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Stelleralides A−C, Novel Potent Anti-HIV Daphnane-Type Diterpenoids from *Stellera chamaejasme* L.

Yoshihisa Asada,[*,†] Aya Sukemori,[‡] Takashi Watanabe,[§] Kuber J. Malla,[∥] Takafumi Yoshikawa,[‡] Wei Li,[*,⊥] Kazuo Koike,[⊥] Chin-Ho Chen,[#] Toshiyuki Akiyama,[▽] Keduo Qian,[▽] Kyoko Nakagawa-Goto,[▽] Susan L. Morris-Natschke,[▽] and Kuo-Hsiung Lee[*,▽,○]

Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, Laboratory for the Studying of Complementary and Medicinal Resources, The Kochi University of Technology, Tosayamada-cho, Kochi, 782-8502, Japan, Department of Plant Resources, G.P.O. Box 2270, Lalitpur, Kathmandu, Nepal, Faculty of Pharmaceutical Sciences, Toho University, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan, Duke University Medical Center, Box 2926, SORF, Durham, North Carolina 27710, United States, Natural Products Research Laboratories, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599-7568, United States, and Chinese Medicine Research and Development Center, China Medical University and Hospital, Taichung, Taiwan

asaday@rs.noda.tus.ac.jp; liwei@phar.toho-u.ac.jp; khlee@unc.edu

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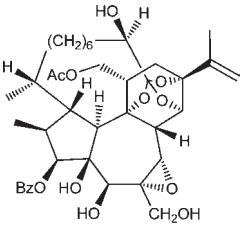
ABSTRACT



4 Stelleralide A

Three novel 1-alkyldaphnane-type diterpenes, stelleralides A−C (4−6), and five known compounds were isolated from the roots of *Stellera chamaejasme* L. The structures of 4−6 were elucidated by extensive spectroscopic analyses. Several isolated compounds showed potent anti-HIV activity. Compound 4 showed extremely potent anti-HIV activity (EC_{90} 0.40 nM) with the lowest cytotoxicity (IC_{50} 4.3 μM) and appears to be a promising compound for development into anti-AIDS clinical trial candidates.

Stellera chamaejasme L. (Thymelaeaceae) is a toxic perennial herb widespread in northern and southwestern China and Nepal. Its roots have been used in traditional Chinese medicine (TCM) as emulgent and dermatological agents. Previous studies on the chemical constituents in the roots of this plant have identified diterpenoids,[1,2] biflavonoids,[3−5] and lignans[6] with antitumor, antimalarial, and antibacterial activities. Highly functionalized daphnane

[†] Tokyo University of Science.
[‡] Kitasato University.
[§] The Kochi University of Technology.
[∥] Department of Plant Resources.
[⊥] Toho University.
[#] Duke University Medical Center.
[▽] University of North Carolina.
[○] China Medical University and Hospital.

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diterpenes from *Trigonostemon thyrsoideum* also showed inhibitory activity against HIV-1,[7] while SJ23B, a jatrophane diterpene from *Euphorbia hyberna*, displayed strong activity in the nanomolar range against HIV in vitro.[8] In addition to the potent antiviral activity of diterpenes, their mechanism of action is also of high interest. For example, prostratin, a tigliane diterpene, interferes with HIV infection by two mechanisms: decreasing the expression of HIV receptors on the surface of healthy cells, and activating the HIV-1 expression of latent HIV hidden in their reservoirs, which is a major stumbling block to the eradication of HIV. The AIDS Research Alliance hoped to complete the final stages of preclinical study on prostratin in 2010 prior to clinical trials.[9]

During our ongoing chemical studies on Nepalese medicinal plants, we also investigated the chemical constituents of the roots of *S. chamaejasme* and isolated eight daphnane-type diterpenes (**1**–**8**), including three new compounds, stelleralides A (**4**), B (**5**), and C (**6**).[10−12] All compounds were screened for anti-HIV activity in MT4 cells.[13,14]

A methanol extract of the roots of *S. chamaejasme* was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was subjected to chromatography on an ODS column, eluting with MeOH and H_2O mixtures in different ratios. Separation of the MeOH-eluted fractions using a combination of silica gel column chromatography (CC), ODS CC, and preparative HPLC afforded eight diterpenes, three new (**4**–**6**) and five known (**1**–**3**, **7**, and **8**) compounds. The known compounds were identified as gnidimacrin (**1**),[15] pimelea factor P2 (**2**),[1,16] wikstroelide F (**3**),[17,18] simplexin (**7**),[1,19,20] and huratoxin (**8**),[16,21,22] by detailed MS and NMR spectroscopic analysis and comparison with literature data. Wikstroelide F (**3**) was isolated for the first time from the genus *Stellera*.

Stelleralide A (**4**)[23] was isolated as a white amorphous powder, $[\alpha]_D^{22}$ +8.1 (*c* 0.13, MeOH). Its molecular formula $C_{39}H_{52}O_{12}$ was determined from the positive-ion HRFABMS data (*m*/*z* 735.3328, $[M + Na]^+$). The ^1H and ^{13}C NMR spectra of **4** and **1** were quite similar, suggesting that **4** is a 1-alkyldaphnane derivative. Detailed comparison of the NMR data revealed that the resonances for two benzoyl moieties in **1** were replaced by those for one benzoyl moiety and one acetyl moiety in **4**. In the HMBC spectrum of **4**, correlations were clearly observed between δ_H 4.92 (H-3) and δ_C 168.3 (Bz-CO) and between δ_H 4.11, 4.88 (H_2-18) and δ_C 170.9 (Ac-CO), indicating that the benzoyl and acetyl moieties are attached at C-3 and C-18, respectively. Thus, the structure of **4** was determined as shown in Chart 1.

Stelleralide B (**5**)[24] was also isolated as a white amorphous powder, $[\alpha]_D^{23}$ −17.0 (*c* 0.12, MeOH), with a molecular formula of $C_{44}H_{54}O_{11}$ determined from

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(10) General methods. IR spectra were recorded on a JASCO DFT/IR 460 spectrometer as KBr disks. Optical rotations were measured with a JASCO DIP-1000 digital polarimeter in a 0.5-dm cell. The ^1H and ^{13}C NMR spectra were recorded using a Varian XL-400 NMR spectrometer in δ (ppm) referenced to the signals of deuterated solvent at 8.60 and 150.0 ppm (pyridine-d_5) and at 7.26 and 77.0 ppm ($CDCl_3$), respectively. FABMS and HRFABMS were conducted using a JEOL JMS-AX 505HA mass spectrometer and a JMS-700 MStation. UV spectra were measured with a Hitachi U-2800 spectrophotometer. CD spectra were recorded using a JASCO J720 spectrometer. Octadecylsilyl silica gel (ODS) (ODS-7515-12A, Senshu Scientific Co., ltd., Tokyo, Japan) and silica gel (silica gel 60, Kanto Chemical Co., Inc., Tokyo, Japan) were used for column chromatography. For HPLC, a Waters 515 HPLC system, equipped with a Shodex RI-72 differential refractometer detector, was used. TLC was conducted on Kieselgel 60 F_{254} and RP-18 F_{254} plates (E. Merck).

(11) Plant material. The roots of *Stellera chamaejasme* were collected from Chele (Alt. 3115 m) to Shyammochem (Alt. 3755 m), upper Mustang, Mustang District, Nepal in August 2004, and identified by Dr. Takahide Kurosawa (Faculty of Symbiotic System Science, Fukushima University). A specimen of the plant [LOM-SP020729(047)] is kept in the herbarium of the University of Tokyo.

(12) Extraction and isolation. The roots (4.2 kg) of *Stellera chamaejasme* were extracted with MeOH. The MeOH extract (840.2 g) was partitioned between EtOAc and H_2O. The EtOAc layer was evaporated under reduced pressure below 40 °C to give a residue (471.6 g), which was subjected to passage over an ODS column, and eluted with a gradient of MeOH−H_2O to give eight fractions (1−8). Fraction 4 (54.6 g) was applied to a silica gel column eluted with a gradient of MeOH−$CHCl_3$, and purification of the fractions by repeated preparative HPLC afforded compounds **1** (120 mg), **2** (160 mg), **3** (13 mg), **4** (4 mg), **5** (5 mg), **6** (51 mg), **7** (8 mg), and **8** (11 mg).

(13) Anti-HIV assay. HIV-1 NL4-3 (multiplicity of infection = 0.001) was used to infect MT4 cells in the presence of various concentrations of compounds. Fresh medium containing appropriate concentrations of the compounds were added to the culture 48 h after infection to maintain normal cell growth. Virus replication was analyzed 4-day postinfection using p24 ELISA kits from Perkin-Elmer. The compound concentration that inhibited HIV-1 replication by 90% (EC_{90}) was calculated by using the biostatistic software Calcusun (Biosoft).

(14) Cytotoxicity studies. Cytotoxicity of the purified natural products to MT4 cells was determined by using a cell viability kit provided by Promega. The CellTiter-Glo Luminescent Cell Viability Assay is a simple method of determining the viability of the cells in culture based on quantitation of ATP in metabolically active cells. The CellTiter-Glo reagent was added to the MT4 cells that were cultured parallel to the antiviral assays. The compound concentration that decreased the cell viability by 50% (IC_{50}) was calculated by using Calcusun (Biosoft).

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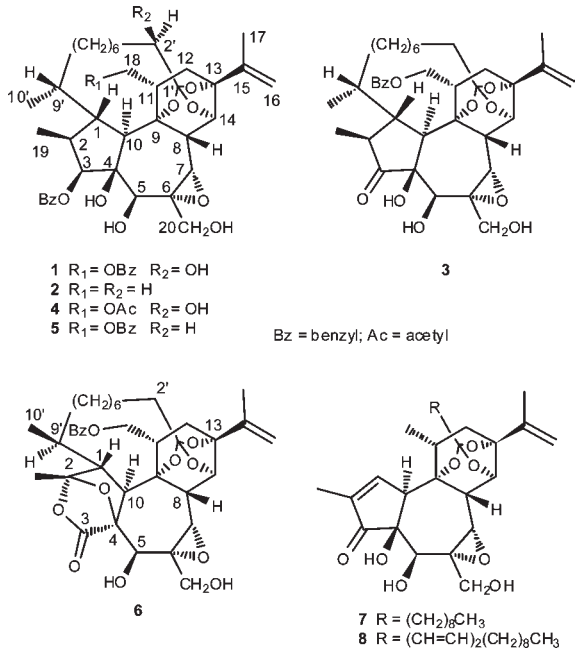
(23) Stelleralide A (**4**). White amorphous powder, $[\alpha]_D^{22}$ +8.1 (*c* 0.13, MeOH); IR (KBr) ν_{max}: 3434, 2928, 1739, 1710, 1602 cm^{-1}. For ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectroscopic data, see Supporting Information. FABMS (positive) *m*/*z* 735 $[M + Na]^+$. HRFABMS (positive) *m*/*z* 735.3328 $[M + Na]^+$ (calcd for $C_{39}H_{52}O_{12}Na$, 735.3357). UV λ nm (log ε): 229 (4.07). CD (MeOH) $\Delta\varepsilon^{24}$ (nm): +0.33 (274), 0 (263), −0.46 (249), 0 (241), +1.0 (231).

(24) Stelleralide B (**5**). White amorphous powder, $[\alpha]_D^{23}$ −17.0 (*c* 0.12, MeOH); IR (KBr) ν_{max}: 3422, 2927, 1718, 1602 cm^{-1}. For ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectroscopic data, see Supporting Information. FABMS (positive) *m*/*z* 781 $[M + Na]^+$. HRFABMS (positive) *m*/*z* 781.3552 $[M + Na]^+$ (calcd for $C_{44}H_{54}O_{11}Na$, 781.5364). UV λ nm (log ε): 229 (4.54). CD (MeOH) $\Delta\varepsilon^{24}$ (nm): −0.62 (349), 0 (325), +2.2 (300).

Chart 1



positive-ion HRFABMS (*m*/*z* 781.3552, $[M+Na]^+$). The ^1H and ^{13}C NMR spectra of **5** resembled those of **1**–**4**, suggesting that **5** is also a 1-alkyldaphnane derivative. A detailed comparison of its ^{13}C NMR spectrum with that of **1** showed that the C-2′ resonance in **1** was shifted upfield to δ_C 33.4 in **5**, indicating that the C-2′ hydroxy group in **1** is replaced by hydrogen in **5**. In confirmation, the molecular formula of **5** ($C_{44}H_{54}O_{11}$) is one oxygen atom lower compared with that of **1** ($C_{44}H_{54}O_{12}$). Thus, the structure of **5** was determined as shown in Chart 1.

Stelleralide C (**6**)[25] was isolated as a white amorphous powder, $[\alpha]_D^{23}$ +14.0 (*c* 0.12, MeOH). Its molecular formula $C_{37}H_{46}O_{11}$ was determined from the positive-ion HRFABMS data (*m*/*z* 689.2932, $[M + Na]^+$). The ^1H and ^{13}C NMR spectra of **6** showed resonances characteristic of the alkyl part of 1-alkyldaphnane derivatives, including a quaternary carbon resonance at δ_C 120.7 (C-1′). However, large differences were observed between the A ring backbone resonances of **6** and those of **1**–**5**. Namely, in the ^{13}C NMR spectrum, **6** showed resonance for an acetal carbon at δ_C 112.1 and a lactone carbonyl carbon at δ_C 175.7. In addition, a singlet methyl at δ_H 1.96 assignable to H-19 was observed in the ^1H NMR spectrum of **6**. The acetal carbon resonance at δ_C 112.1 was assigned to C-2, because HMBC correlations (Figure 1) with protons at δ_H 3.15 (H-1), 3.49 (H-10), and 1.96 (H-19) were clearly observed. Similarly,

(25) Stelleralide C (**6**). White amorphous powder, $[\alpha]_D^{23}$ +14.0 (*c* 0.12, MeOH); IR (KBr) ν_{max}: 3424, 2925, 1792, 1719, 1603 cm^{-1}. For ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$ and C_5D_5N) spectroscopic data, see Supporting Information. FABMS (positive) *m*/*z* 689 $[M + Na]^+$. HRFABMS (positive) *m*/*z* 689.2932 $[M + Na]^+$ (calcd for $C_{37}H_{46}O_{11}Na$, 689.2938). UV λ nm (log ε): 229 (4.11). CD (MeOH) $\Delta\varepsilon^{24}$ (nm): +5.3 (226).

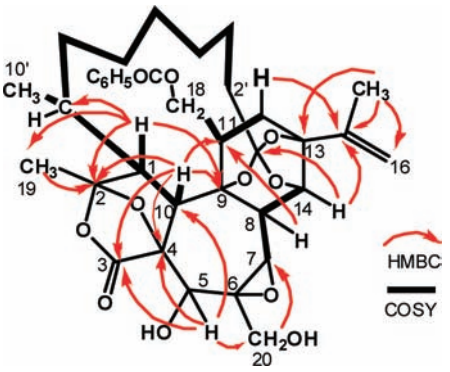


Figure 1. Key HMBC and $^1H-^1H$ correlations of **6**.

the lactone carbonyl resonance at δ_C 175.7 was assigned to C-3 based on the HMBC correlations (Figure 1) with protons at δ_H 5.42 (H-5) and 3.49 (H-10). In addition, the presence of a β-oriented 2,4-epoxide moiety was deduced from spectroscopic evidence, in which resonances assignable to H-2 and H-4 were not observed in ^1H NMR spectrum and the resonance for H-5α (δ_H 5.42) was shifted to lower field as compared with those of **1**–**5**, due to the anisotropic effect of the adjacent carbonyl group attached to C-4. In the ROESY spectrum (Figure 2) of **6**, the presence of correlations of δ_H 1.96 (H_3-19) with δ_H 1.10 (H_3-10′) and 3.15 (H-1), and the lack of a correlation between δ_H 1.96 (H_3-19) and 1.42 (H-9′), indicated a β-orientation of the 19-methyl moiety. Furthermore, the correlations between H_3-10′/H-1 and H-10/H-9′ in the ROESY spectrum indicated a 9′*S* configuration in **6**.

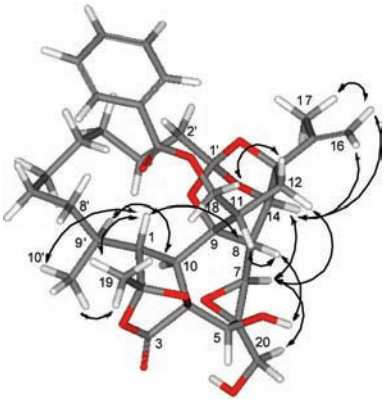


Figure 2. Key correlations in ROESY spectrum of **6**.

The isolated compounds (**1**–**8**) were evaluated for anti-HIV activity against NL4-3 in MT4 cells, as well as cytotoxicity. The data (EC_{90} and IC_{50} values, respectively) are listed in Table 1. New compound **4** and the known compound **1** were the most potent compounds, with anti-HIV EC_{90} values of less than 1 nM (0.40 and 0.41 nM, respectively), where EC_{90} was the compound

Table 1. Anti-HIV Activity of **1**–**8**

no.	anti-HIV (NL4–3) EC_{90} (μM)	cytotoxicity (MT4) IC_{50} (μM)
1	0.00041	2.8
2	0.0014	3.2
3	0.0017	7.3
4	0.00040	4.3
5	0.0016	14.5
6	0.091	3.4
7	0.0080	22.5
8	0.0058	9.4

concentration that inhibited HIV-1 replication by 90%. Structurally, the two compounds differed only in the C-18 substituent (OAc in **4** and OBz in **1**). Compound **4** (IC_{50} 4.3 μM) was less cytotoxic than **1** (IC_{50} 2.8 μM). Compounds **2**, **3**, and **5** showed lower, but significant, anti-HIV potency with EC_{90} values between 1.4 and 1.7 nM. All three compounds are unsubstituted at C-2′, while the more potent **1** and **4** contain an OH group at this position. Unlike in the other compounds, the long C-1′ alkyl chain of **7** and **8** does not form a macrocyclic ring at C-1, and these two compounds were less potent. The least potent compound was **6**, which contains a 2,4-epoxide moiety and lactone ring.

In conclusion, eight 1-alkyldaphnane type diterpenes, including three new compounds, stelleralides A–C, were isolated from the roots of *Stellera chamaejasme*. Two compounds, new **4** and known **1**, exhibited extremely potent anti-HIV EC_{90} values of less than 1 nM. The structural difference between these two and several less potent compounds was the presence of a C-2′ OH group. Synthesis of structurally modified analogs will be pursued to establish structure–activity relationship (SAR) correlations and optimize these lead compounds as anti-AIDS clinical trial candidates.

The daphnane diterpenes described in this study share some structural similarity to other diterpenes, such as prostratin (12-deoxyphorbol-13-acetate), DPP (12-deoxyphorbol-13-phenylacetate), and ingenol derivatives. Prostratin has been well documented for its anti-HIV-1 activity.[26–28] DPP is a tigliane diterpene, similar to prostratin. DPP exhibited more potent anti-HIV-1 activity than prostratin.[29] In addition to the tigliane diterpenes, ingenol derivatives were reported to have anti-HIV-1 activity comparable to that of DPP.[30,31] The anti-HIV-1 activity of these compounds was, at least in part, due to their ability to activate protein kinase C and down-regulate HIV-1 receptors, CD4, and chemokine receptors.[26,28,30] The mechanism of action of the daphnane diterpenes reported here is currently under investigation. However, due to their structural similarity to other phorboids, it is likely that activation protein kinase C and down regulation of HIV-1 cellular receptors might be responsible for their potent anti-HIV-1 activity.

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Supporting Information Available. Tables of ^1H, ^{13}C, and HMBC NMR spectroscopic data for **4**–**6**. Copies of ^1H and ^{13}C spectra for **1**–**8**. Copies of 2D NMR spectra and MS data for **4**–**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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