A Lanostane Triterpenoid and Three Cholesterol Sterols from *Tilia kiusiana*

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Kiusianins A–D (1–4) were isolated from the leaves of a Japanese endemic plant, *Tilia kiusiana*, together with 14 known compounds. The structures of a new lanostane-type triterpenoid 1 and three new cholesterol-type sterols 2–4 were elucidated by spectroscopic methods, including two dimensional (2D) NMR. All the compounds isolated were evaluated for their cytotoxicity against two human cancer cell lines, HeLa and HL-60.

Key words *Tilia kiusiana*; Tiliaceae; triterpene; sterol; cytotoxicity

*Tilia kiusiana* is an endemic Japanese plant species found on the islands of Honshu, Shikoku, and Kyushu in Japan. For our screening program to discover new biologically active compounds from Japanese trees, we have constructed an extracts library having more than one hundred species. All the samples in the library were tested for activity against murine leukemia P388 cells. We investigated the constituents of the leaves of the Japanese tree, *Tilia kiusiana* MAKINO et. SHIRAS (Tiliaceae), because its methanol extract showed cytotoxic activity against P388 cells at 30 μg/mL. This pharmacological property of *T. kiusiana* led us to investigate the components of *Tilia kiusiana*, resulting in the isolation of four new compounds 1–4 named kiusianins A–D.

Herein, we describe the isolation and structure elucidation of kiusianins A–D (1–4) and the cytotoxic evaluation of all the isolated compounds against two human cancer cell lines, cervical cancer cells HeLa and promyelocytic leukemia cells HL-60.

The MeOH extract of the leaves of *T. kiusiana* was partitioned between n-hexane and 90% MeOH. The MeOH-soluble material was purified by octadecyl silica (ODS) column chromatography followed by silica gel column chromatography and reverse-phase HPLC to afford new triterpenoid 1 and three new sterols 2–4. The structure of 1 was deduced from the detailed analysis of the 1H- and 13C-NMR and distortionless enhancement by polarization transfer (DEPT), and further aided by two dimensional (2D) NMR experiments (1H-1H correlation spectroscopy (COSY), 1H-detected heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBD)). The 1H- and 13C-NMR and HMQC spectra (Tables 1, 2) of 1 indicated the presence of one aldehyde group (δH 9.40, s, H-26; δC 195.4, C-26), whose proton signal had an HMBC correlation with C-25 of one trisubstituted double bond attached to a methyl group [δH 6.50, brt, J = 7.6 Hz, H-24; δC 155.5, C-24; δH 13.92, C-25; δC 175.6, s, H-12; δC 9.2, C-27)], indicating the presence of a 6-methyl-2-en-1-al side chain. NMR analysis further identified the presence of one sp3 oxymethine (δH 79.3, C-3), one trisubstituted double bond [δH 5.27, dd, J = 6.8 and 2.9 Hz, H-7, δC 118.1, C-7; δC 145.7, C-8], two sp3 methines (δH 50.7, C-5; δC 36.1, C-20), four sp3 quaternary carbons [δH 39.0 (C-4), 35.0 (C-10), 51.2 (C-13), 43.6 (C-14)], nine methylenes, and five methyl groups [δH 0.82, δC 21.9 (C-18); δH 0.75, δC 13.1 (C-19); δH 0.98, δC 27.3 (C-28); δH 0.97, δC 27.6 (C-29); δH 0.86, δC 14.7 (C-30)]. From the data provided, three of the seven degrees of unsaturation were accounted for; therefore, compound 1 must contain four rings.

The 1H-13C COSY spectrum revealed the presence of four segments; C-1–C-3, C-5–C-7, C-9–C-12, and C-15–C-24 (bold lines in Fig. 2). The HMBC correlations (Fig. 1) from the remaining five methyl signals (H-18, H-19, H-28, H-29, and H-30) to the corresponding quaternary carbons and carbons of each segments (Fig. 2) means that 1 is lanostane-type triterpenoid having the same C-24–C-27 side chain as that in previously known compound 5. Furthermore, the nuclear Overhauser effect spectroscopy (NOESY) correlation of H-3/ H-5 and the coupling constant of H-3 (δH 3.24, dd, J = 11.4, 4.1 Hz) indicated that 3-OH was in an equatorial β-orientation. For the other NOESY correlations (Fig. 3), the cross-peaks of H-19/H-30, H-3/H-29, and H-3/H-5/H-9 indicated that H-3 was in a β-axial configuration and H-5, H-9, and H-29 were in an α-axial configuration. Moreover, the consecutive cross-peaks of H-19/H-18/H-21 and H-17/H-28 indicated that H-28 and H-17 were in an axial α-configuration, and the side chain was β-oriented. Meanwhile, the NOESY correlation

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between H-24 and H-26 indicated an E-geometry for the Δ^24,25 double bond. Thus, the relative configuration of compound 1 was assigned as 3β-hydroxy-lanostane-7,24E-dien-26-al and named kiusianin A.

The molecular formula of compound 2 was established as C_{27}H_{44}O_{2} by HR-EI-MS, indicating seven degrees of unsaturation. The IR spectrum revealed the presence of a hydroxyl group, an α,β-unsaturated ketone and an olefin group at 3410, 1732, and 1667 cm⁻¹, respectively. The ^1H- and ^13C-NMR, and HMOC spectra of 2 indicated the presence of one ketone carbonyl, two sp² quaternary carbons, two sp³ quaternary carbons, two sp² methines, five sp³ methines, ten methylenes, one oxymethylene, and four methyls. Because three of the seven degrees of unsaturation have been accounted for, it was conducted that compound 2 contained four rings. The spectroscopic data disclosed that compound 2 was closely related to cholestan-4,20(22)-diene-3-one. The only difference was that the olefinic methyl group at C-20 was replaced by a hydroxyl methyl group. This was supported by the HMBC correlation of H_{2}-21 (\(\delta_H\) 4.04 br d, 4.18 br d, ABq, J=11.5 Hz; \(\delta_C\) 61.2) to C-17 and C-22 (\(\delta_C\) 131.4) as well as of H-22 (\(\delta_H\) 5.42, t, J=7.3 Hz) to C-17 and C-21. The aforementioned data and detailed analysis of ^1H-1H COSY and HMBC analyses (Fig. 2) suggested that 2 was 21-hydroxy-4,20(22)-diene-3-one. The NOESY correlations indicated that the relative configuration of 2 was identical to that of cholesterol. An E-geometry for the Δ^20,22 double bond was evident from the observation of NOE between H-21 and H-23. Thus, the relative configuration of compound 2 was assigned as 21-hydroxy-cholestan-4,20-dien-3-one and named kiusianin B.

Compound 3 was formulated as C_{27}H_{42}O_{3} by HR-EI-MS, and its IR spectrum showed the presence of a hydroxyl group, an olefin, and a ketone at 3392, 1732, and 1667 cm⁻¹, respectively. The NMR data (Tables 1, 2) disclosed that 3 was cholest-7-en-3-one with one hydroxyl group attached. The position of the hydroxyl group was unambiguously as-
signed to C-6 on the basis of HMBC correlation of H-5 ($\delta_H$ 2.25) to C-6 ($\delta_C$ 70.8) and $^1H–^1H$ COSY cross-peak of H-6/H-7. The NOESY spectrum of 3 showed cross-peaks of H-5/H-9 and H-6, indicating that the hydroxyl group was in a $\beta$-configuration. The other NOESY correlations (Fig. 3) showed that the relative configuration of 3 was identical to that of cholesterol. Thus, the relative configuration of compound 3 was elucidated as 6$\beta$-hydroxy-cholest-7-en-3-one and named kiusianin C.
The molecular formula of compound 4 was established as C_{25}H_{32}O_{2} by HR-EL-MS. The IR spectrum showed the presence of a hydroxyl group, a ketone carbonyl group, and an olefin at 3398, 1730, 1665, and 1632 cm\(^{-1}\). The presence of a hydroxyl group, a ketone carbonyl group, and an olefin was confirmed by the presence of a hydroxyl group, a ketone carbonyl group, and an olefin at 3398, 1730, 1665, and 1632 cm\(^{-1}\). The NOESY data supported the relative configuration of 4 was the same as that of 3. Thus, the relative configuration of compound 4 was elucidated as 6β-hydroxy-cholest-7,20-diene-3-one and named kiusianin D.

All the compounds isolated in the present phytochemical study were evaluated for cytotoxicity (Table 3). As results, guggulsterol III (10) and linarin (17) exhibited significant cytotoxicity in vitro against human promyelocytic leukemia cells (IC\(_{50}\) 10 = 5.4 μM; IC\(_{50}\) 17 = 10.0 μM). New compounds 2–4 showed moderate cytotoxicity against HL-60 cells (IC\(_{50}\) 2 = 11.9 μM; IC\(_{50}\) 3 = 11.1 μM; IC\(_{50}\) 4 = 14.1 μM), and a new compound 1 and known compounds 8, 9, and 11–15 showed weak activity against HL-60 cells with IC\(_{50}\) values in the range of 20.5–30.6 μM. New compounds 1 and 2 known compounds 10, 14–15, and 17 showed weak activity against HL-60 cells with IC\(_{50}\) values in the range of 22.7–30.6 μM. On the other hand, compounds 5, 16, and 18 did not show cytotoxicity against HeLa and HL-60 cells (IC\(_{50}\) > 60 μM).

### Experimental

**General** 1H- and 13C-NMR spectra were obtained on Bruker AVANCE 500 with cryo-platoform and AMX500 spectrometer using tetramethylsilane as the internal standard. HR-EL-MS spectrum was obtained on JEOL JMS-GC Mate spectrometer. UV and IR spectra were obtained on Shimadzu UV-260 and JASCO FT/IR-5300 spectrometers, respectively. Column chromatography was performed using ODS (YMC ODS-AM) and silica gel (Wagogel C-300, Wako Pure Chemical Industries, Ltd. (Osaka, Japan)). MPLC was performed using silica-gel (Lobar\(^{4}\) Fertigsäule Größe B, LiChroprep\(^{5}\) Si 60 (40–63 μm), Merck (310×25 mm)). HPLC was performed using YMC-Pack ODS-AM (250×10 mm) and YMC-Pack SIL (250×10 mm). Fractions were monitored by TLC, and spots were visualized using the anisaldehyde reagent.

**Plant Material** Plants of T. kiusiana were collected from Tama Forest Science Garden, Forestry and Forest Products Research Institute, Tokyo, Japan, in September 2008. The plant was identified by K. Iwamoto (Tama Forest Science Garden, Forestry and Forest Products Research Institute). A voucher specimen (JSS06022-1) has been deposited at the Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University.

**Extraction and Isolation** The leaves of T. kiusiana (dry weight, 187 g) were crushed and then extracted with MeOH at room temperature for two weeks and then evaporated in vacuo. The MeOH extracts (13 g) were chromatographed using an ODS column (CH\(_3\)CN/H\(_2\)O, 20:80→100:0, MeOH and CHCl\(_3\)) to obtain seven fractions (F1–1–7). The F1-3 fraction (CH\(_3\)CN–H\(_2\)O, 60:40, 240 mg) was purified using silica gel column chromatography (c.c.) (CHCl\(_3–MeOH, 90:10→0:100) and HPLC (MeOH–H\(_2\)O, 70:30)) to afford compound 17 (3.4 mg). The F1-4 fraction (CH\(_3\)CN–H\(_2\)O, 80:20, 150 mg) was separated by silica gel c.c. (CHCl\(_3–MeOH, 100:0→0:100) to obtain nine fractions (F2–1–9). The F2-2 fraction (18 mg) was further purified by HPLC (MeOH–H\(_2\)O, 70:30) to isolate 7 (3.5 mg). The F2-6 fraction (5.0 mg) was further purified on HPLC (MeOH–H\(_2\)O, 85:15) to isolate 8 (1.5 mg). The F1-6 fraction (MeOH, 1.8 g) was separated using fractionated with a silica gel c.c. (CHCl\(_3–MeOH, 100:0→0:100) to obtain six fractions (F3–1–6). The F3-2 fraction (410 mg) was further purified using silica gel c.c. (CHCl\(_3–MeOH, 98:2→0:100) to obtain four fractions (F4–1–4). The F4-3 fraction (15 mg) was further purified using HPLC (MeOH–H\(_2\)O, 95:5) to isolate 15 (3.3 mg). The F4-3 fraction was further purified using HPLC (MeOH–H\(_2\)O, 95:5) to afford kiusianin A (1, 4.3 mg) and 5 (2.6 mg).

The second portion of MeOH extracts (8.4 g) was partitioned between n-hexane and 90% aqueous MeOH. The 90% aqueous MeOH-soluble materials (5.0 g) was subjected to silica gel column chromatography (n-hexane–EtOAc, 80:20→0:100, and then EtOAc–MeOH, 95:5→0:100) to obtain fractions (F5–1–13). The F5-2 fraction (456 mg) was separated using silica gel c.c. (n-hexane–EtOAc, 90:10→0:100, and then MeOH) to obtain eight fractions (F6–1–8). The F6-1 fraction (120 mg) was separated using silica gel c.c. (CHCl\(_3–MeOH, 98:2→0:100) to obtain five fractions (F7–1–5). The F7-2 fraction (15 mg) was further purified by HPLC (MeOH–H\(_2\)O, 93:7) to afford 9 (2.4 mg) and 11 (0.60 mg). The F7-4 fraction was purified using HPLC (MeOH–H\(_2\)O, 90:10) to afford 14 (7.5 mg). The F6-3 fraction (95 mg) was subjected to a silica gel c.c. (n-hexane–EtOAc, 70:30→0:100) to obtain two fractions (F8–1–2). The F8-1 fraction (63 mg) was separated using HPLC (n-hexane–EtOAc, 70:30) to obtain four fractions (F9–1–4). The F9-2 fraction (7.5 mg) was purified using HPLC (MeOH–H\(_2\)O, 95:5) to afford 6 (4.5 mg). The F9-4 fraction...
(28 mg) was separated on ODS column (MeOH–H2O, 30:70→0/100, MeOH and CHCl3) to obtain five fractions (F10-1–5). The F10-4 fraction (4 mg) was purified using HPLC (MeOH–H2O, 85:15) to afford kiusianin B (2, 0.50 mg). The F10-5 fraction (6 mg) was further purified using HPLC (CH3CN) to afford compound 12 (1.2 mg). The F6-4 fraction (61 mg) was subjected to silica gel c.c. (CHCl3–MeOH, 100:0→0:100) to obtain five fractions (F11-1–5). The F11-4 fraction (15 mg) was separated using silica gel c.c. (CHCl3–MeOH, 100:0→0:100) to obtain three fractions (F12-1–3). The F11-2 fraction (3 mg) was further purified using HPLC (MeOH–H2O, 95:5) to afford compound 13 (1.0 mg). The F6-5 fraction (28 mg) was separated using HPLC (MeOH–H2O, 95:5) to afford compound 16 (0.88 mg). The final por...