an oxygenated methine carbon at δ 80.0, an anomeric carbon at δ 101.6, five other sugar carbon signals, and aliphatic chain carbons. Based on these results, compound **1** was considered to be comprised of a sitosterol moiety, a sugar moiety, and an aliphatic chain. Aglycon (**1c**), which was obtained from an alkaline hydrolysis of compound **1** followed by an enzymatic hydrolysis of compound **1a**, was identified as β-sitosterol by TLC analysis with an authentic sample (Rf = 0.56, $CHCl_3$-MeOH 20:1) and by comparison of its spectral data with previously reported values (Grag and Nes, 1984). The glycosidic linkage was determined to be in the β conformation based on the $J_{1,2}$ value of the anomeric proton (δ 4.42, *J* = 7.6 Hz, H-1'). Enzymatic hydrolysis of **1a** yielded a monosaccharide unit, which was identified as D-glucose by HPLC comparison with an authentic sample ($t_R$ 17.5 min). The attachment position of the sugar on the aglycon was deduced from the $^{13}$C-NMR and HMBC spectra. The $^{13}$C-NMR spectrum revealed the presence of a signal shifted downfield at δ 80.0 ppm, which was determined to be the C-3 of aglycon. This observation was further supported by the HMBC correlation between $\delta_H$ 4.42 (H-1') and $\delta_C$ 80.0 (C-3) (Fig. 2).

Alkaline hydrolysis of compound **1** yielded **1a** and **1b**. Compound **1a** was identified as b-sitosterol glucoside by TLC analysis with an authentic sample (Rf = 0.54, $CHCl_3$-MeOH 10:1) and by comparison of its spectral data with reported values (Chang *et al.*, 1981). The FAB-MS of **1b** showed a molecular ion peak at *m/z* 397 $[M+H]^+$, corresponding to the formula $C_{26}H_{52}O_2$. It was identified as hexacosanoic acid based on $^1$H-, $^{13}$C-NMR and FAB-MS data (Trag *et al.*, 2005). The attachment position of the hexacosanoic acid was determined based on its $^{13}$C-NMR and HMBC spectra. The C-3 of glucose was shifted upfield to δ 73.9 ppm, in comparison to that of the corresponding signal of b-sitosterol glucoside (Chang *et al.*, 1981). This finding was further supported by the HMBC correlation observed between $\delta_H$ 3.66 (H-3') and $\delta_C$ 174.9 (C-1") (Fig. 2). Based on these results, compound **1** was identified as b-sitosterol glucoside-3'-*O*-hexacosanoicate (Fig. 1), a novel natural compound.

There are a number of reports on the isolation of acylated sterol glucosides from the high plants (Ulubelen *et al.*, 1981; Marina *et al.*, 1991). Generally, the sterol glycoside moiety was determined to be either β-sitosterol glucoside, stigmasterol glucoside, or α-spinasterol glucoside. The acyl moiety was found to be either palmitic acid, stearic acid, oleic acid, or linoleic acid, which was attached to the 6th position or to the other hydroxyl group of glucose. However, the compound reported in this study is the first example of an acylated sterol glucoside possessing hexacosanoic acid.

All isolated compounds were tested for their classical pathway complement inhibitory activity *in vitro* following the protocol previously described. The results ($IC_{50}$ values) are summarized in Table I. Compound **1**, which is the new

**Table I.** Inhibitory Effects of compounds **1-5** on the Complement System of the Classical Pathway *in vitro*

| Compounds | $IC_{50}$ (μM) |
|---|---|
| β-Sitosterol glucoside-3'-*O*-hexacosanoicate (**1**) | 0.2 ± 0.08[a] |
| β-Amyrin acetate (**2**) | >500 |
| 3β,24-Dihydroxytaraxer-14-ene (**3**) | >500 |
| Cleomiscosin A (**4**) | >500 |
| Cleomiscosin C (**5**) | >500 |
| Tiliroside[b] | 78.1 ± 2.4 |

[a] Data are expressed as the mean ± S.E.M. of three experiments.
[b] Positive control.

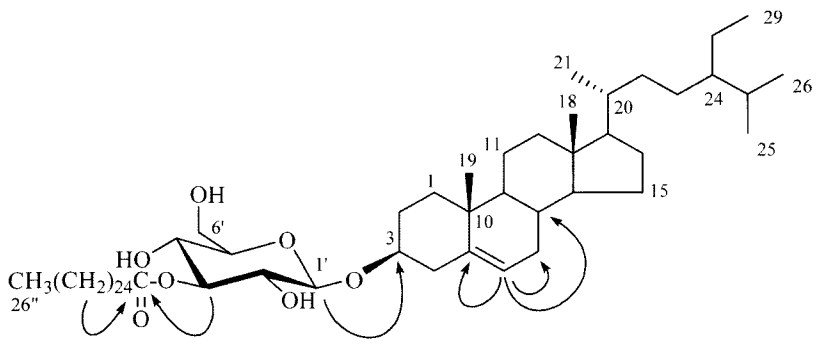


**Fig. 2.** Selected correlations in HMBC spectrum of compound **1**

acylated sterol glucoside, showed the more potent anti-complement activity, with an $IC_{50}$ value of 0.2 μM, when compared to tiliroside ($IC_{50}$ = 78.1 μM). According to previous reports, β-sitosterol, which is the aglycon of compound **1** (Park *et al.*, 2004), and b-sitosteryl 3-*O*-β-D-glucopyranoside, which is the primary moiety of compound **1** (Yoon *et al.*, 2005), did not show activity in the same assay system; however, β-sitosteryl-3-*O*-β-D-glucopyranosyl-6'-*O*-palmitate, which is acylated at the C-6' position of the sugar, did show potent activity, with an $IC_{50}$ value of 1.0 μM (Yoon *et al.*, 2005). These results demonstrate the effect that the acylated sugar moiety may have on the structure-activity relationship.

The sum of these data suggests that the new acylated sterol glucoside (**1**) might be a good candidate as a compound for use in the inhibition of the unwanted and excessive activation of the complement system.

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