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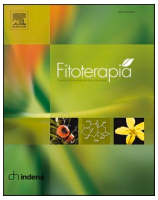

# LC-MS identification, isolation, and structural elucidation of anti-HIV macrocyclic daphnane orthoesters from *Edgeworthia chrysantha*

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ABSTRACT

The occurrence of macrocyclic daphnane orthoesters (MDOs) with a 1-alkyl group originating from a $C_{14}$ aliphatic chain is extremely limited in the plant kingdom and has only been isolated from *Edgeworthia chrysantha*. In the present study, LC-ESI-MS/MS analysis was performed on different parts of *E. chrysantha*, including flower buds, flowers, leaves, and stems, and resulted in the identification of seven MDOs in all the four plant parts, including two previously unreported compounds **1** and **7**. Further LC-MS guided isolation was carried out to afford compounds **1** and **7**, and their structures were determined by various spectroscopic analyses. These compounds were also evaluated for anti-HIV activity, thus expanding insights into the structure-activity relationships for MDOs.

## 1. Introduction

*Edgeworthia chrysantha* Lindl. is a deciduous shrub in the family Thymelaeaceae. It exhibits trichotomized branches and produces yellow flowers that typically bloom from late winter to early spring. It is mainly distributed in South Central China, Southeast China, Myanmar, Georgia, Korea, and Japan [1]. *E. chrysantha* is also known as Oriental paperbush, and as its name suggests, the bast fiber made from this plant is used in Japan as a raw material for Washi (traditional Japanese paper) and banknote paper. In China, the buds and roots of *E. chrysantha* have been used in traditional Chinese medicines for the treatment of hoarseness, bruises, arthralgia, neuralgia, and eye diseases such as photophobia, epiphora, and visual impairment [2]. The chemical constituents of *E. chrysantha* have been reported to include coumarins, flavonoids, and sterols [3–6]. Our previous study also reported the presence of macrocyclic daphnane orthoesters (MDOs) in the flower buds [7].

MDOs are a class of 1-alkyldaphnane exclusively isolated from plants of the Thymelaeaceae family. These compounds are characterized by a macrocyclic ring formed by an orthoester-linked aliphatic chain straddling the diterpene skeleton. MDOs have been shown to have potent antineoplastic activity and the ability to eliminate latent HIV-1 cells, making them attractive candidates for anticancer and anti-HIV drugs [8–13]. The most common MDOs are those with a 1-alkyl group originating from a $C_{10}$ aliphatic chain, and to date, over 50 compounds of this type have been reported from the genera *Daphne*, *Daphnopsis*, *Dirca*, *Gnidia*, *Pimelea*, *Stellera*, *Synaptolepis*, and *Wikstroemia* [14,15]. However, the occurrence of other MDO types, characterized by a 1-alkyl group originating from a $C_{16}$ or $C_{14}$ aliphatic chain, is quite limited. The former type has only been reported in species of *Synaptolepis* [16–18], while the latter type has exclusively been isolated from *Edgeworthia chrysantha* [7]. As part of our ongoing investigation of searching for novel biological diterpenoids from plants of the Thymelaeaceae family [19–26], herein, we reported liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) identification of MDOs in various parts (flower buds, flowers, leaves, and stems) of *E. chrysantha*, and the isolation and structural elucidation of two previously unreported MDOs (**1** and **7**) (Fig. 1). Their anti-HIV activity was also evaluated.

## 2. Materials and methods

### 2.1. General experimental procedures

The following instruments were used for physicochemical and spectroscopic measurement: a JASCO P-2200 polarimeter to measure optical rotations in a 0.5 dm cell; a JASCO V-730BIO spectrophotometer

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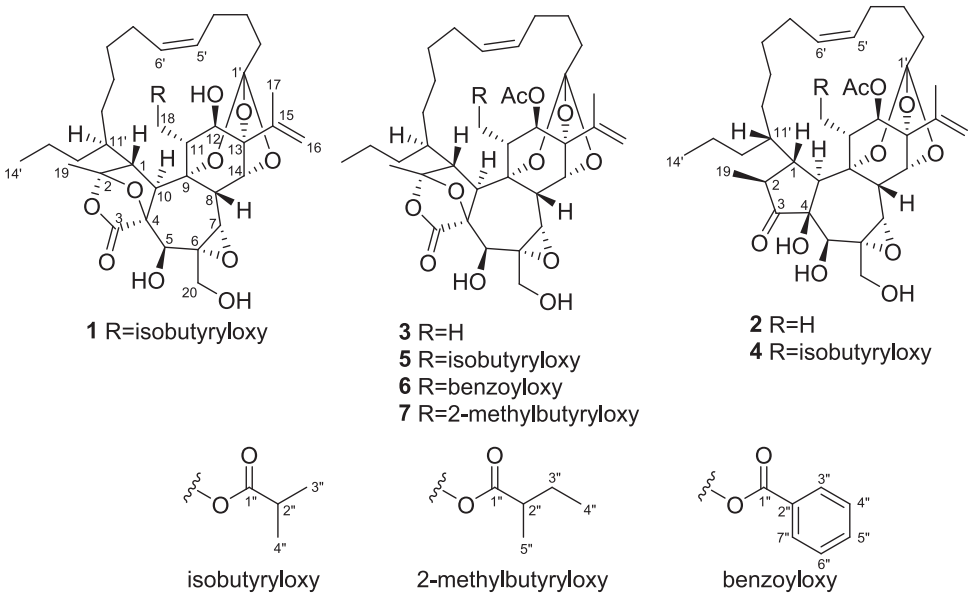


**Fig. 1.** Structures of compounds **1**–**7**.

to measure UV spectra; a JASCO J-1500 spectropolarimeter to measure ECD spectra in a 10 mm cell; a JASCO FT/IR-4100 Fourier transform infrared spectrometer to measure IR spectra by the KBr disk method; a JEOL ECA-500 spectrometer to measure NMR spectra, using $CDCl_3$ as the internal reference; and a Q-Exactive Hybrid Quadrupole Orbitrap mass spectrometer to measure HRESIMS spectra.

Diaion HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan) and ODS silica gel (Chromatorex DM1020T, Fuji Silysia Chemical Ltd., Aichi, Japan) were used for column chromatography. HPLC were performed on a Gradient HPLC system (two JASCO/PU-2080 Plus Intelligent HPLC Pumps, a MX-2080-32 Dynamic Mixer, a Jasco UV-970 Intelligent UV/Vis detector, and a Fraction collector-SSC-6800), or a preparative HPLC system (a Waters 515 HPLC pump, an ERC RefractoMax520 differential refractometer detector, and a Shimadzu SPD-10 A UV–vis detector). A RP-$C_{18}$ silica gel column (YMC-Actus Triart $C_{18}$, 5 μm, 150 × 20 mm) at a flow rate of 8.0 mL/min and a silica gel column (YMC-Pack SIL, 5 μm, 250 × 20 mm) at a flow rate of 5.0 mL/min were used for HPLC separation.

### 2.2. Plant material

The plants of *Edgeworthia chrysantha* Lindl. were cultivated at Toho University Medicinal Plant Garden, Chiba, Japan. Different plant parts were collected: leaves and stems in December 2018, flowers in March 2020, and flower buds in February 2021. The plant materials were identified by one of the authors, W. L. Voucher specimens [THMPG-1 (leaves), THMPG-2 (stems), THMPG-3 (flowers), and THMPG-4 (flower buds)] have been deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Toho University, Japan.

### 2.3. LC-MS analysis

LC-MS analysis was carried out using a Vanquish UHPLC system connected to a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) with an ESI source, with the same conditions as previously described (Otsuki et al., 2021). Briefly, a YMC-Triart C18 column (2.1 × 150 mm, 1.9 μm) was used and the column temperature was set at 40 °C. A mobile phase composed of A (0.1 vol% formic acid in distilled water) and B (0.1% formic acid in acetonitrile) was used for gradient elution: 0–15 min at 50–100% B at a flow rate of 0.4 mL/min. ESI-MS data were obtained in the Full MS and Full MS/data dependent (dd)-MS/MS modes. The extracted ion chromatogram was generated by extracting the following mass ranges at 5 ppm mass tolerance: *m/z* 703.3688 $[M + H]^+$ (**1**), *m/z* 645.3633 $[M + H]^+$ (**2**), *m/z* 659.3426 $[M + H]^+$ (**3**), *m/z* 731.4001 $[M + H]^+$ (**4**), *m/z* 745.3794 $[M H]^+$ (**5**), *m/z* 779.3637 $[M + H]^+$ (**6**), and *m/z* 759.3950 $[M + H]^+$ (**7**).

### 2.4. Extraction and isolation

The air-dried flowers of *E. chrysantha* (330 g) were extracted with MeOH at room temperature to afford the MeOH extract (60.6 g). Partition between EtOAc and $H_2O$ afforded the EtOAc fraction (12.3 g), which was subjected to a Diaion HP-20 column and eluted with a stepwise gradient of MeOH−$H_2O$ (from 5:5 to 10:0, *v/v*) to afford four fractions (E1 to E4). Fraction E3 (4.93 g) was separated by gradient HPLC with a gradient of MeOH–$H_2O$ (from 7:3 to 10:0, *v/v*), to afford twelve fractions (E3-1 to E3-12). Fraction E3-10 (336.0 mg) was separated by RP-HPLC ($CH_3CN$−$H_2O$ = 75:25, *v/v*) and NP-HPLC ($CHCl_3$–MeOH = 97:3) to give **1** (1.6 mg).

The MeOH extract (499 g) was obtained from the air-dried stems (4.83 kg) by the same extraction procedure as flowers, then partitioned between EtOAc and $H_2O$ to afford the EtOAc fraction (70.5 g). Further Diaion HP-20 column chromatography afforded fraction E3 (22.5 g), which was subjected to an ODS column and eluted with a stepwise gradient of MeOH−$H_2O$ (from 7:3 to 10:0, *v/v*). Fraction E3-4 (5.14 g) was separated by gradient HPLC with a gradient of MeOH–$H_2O$ (from 7:3 to 10:0, *v/v*) to afford fraction E3-4-11 (373 mg), then by RP-HPLC ($CH_3CN$−$H_2O$ = 80:20, *v/v*) and NP-HPLC ($CHCl_3$–MeOH = 98:2, *v/v*) to give **7** (1.4 mg).

#### 2.4.1. Edgeworthianin F (1)

Colorless solid; $[\alpha]_D^{25}$ + 14.4 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 221 (2.98) nm; ECD (MeOH): $[\theta]^{25}$ (nm) 8290 (222); IR (KBr) $\nu_{max}$ 3425, 2960, 2927, 2856, 1794, 1741, 1631, 1560, 1542, 1459, 1397, 1384, 1261, 1227, 1184 $cm^{-1}$; $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 1; HRESIMS (positive) *m/z* 703.3674 $[M + H]^+$ (calcd for $C_{38}H_{55}O_{12}$, 703.3688).

#### 2.4.2. Edgeworthianin G (7)

Colorless solid; $[\alpha]_D^{25}$ + 13.9 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 221 (3.13) nm; ECD (MeOH): $[\theta]^{25}$ (nm) 11,158 (223); IR (KBr) $\nu_{max}$

**Table 1**
$^{1}H$ (500 MHz) and $^{13}C$ (125 MHz) NMR spectroscopic data of compounds **1** and **7** ($CDCl_3$).

| No. | **1** | | | **7** | | |
|---|---|---|---|---|---|---|
| | $\delta_H$ ($J$ in Hz) | $\delta_C$, type | | $\delta_H$ ($J$ in Hz) | $\delta_C$, type | |
| 1 | 2.69, dd (6.0, 5.1) | 51.6 | CH | 2.72, t (6.0) | 51.7 | CH |
| 2 | | 112.7 | C | | 112.8 | C |
| 3 | | 173.8 | C | | 173.6 | C |
| 4 | | 86.6 | C | | 86.5 | C |
| 5 | 4.90, s | 68.6 | CH | 4.88, s | 68.7 | CH |
| 6 | | 59.3 | C | | 59.3 | C |
| 7 | 3.48, br s | 59.2 | CH | 3.53, br s | 58.9 | CH |
| 8 | 4.09, br d (2.6) | 33.1 | CH | 3.90, d (2.9) | 33.6 | CH |
| 9 | | 79.5 | C | | 79.2 | C |
| 10 | 3.05, d (5.1) | 51.3 | CH | 3.06, d (5.5) | 51.1 | CH |
| 11 | 2.02, m | 52.0 | CH | 2.03, d (7.1) | 50.6 | CH |
| 12 | 4.38, s | 74.4 | CH | 5.36, s | 74.4 | CH |
| 13 | | 84.4 | C | | 83.2 | C |
| 14 | 4.60, d (2.6) | 79.4 | CH | 4.63, d (2.9) | 79.3 | CH |
| 15 | | 144.4 | C | | 142.7 | C |
| 16 | 5.07, br s | 112.7 | $CH_2$ | 4.94, s<br>4.98, br s | 113.2 | $CH_2$ |
| 17 | 1.84, s | 18.6 | $CH_3$ | 1.79, br s | 18.5 | $CH_3$ |
| 18 | 4.26, d (12.6)<br>4.38, dd (12.6, 6.3) | 67.4 | $CH_2$ | 4.20, d (12.9)<br>4.61, dd (12.9, 7.1) | 65.7 | $CH_2$ |
| 19 | 1.78, s | 19.1 | $CH_3$ | 1.78, s | 18.9 | $CH_3$ |
| 20 | 3.70, d (12.3)<br>4.07, d (12.3) | 63.3 | $CH_2$ | 3.72, d (12.6)<br>4.10, d (12.6) | 63.1 | $CH_2$ |
| 1′ | | 119.9 | C | | 120.1 | C |
| 2′ | 1.83, 1.91, m | 34.9 | $CH_2$ | 1.87, 1.92, m | 34.8 | $CH_2$ |
| 3′ | 1.63, m | 24.2 | $CH_2$ | 1.62, m | 24.2 | $CH_2$ |
| 4′ | 1.99, 2.14, m | 26.8 | $CH_2$ | 1.99, 2.15, m | 26.8 | $CH_2$ |
| 5′ | 5.44, dt (10.6, 7.3) | 129.0 | CH | 5.44, dt (10.9, 7.2) | 129.0 | CH |
| 6′ | 5.36, dt (10.6, 7.4) | 131.2 | CH | 5.37, dt (10.9, 7.5) | 131.2 | CH |
| 7′ | 2.00, m | 25.6 | $CH_2$ | 2.01, m | 25.7 | $CH_2$ |
| 8′ | 1.40, m | 28.1 | $CH_2$ | 1.39, m | 28.1 | $CH_2$ |
| 9′ | 1.26, 1.40, m | 25.1 | $CH_2$ | 1.25, 1.36, m | 25.2 | $CH_2$ |
| 10′ | 1.34, 1.68, m | 30.0 | $CH_2$ | 1.33, 1.64, m | 30.1 | $CH_2$ |
| 11′ | 1.50, m | 38.0 | CH | 1.50, m | 37.8 | CH |
| 12′ | 1.36, m | 33.0 | $CH_2$ | 1.35, m | 33.1 | $CH_2$ |
| 13′ | 1.30, m | 19.2 | $CH_2$ | 1.28, m | 19.3 | $CH_2$ |
| 14′ | 0.87, t (6.9) | 14.4 | $CH_3$ | 0.88, t (6.6) | 14.4, | $CH_3$ |
| $OCOCH_3$ | | | | | 168.9 | C |
| | | | | 1.95, s | 21.1 | $CH_3$ |
| 1″ | | 177.0 | C | | 176.4 | C |
| 2″ | 2.54, sept (6.9) | 34.1 | CH | 2.39, sext (6.9) | 41.2 | CH |
| 3″ | 1.17, d (6.9) | 18.9 | $CH_3$ | 1.47, 1.71, m | 26.5 | $CH_2$ |
| 4″ | 1.18, d (6.9) | 19.0 | $CH_3$ | 0.90, t (7.5) | 11.7 | $CH_3$ |
| 5″ | | | | 1.19, d (6.9) | 16.6 | $CH_3$ |

3429, 2958, 2925, 2854, 1794, 1737, 1631, 1602, 1572, 1466, 1397, 1341, 1261, 1189, 1156, 1098, 1032 $cm^{-1}$; $^{1}H$ and $^{13}C$ NMR spectroscopic data, see Table 1; HRESIMS (positive) $m/z$ 759.3939 $[M + H]^+$ (calcd for $C_{41}H_{59}O_{13}$, 759.3950).

### 2.5. Anti-HIV assay and cytotoxicity study

MT4 lymphocytes were infected with HIV-$1_{NL4-3}$ Nanoluc-*sec* virus in the presence of compounds at various concentrations in 96-well plates [27]. On the third day post-infection, supernatant samples were harvested and luciferase activity was measured using the Nano-Glo Luciferase Assay System (Promega). Cytotoxicity of the compounds on MT4 cells was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). $EC_{50}$ and $IC_{50}$ were calculated by using CalcuSyn (Biosoft) and were defined as the concentration of compound that reduced the luciferase activity by 50% and the cell viability by 50%, respectively. Edgeworthianins A (**3**) and B (**5**) were isolated from *E. chrysantha* in our previous study and used as reference compounds in the assays [7].

## 3. Results and discussion

### 3.1. LC-MS analysis of the flower buds, flowers, leaves, and stems of E. chrysantha

Liquid chromatography–mass spectrometry (LC-MS) analysis was performed on different parts of *E. chrysantha* including flower buds, flowers, leaves, and stems. Each plant part was extracted with MeOH, then partitioned between EtOAc and $H_2O$. The EtOAc fractions were pretreated with a Sep-Pak C18 Cartridge and applied to the LC-MS analysis. Ultra-high performance liquid chromatography coupled with Q-Exactive hybrid quadrupole-orbitrap mass spectrometer was used for qualitative analysis with an ESI source and full MS and data-dependent MS/MS (dd-$MS^2$) scans at higher energy collision–induced dissociation (HCD). Five MDOs obtained in our previous study, including edgeworthianin D (**2**), edgeworthianin A (**3**), edgeworthianin E (**4**), edgeworthianin B (**5**), and edgeworthianin C (**6**) [7], were also subjected to the LC-MS analysis for reference. As a result, comparison of the molecular formula calculated by high-resolution accurate mass and the retention time with previously isolated MDOs led to the identification of MDOs **2**–**6** in all four parts of *E. chrysantha*. Further analysis of the ESI-MS/MS spectra of the detected peaks in the flower buds, which had a high content of known MDOs, revealed two additional MDOs **1** and **7**. Their exhibited an ESI-MS/MS fragmentation pattern remarkably similar to MDOs **5** and **6**, but possessed different molecular formulas from **5** and **6**, and therefore, they were postulated to be previously unreported MDOs (Fig. S1 and Table S1). The extracted ion chromatograms, generated by extracting the accurate mass calculated from the molecular formula for the abovementioned seven MDOs, demonstrated the presence of common MDOs in all four parts of *E. chrysantha*, despite variations in their content (Fig. 2 and Table S2).

### 3.2. ESI-MS/MS fragmentation elucidation of compounds 1 and 7

Fragmented ion data obtained from ESI-MS/MS spectra provided valuable information for structural characterization [28,29]. Our previous study demonstrated that MDOs exhibited abundant product ions in positive ion ESI-MS/MS, which were applicable to the structural elucidation of MDOs [24]. Hence, the ESI-MS/MS fragmentation pathway was employed to elucidate the structure for compounds **1** and **7** (Fig. 3).

The ESI-MS/MS spectra of peaks 1 and 7 were obtained from the protonated molecule as precursor ion, and the product ions observed at $m/z$ 450–600 and $m/z$ 250–400 for both peaks 1 and 7 were remarkably similar to peaks 5 and 6 (Table S1). The fragmentation pathway for these ions were interpreted as shown in Fig. 3. Initially, the elimination of the substituent attached to C-18 and the dissociation of the orthoester moiety occurred. Subsequently, fragmentation was carried out via two major routes, A and B. In route A, following the elimination of the substituent attached to C-12, consecutive losses of $H_2O$ and CO from the diterpene skeleton produced a series of $C_{34}$ to $C_{32}$ fragment ions in the range of $m/z$ 450–600. In addition, the 1,3-shift rearrangement of the double bond following the opening of the ether moiety in the bicyclo [2.2.1]heptane A-ring allowing a $CO_2$ elimination through the retro-ene reaction. On the other hand, in route B, the 1-alkyl moiety at the A-ring was eliminated as a $C_{14}H_{22}O$ unit by the retro-ene reaction, which was followed by the elimination of the substituent attached to C-12. Subsequently, similar to route A, successive losses of $H_2O$, CO, and $CO_2$ from the diterpene skeleton generated a series of $C_{20}$ to $C_{18}$ fragment ions in the range of $m/z$ 250–400.

Elucidation of the ESI-MS/MS spectra based on the above fragmentation pathway suggested that compounds **1** and **7** have the same diterpenoid skeletal structure as compounds **5** and **6**, but the substituents attached to C-12 and C-18 were different. Namely, a neutral loss of $C_4H_8O_2$ from the precursor ion was observed in MS/MS spectrum of compound **1**, indicating the presence of an isobutyryloxy group at C-18. On the other hand, compound **7** showed a neutral loss of $C_5H_{10}O_2$

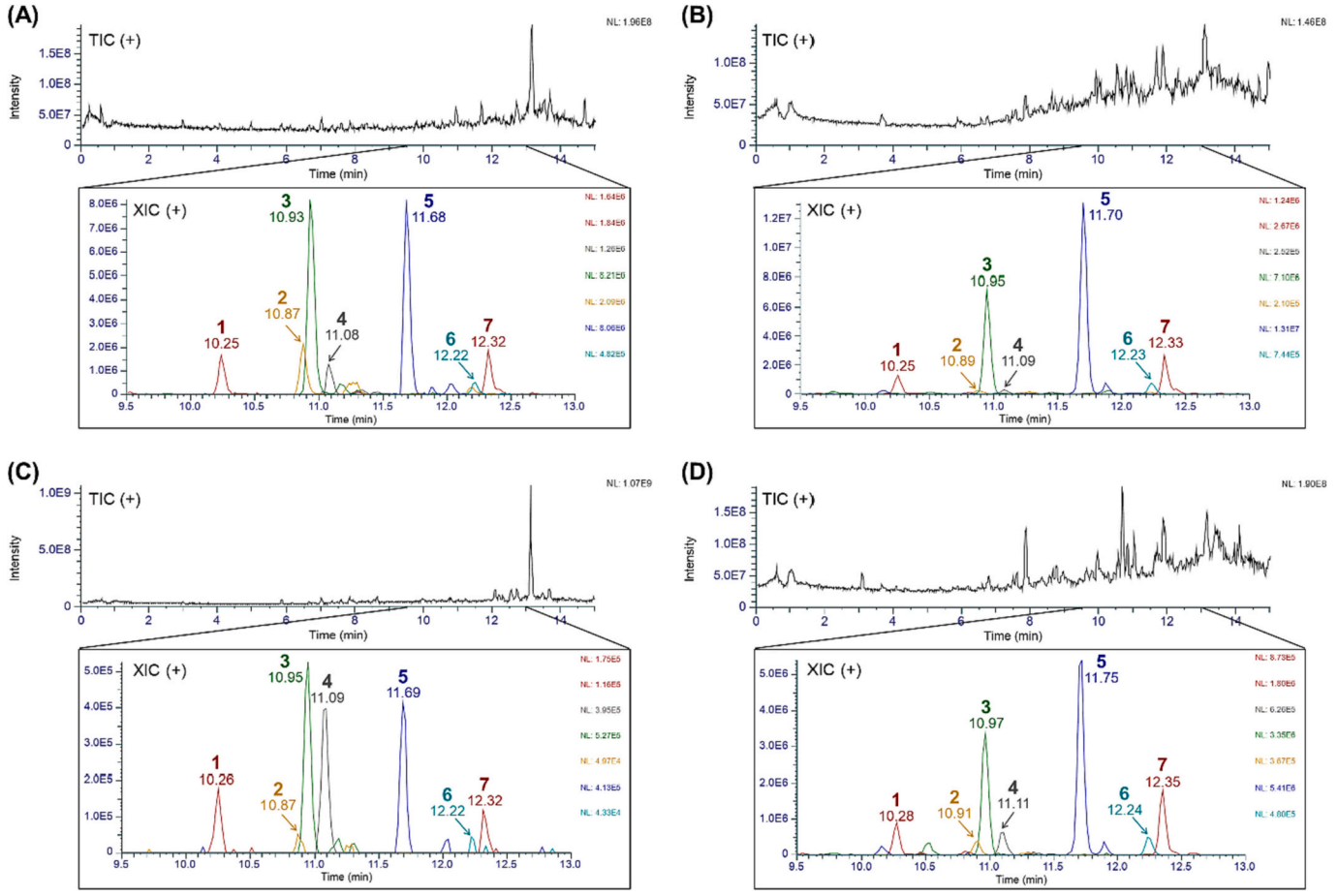


**Fig. 2.** Total ion chromatogram and extracted ion chromatogram in the positive ion mode of peaks **1**–**7** from the EtOAc fraction of *E. chrysantha*; (A) flower buds, (B) flowers, (C) leaves, and (D) stems.

from the precursor ion, followed by a neutral loss of $C_2H_4O_2$, suggesting the presence of a pentanoyloxy moiety or its structural isomer at C-18, and an acetyloxy group at C-12.

### 3.3. LC-MS-guided isolation of compounds *1* and *7*

Compounds **1** and **7** were isolated by LC-MS-guided separation from the flowers and stems of *E. chrysantha*, respectively. The dried flowers or stems were extracted with MeOH, then partitioned between EtOAc and $H_2O$. The EtOAc fraction was further separated by Diaion HP-20 column chromatography and repeated preparative HPLC. During the isolation procedure, each fraction was monitored by LC-MS analysis and further separation was conducted for the fractions containing peaks 1 or 7. As a result, the compound in accordance with peak 1 was isolated from the flowers and named as edgeworthianin F (**1**), while the compound in accordance with peak 7 was isolated from the stems and was named as edgeworthianin G (**7**).

### 3.4. Structural elucidation of compounds *1* and *7*

Edgeworthianins F (**1**) and G (**2**) were both obtained as colorless solids, showing $[\alpha]_D^{26}$ + 14.4 (*c* 0.10, MeOH) and + 13.9 (*c* 0.10, MeOH), respectively. Their $^1$H and $^{13}$C NMR data showed the characteristic resonances for an isopropenyl moiety, a methine proton at H-1, and a typical orthoester carbon at C-1′, suggesting that they were MDO diterpenoids (Table 1). In addition, the characteristic resonances of the macrocyclic ring formed from $C_{14}$ unsaturated aliphatic chain were observed for in **1** and **7**, suggesting that their macrocyclic ring were formed from unsaturated aliphatic chain [7].

The molecular formula of edgeworthianin F (**1**) was determined as $C_{38}H_{54}O_{12}$ by a HRESIMS protonated molecular ion at *m/z* 703.3674 $[M + H]^+$ (calcd for $C_{38}H_{55}O_{12}$, 703.3688). The IR absorptions revealed the presence of hydroxy (3425 cm$^{-1}$) and carbonyl (1741 cm$^{-1}$) functionalities. In the $^1$H and $^{13}$C NMR spectra, the characteristic resonances for a methyl singlet at $\delta_H$ 3.70 (3H, s, H-19), an acetal carbon at $\delta_C$ 112.7 (C-2), and a lactone carbonyl carbon at $\delta_C$ 173.7 (C-3) suggested that the presence of a bicyclo[2.2.1]heptane ring structure in A-ring. The position was further confirmed by HMBC correlations from H-1, $H_3$-19 to C-2, and H-10, H-5 to C-3. Additional resonances due to polyoxygenated functionalities in the daphnane skeleton were observed for an epoxy group at $\delta_H$ 3.48 (1H, s, H-7), $\delta_C$ 59.3 (C-6) and 59.2 (C-7), three oxygenated methines at $\delta_H$ 4.90 (1H, s, H-5), 4.38 (1H, s, H-12), and 4.60 (1H, d, $J$ = 2.6 Hz, H-14), an oxygenated methylene at $\delta_H$ 3.70 (1H, d, $J$ = 12.3 Hz, H-20a) and 4.07 (1H, d, $J$ = 12.3 Hz, H-20b), and three oxygenated tertiary carbons at $\delta_C$ 86.6 (C-4), 79.5 (C-9), and 84.4 (C-13). Their positions were assigned by comprehensive analyses of the $^1$H—$^1$H COSY and HMBC spectra (Fig. 4). Furthermore, the presence of isobutyryloxy moiety was indicated by the resonances of geminal dimethyl protons at $\delta_H$ 1.17 (3H, d, $J$ = 6.9 Hz, H-3″) and 1.18 (3H, d, $J$ = 6.9 Hz, H-4″), a methine proton at $\delta_H$ 2.54 (1H, sept, $J$ = 6.9 Hz, H-2″), and an ester carbonyl carbon at $\delta_C$ 177.7 (C-1″), and its position was confirmed by the HMBC correlation from $H_2$-18 to C-1″.

In the macrocyclic ring part, the proton resonances were observed for two olefinic protons at $\delta_H$ 5.44 (1H, dt, $J$ = 10.6, 7.3 Hz, H-5′) and 5.36 (1H, dt, $J$ = 10.6, 7.4 Hz, H-6′) and *n*-propyl moiety, including three methylenes at $\delta_H$ 1.50 (1H, m, H-11′), 1.36 (2H, m, H-12′), and 1.30 (2H, m, H-13′) and a terminal methyl group at $\delta_H$ 0.87 (3H, t, $J$ = 6.9 Hz, H-14′), which indicated the macrocyclic ring formed from a $C_{14}$

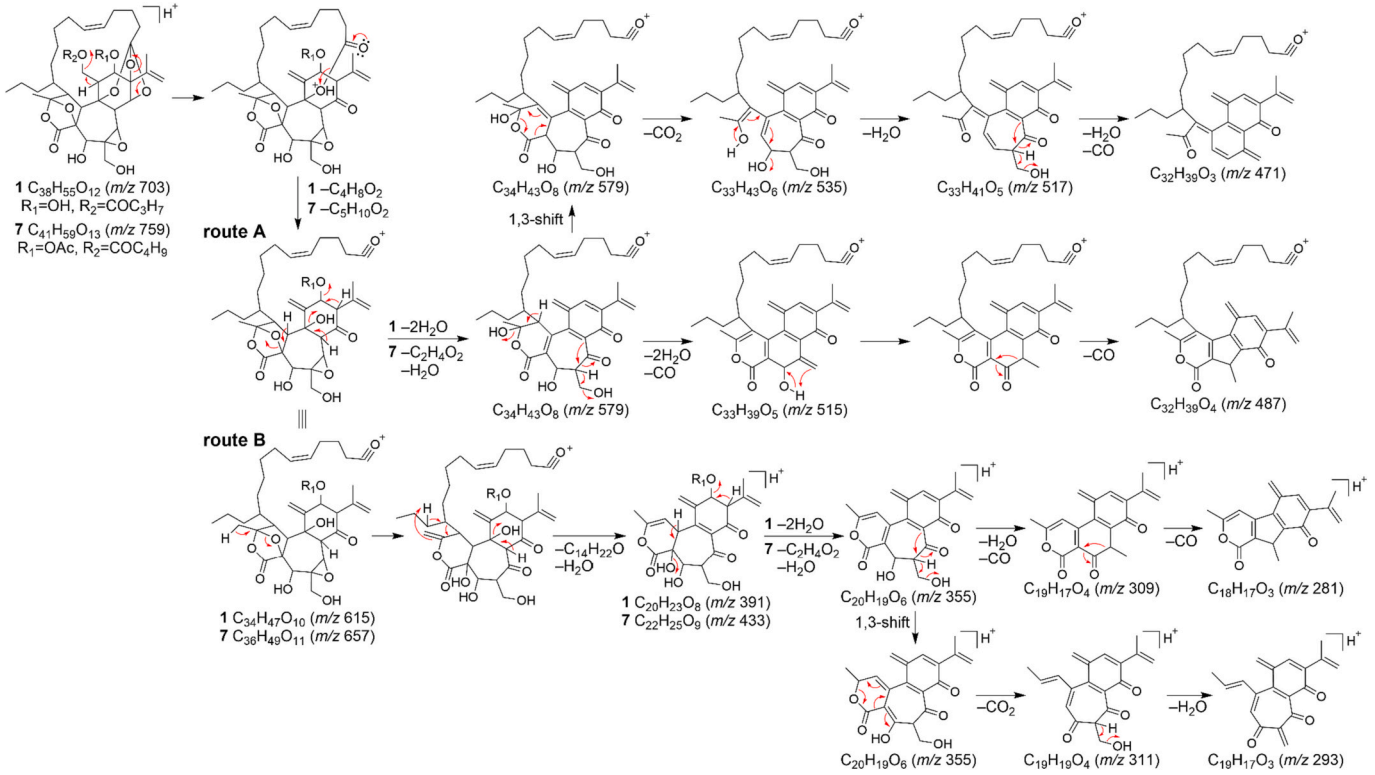


**Fig. 3.** Proposed ESI-MS/MS fragmentation pathways of compounds **1** and **7**.

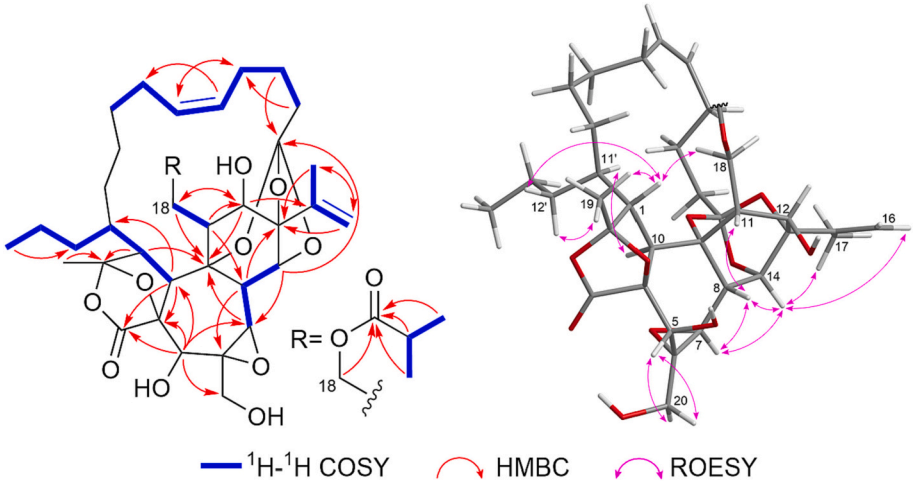


**Fig. 4.** Key $^{1}$H—$^{1}$H COSY, HMBC, and ROESY correlations of compound **1**. The $^{1}$H—$^{1}$H COSY correlations from $H_2$-8′ to H-11′ were difficult to accurate assignment due to the overlapping proton resonances and were not shown in the figure.

unsaturated aliphatic chain. The configuration of the olefin group was determined as *cis* by the small coupling constant $J_{5',6'} = 10.6$ Hz, and the position was assigned to be $\Delta^{5',6'}$ by the $^{1}$H – $^{1}$H COSY correlations from $H_2$-2′ to $H_2$-8′, as well as the HMBC correlation from H-14 and H-3′ to C-1′. The connection of C-11′ and C-1 was deduced by the $^{1}$H—$^{1}$H COSY correlations from H-11′ to H-1 and the HMBC correlations from H-10 to C-11′.

The relative configurations of **1** were determined by detailed interpretation of the ROESY spectrum and proton couplings. The ROESY correlations between H-7/H-14, H-8/H-11, H-14/$H_2$–16, H-14/$H_3$–17 indicated that H-7, H-8, H-11, H-14, and the isopropenyl moiety at C-13 have β-orientations, while H-5 and H-10 have α-orientations (Fig. 4). Furthermore, the β-orientation of H-1 and α-orientation of H-11′ were determined by the ROESY correlations between $H_2$-18/H-1, H-1/$H_2$-12′, $H_3$-19/$H_2$-12′, and H-10/H-11′. The characteristic proton resonance of H-7 as a broad singlet and H-12 as a singlet indicated that the dihedral angle between H-7/H-8 and H-11/H-12 is close to 90°, which supported the aforementioned determinations [7].

The molecular formula of edgeworthianin G (**7**) was established as $C_{41}H_{58}O_{13}$ from the HRESIMS positive ion peak at *m/z* 759.3939 $[M + H]^+$ (calculated for $C_{41}H_{59}O_{13}$, 759.3950). Comparison of the NMR data of **7** and **1** suggested that they have similar MDO structures, but differ in the acyloxy moieties attached to C-12 and C-18 (Table 1). The acetyloxy moiety was evident from the proton resonance for an acetyl methyl at $\delta_H$ 1.95 (3H, s, OCOC$H_3$). The 2-methylbutyloxy moiety was identified from the proton resonances for a triplet methyl at $\delta_H$ 0.90 (3H, t, $J = 7.5$

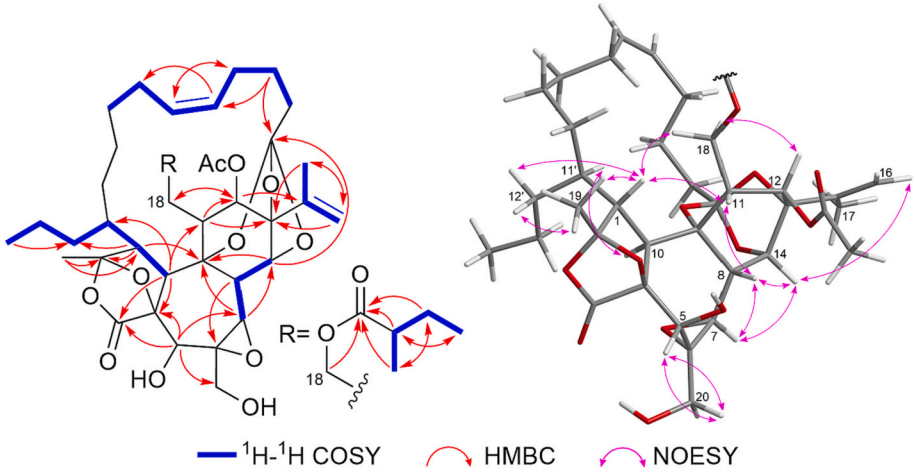


**Fig. 5.** Key $^1$H—$^1$H COSY, HMBC, and NOESY correlations of compound **7**. The $^1$H—$^1$H COSY correlations from $H_2$-8′ to H-11′ were difficult to accurate assignment due to the overlapping proton resonances and were not shown in the figure.

**Table 2**
Anti-HIV and cytotoxic activities of compounds **1**, **3**, **5**, and **7**.

| Compound | anti-HIV (NL4–3) $EC_{50}$ (μM) | cytotoxicity (MT4) $IC_{50}$ (μM) |
|---|---|---|
| **1** | 1.61 ± 0.41 | 31.6 ± 3.56 |
| **3** | 0.85 ± 0.23 | > 30.0 |
| **5** | 0.10 ± 0.02 | > 4.0 |
| **7** | 7.03 ± 1.22 | 23.2 ± 2.77 |

The values are means ± SD ($n$ = 3).

Hz, H-4″), a doublet methyl at $\delta_H$ 1.19 (3H, d, $J$ = 6.9 Hz, H-5″), a methylene at $\delta_H$ 1.47 (1H, m, H-3″a) and 1.71 (1H, m, H-3″b), and a methine at $\delta_H$ 2.39 (1H, sext, $J$ = 6.9 Hz, H-2″). Its structure was also confirmed by the $^1$H—$^1$H COSY correlations from H-2″ to $H_3$-5″ and H-2″ to $H_3$-4″ and the HMBC correlations from $H_2$-3″ and $H_3$-5″ to C-1″ (Fig. 5). The position of the acyloxy moieties were determined to be the acetyloxy moiety attached to C-12 and the 2-methylbutyloxy moiety attached to C-18 by observing of the HMBC correlations from H-12 and H-18 to the respective ester carbonyl carbons. Moreover, detailed analysis of proton couplings and a NOESY spectrum of **7** indicated that the relative configurations of **7** were the same as those of **1** (Fig. 5). Due to the limited isolation yield of **7**, it was not feasible to determine the absolute configuration of C-2″.

The ECD spectra of compounds **1** and **7** showed a positive Cotton effect at around 230 nm, which could be assigned to be the n → π* transition of the A-ring functionalities [7]. This Cotton effect was the same as for daphnepedunin E [26] and stelleralide D [30], which were known MDOs with a bicyclo[2.2.1]heptane ring structure in A-ring, and the absolute configuration of the the daphnane skeleton part of these compounds was considered in agreement with pimelotide A, whose absolute configuration was determined by X-ray crystallographic data [26,31]. Thus, the structure of edgeworthianins F (**1**) and G (**7**) were determined as shown in Fig. 1.

### 3.5. Anti-HIV activity

Given that MDOs have previously reported remarkable anti-HIV activity, the newly isolated MDOs **1** and **7** were evaluated for their activity against HIV-1 infection of human T-lymphocyte cell line MT4 (Table 2) [13,21,24,26]. Edgeworthianins A (**3**) and B (**5**), which reported anti-HIV activity in our previous study, were also evaluated together as reference compounds [7]. As a result, all compounds exhibited anti-HIV activity with $EC_{50}$ value range of 0.10–7.03 μM. In comparison with MDOs **5** ($EC_{50}$ = 0.10 μM) and **1** ($EC_{50}$ = 1.61 μM), there was a significant reduction in efficacy due to the replacement of the acetyloxy moiety at C-12 with a hydroxyl group. Furthermore, our previous study demonstrated that irrespective of the structure of the A ring, the isobutyryloxy moieties at C-18 showed stronger anti-HIV activity than no substituent or the benzoyloxy moieties at C-18, suggesting that aliphatic substituents may enhance the anti-HIV activity [7]. However, in the present study, MDOs **5** and **7** ($EC_{50}$ = 7.03 μM) indicated a substantial decrease in anti-HIV activity when the 2-methylbutyloxy moiety at C-18.

## 4. Conclusion

The distribution of MDOs with a 1-alkyl group originating from a $C_{14}$ aliphatic chain in the plant kingdom is extremely limited in both number and structural diversity. In the present study, LC-ESI-MS/MS analysis resulted in the presence of seven MDOs in different parts (flower buds, flowers, leaves, and stems) of *E. chrysantha*, including two previously unreported MDOs, **1** and **7**. The structures of **1** and **7** were estimated through ESI-MS/MS fragmentation elucidation and validated by various spectroscopic analyses. The isolated MDOs, **1** and **7**, were also evaluated for anti-HIV activity, broadening the comprehension of structure-activity relationships for MDOs.

## CRediT authorship contribution statement

**Kouharu Otsuki:** Methodology, Investigation, Formal analysis, Writing – original draft, Project administration, Funding acquisition. **Tsubasa Kobayashi:** Investigation. **Kazuki Nakamura:** Investigation. **Takashi Kikuchi:** Methodology, Writing – review & editing. **Li Huang:** Investigation. **Chin-Ho Chen:** Methodology, Investigation, Writing – review & editing, Funding acquisition. **Kazuo Koike:** Conceptualization, Project administration. **Wei Li:** Conceptualization, Methodology, Supervision, Project administration, Writing – review & editing, Funding acquisition.

## Declaration of competing interest

The authors declare no conflicts of interest.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2023.105731.

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