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A NEW E-RING γ-LACTONE PENTACYCLIC TRITERPENE FROM *Lysimachia clethroides* AND ITS CYTOTOXIC ACTIVITIES

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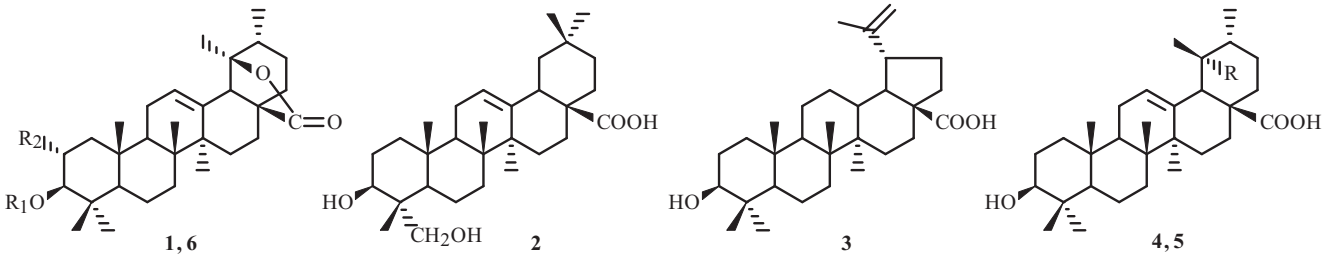
A new E-ring γ-lactone pentacyclic triterpene, 3β-myristoxyurs-12-en-19,28-olide, has been isolated from the chloroform extract of Lysimachia clethroides *Duby, together with four known pentacyclic triterpenoid acids. The structures of the compounds were elucidated by comprehensive spectroscopic analyses. All of the compounds showed various degrees of cytotoxic activities in MTT assay.*

Keywords: *Lysimachia clethroides*, cytotoxicity, pentacyclic triterpene, γ-lactone, 3β-myristoxyurs-12-en-19,28-olide.

Lysimachia clethroides Duby is a perennial herb indigenous to many parts of the tropics and subtropics, including the East and South China regions. Previous studies on constituents from the *Lysimachia* genus led to the isolation of different compounds such as flavones, triterpenoids, and benzoquinones [1–4]. The herb *Lysimachia clethroides* Duby has long held a place in natural medicine in the subtropical regions where it grows. People in the East and South China regions use *Lysimachia clethroides* Duby for edema, pyretic stranguria, jaundice, furuncle, and leukemia in folklore [5]. Our previous studies have resulted in the isolation of a series of triterpenoids from *Lysimachia clethroides* Duby [6]. In the course of our investigation for bioactive agents from the chloroform extract of *Lysimachia clethroides* Duby, a new E-ring γ-lactone pentacyclic triterpene, 3β-myristoxyurs-12-en-19,28-olide (**1**), together with four known compounds, hederagenin (**2**) [7], betulinic acid (**3**) [8], ursolic acid (**4**) [9], and 19α-hydroxyurs-12-en-28-oic acid (**5**) [10], were obtained from its chloroform extract. We now report the isolation and structure elucidation of the new compound (**1**) and the cytotoxic activities of the isolated compounds.



1, 6 **2** **3** **4, 5**

1: R_1 = -C(O)-$(CH_2)_{12}$-CH_3, R_2 = H
6: R_1 = H, R_2 = OAc

1 —saponification→ **5**

4: R = H
5: R = OH

3β-Myristoxyurs-12-en-19,28-olide (**1**) was obtained as a white powder. The molecular formula was determined to be $C_{44}H_{72}O_4$ by high-resolution (HR) – electrospray ionization (ESI) – mass spectrometry, which showed a series of quasi-molecular ion peaks at *m/z* 665.5502 $[M + H]^+$, 687.5322 $[M + Na]^+$, and 703.5061 $[M + K]^+$. Its IR spectrum exhibited absorption bands at 1388 and 1372 (gem-dimethyl), 1764 (γ-lactone) and 1722 (ester), and 1604 cm^{-1} (trisubstituted double bond). Interpretation of the ^1H and ^{13}C NMR spectral data together with 2D NMR data led to structure **1**. The ^1H NMR spectrum of **1** showed singlet resonances of six tertiary methyl groups at δ 0.72, 0.81, 1.12, 1.19, 1.28, and 1.43 and a secondary methyl signal at 1.14 (d, J =7.5 Hz) corresponding to Me-30, as well as a proton signal at 4.25 (dd, J = 9.8, 4.5 Hz) corresponding to H-3.

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TABLE 1. *In vitro* Cytotoxic Activities of Triterpenoids from *Lysimachia clethroides* (IC_{50} values in μg/mL)

Compound	A549	SK-MES-1	NCI-H446
1	22.16 ± 3.70	19.92 ± 3.66	26.25 ± 3.12
2	11.05 ± 2.05	9.57 ± 0.88	7.83 ± 1.14
3	26.86 ± 5.31	21.89 ± 3.18	24.17 ± 4.10
4	18.12 ± 3.23	16.24 ± 3.18	16.98 ± 2.86
5	14.96 ± 2.62	17.85 ± 2.62	19.22 ± 3.42
Norcantharidin	0.76 ± 0.011	0.92 ± 0.015	0.52 ± 0.007

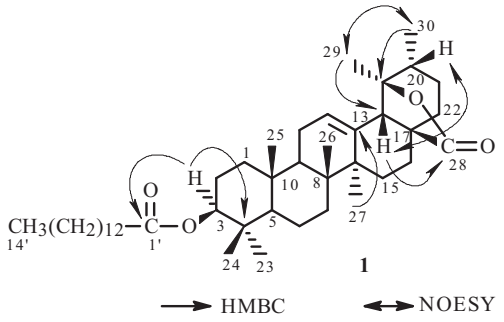

Fig. 1. HMBC and NOESY correlations of compound **1**.

Close inspection of the ^{1}H NMR revealed the presence of a singlet signal at δ 2.50 attributed to H-18, compatible with the absence of a proton at the C-19 position, thus confirming the tertiary nature of that carbon atom. Finally, the vinylic proton at δ 5.12 appeared as a triplet (J = 3.6 Hz), compatible with the existence of the allylic methylene group at C-11, which suggested that compound **1** belongs to the Δ^{12} ursane-type pentacyclic triterpenes. The lactone group was established at C-19 and C-28 on the basis of down-field shifts of C-19 at δ 80.6 and C-28 at δ 183.2 [11]. The myristoyl group appeared to be located at C-3 based upon the downfield shift of H-3 at δ 4.25. The proposed structure of compound **1** was verified by a heteronuclear multiple bond connectivity (HMBC) experiment (Fig. 1). The EI-MS of compound **1** corroborated the above structure, with important fragments at *m/z* 211 (myristoyl group), 246 and 418 (Retro Diels-Alder fragmentations), and 637 (M + 1 – CO).

The configuration of the lactone ring attached to C-19 was envisaged to be β, as the result of the epimerization at C-19 concomitant to the change in conformation of ring E from chair as in 19α-hydroxyurs-12-en-28-oic acid (**5**) to inversed boat in 3β-myristoxyurs-12-en-19,28-olide (**1**). This was corroborated by molecular model building, which showed the inversed boat conformation to be the most plausible for the formation of the lactone ring [11]. As a consequence of the above epimerization, the Me-29 group had to be α. According to the Me-30 group, it has the same orientation as in other similar derivatives. The configurations of the lactone ring, Me-29 group, and the Me-30 group in compound **1** were further supported by its nuclear overhauser effect spectroscopy (NOESY) spectrum (Fig. 1).

This structure was also confirmed by alkaline hydrolysis of compound **1**, which led to 19α-hydroxyurs-12-en-28-oic acid (**5**) and myristic acid [12]. During the course of saponification of compound **1**, the β configuration of the lactone group at C-19 turn into an α configuration of the hydroxyl group as the result of the Walden inversion at C-19. In the light of the above evidence, compound **1** was identified as 3β-myristoxyurs-12-en-19,28-olide. The structure of compound **1** was also confirmed by the comparison of ^{13} C NMR spectral data with those of serratolide (**6**) [11] under the same test condition.

Cytotoxic activities of compounds **1**–**5** were determined by MTT assay and expressed as IC_{50} ± SD (Table 1), and all these compounds showed some cytotoxic activities against human lung adenocarcinoma A549 cell, human lung squamous carcinoma SK-MES-1 cell, and human small cell lung cancer NCI-H446 cells. The cytotoxic activities of **2** were stronger than those of **1**, **3**, **4**, and **5** and worse than those of norcantharidin in A549, SK-MES-1, or NCI-H446 cells. Our results suggested that pentacyclic triterpenoids might be, at least in part, responsible for the proposed therapeutic effect of *Lysimachia clethroides* Duby.

EXPERIMENTAL

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus. IR spectra were taken on a Perkin-Elmer 983 G spectrometer. ^{1}H, ^{13}C NMR, and 2D NMR spectra were recorded on a Varian Inova 600 spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as internal standard. EI-MS were determined on a Micromass Zabspec spectrometer. Semipreparative HPLC was carried out on a column of ODS (250 × 9.4 mm i.d., Agilent Zorbax SB-Phenyl, Palo Alto, USA) with a Waters 2996 detector at a flow rate of 2 mL/min and wave length for detection of 203 nm. Medium-pressure liquid chromatography (MPLC) was carried out on a column of silica gel H (460 × 26 mm i.d., Buchi Borosilikat 4.6, Flawil, Swiss). Silica gel (200–300 mesh) for column chromatography and precoated plates of silica gel used for TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, China. Compounds on the TLC were colored by a 10% sulfuric acid-alcohol solution.

Plant Material. The entire plants of *Lysimachia clethroides* Duby were collected in Hubei Province of China in May 2007 and identified by Prof. Chunyu Liu. A voucher sample (No. 01-03-24-08) was deposited in the Herbarium of the College of Pharmacy, Soochow University.

Extraction and Isolation. The dried plant material (10 kg) was extracted with 75% EtOH (120 L) three times under reflux, and the solvent was subsequently removed under reduced pressure to give the EtOH extract (0.91 kg), which was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction was further partitioned between petroleum ether and 90% MeOH (v/v). The petroleum ether fraction (20.5 g) was chromatographed over a silica gel column (5.0 × 60.0 cm) eluted with petroleum ether–EtOAc (0–100%) to afford 12 fractions. Fraction 3 was isolated by MPLC eluted with petroleum ether–EtOAc (90:10) to give 3β-myristoxyurs-12-en-19,28-olide (**1**, 38.3 mg, yield 3.83 mg/kg). Fraction 5 was further purified by MPLC on silica gel (500–600 mesh) eluted with a cyclohexane–EtOAc gradient (ratios of cyclohexane–EtOAc from 9:1 to 7:3) to yield pure betulinic acid (**3**, 105 mg, yield 10.5 mg/kg), and pure ursolic acid (**4**, 508 mg, yield 50.8 mg/kg). Fraction 7 was subjected to semipreparative RP-HPLC with 90% acetonitrile to give hederagenin (**2**, 12.8 mg, yield 1.28 mg/kg) and 19α-hydroxyurs-12-en-28-oic acid (**5**, 23.5 mg, yield 2.35 mg/kg).

3β-Myristoxyurs-12-en-19,28-olide (1). White powder ($CHCl_3$), mp > 300°C ($CHCl_3$). IR (KBr, ν_{max}, cm^{-1}): 1764 (γ-lactone), 1722 (ester), 1604 (>C=CH-), 1388–1372 (gem-dimethyl), 1200 (C-O), 862, and 764. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.72 (3H, s, Me-26), 0.81 (3H, s, Me-24), 0.89 (3H, t, J = 7.2, Me-14′), 1.12 (3H, s, Me-23), 1.14 (3H, d, J = 7.5, Me-30), 1.19 (3H, s, Me-25), 1.25 (20H, m, H-4′-13′), 1.28 (3H, s, Me-27), 1.43 (3H, s, Me-29), 2.35 (2H, t, J = 7.5, H-2′), 2.50 (1H, s, H-18), 4.25 (1H, dd, J = 9.8, 4.5, H-3), 5.12 (1H, t, J = 3.6, H-12). ^{13}C NMR ($CDCl_3$, 125 MHz, δ): 38.5 (C-1), 26.3 (C-2), 81.3 (C-3), 42.7 (C-4), 55.3 (C-5), 18.6 (C-6), 37.9 (C-7), 39.7 (C-8), 47.7 (C-9), 38.0 (C-10), 23.7 (C-11), 128.6 (C-12), 138.6 (C-13), 41.6 (C-14), 28.1 (C-15), 24.7 (C-16), 47.5 (C-17), 52.0 (C-18), 80.6 (C-19), 39.3 (C-20), 31.9 (C-21), 37.7 (C-22), 28.2 (C-23), 17.1 (C-24), 15.6 (C-25), 16.7 (C-26), 23.9 (C-27), 183.2 (C-28), 16.7 (C-29), 21.3 (C-30), 171.0 (C-1′), 35.2 (C-2′), 31.9 (C-3′), 29.7 (C-4′-7′), 29.4 (C-8′-10′), 29.1 (C-11′), 24.7 (C-12′), 22.7 (C-13′), 14.1 (C-14′). EI-MS (*m/z*, I_{rel}, %): 637 (3), 453 (100), 425 (32), 418 (17), 246 (45), 211 (38) 207 (21). HR-ESI-MS *m/z* 665.5502 ($[M + H]^+$, calcd for $C_{44}H_{73}O_4$, 665.5509).

***In vitro* Cytotoxic Bioassays.** To evaluate the cytotoxic effect of the compounds on A549, SK-MES-1, and NCI-H446 bronchogenic carcinoma cell lines, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed [13]. Cells were plated into 96-well flat-bottomed cultured plates at a concentration 5×10^4 cell per well in complete RPMI 1640 culture medium. Twenty-four hours after plating, the medium containing fetal calf serum was removed, and test solutions were given to cells in various final concentrations such as 5, 10, 25, 50, and 100 μg/mL. After incubation with the drugs for 24 h, the MTT solution was added to the wells, and the plates were incubated at 37°C for 4 h. The active control group was treated with norcantharidin. The amount of formazan was determined by photometry at 540 nm. The results were expressed as percentage of the absorbance in control cells compared to that in the drug-treated cells. The IC_{50} of **1–5** is shown in Table 1. The results showed that these compounds can inhibit the growth of A549, SK-MES-1, and NCI-H446 cells. As shown in Table 1, after 24 h incubation with A549, the IC_{50} of **1–5** was 22.16 ± 3.70, 11.05 ± 2.05, 26.86 ± 5.31, 18.12 ± 3.23, and 14.96 ± 2.62 μg/mL, respectively. In SK-MES-1 cells, the IC_{50} was 19.92 ± 3.66, 9.57 ± 0.88, 21.89 ± 3.18, 16.24 ± 3.18, and 17.85 ± 2.62 μg/mL, respectively. In NCI-H446 cells, the IC_{50} was 26.25 ± 3.12, 7.83 ± 1.14, 24.17 ± 4.10, 16.98 ± 2.86, and 19.22 ± 3.42 μg/mL, respectively.

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