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Mufolinin A, an unprecedented ring A-*seco* 10-ethyllimonoid from *Munronia unifoliolata*

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ABSTRACT

Mufolinin A (**1**), a ring A-*seco* rearranged limonoid with an unprecedented ethyl at C-10 and novel 6/6/6/5 fused-ring skeleton, together with three new potential precursors (ring A-*seco* limonoids, **2**–**4**) were isolated from *Munronia unifoliolata*. Their structures and absolute configurations were confirmed by nuclear magnetic resonance (NMR), high resolution electrospray ionization mass spectroscopy (HRESIMS), X-ray crystallography, electronic circular dichroism (ECD) calculations and NMR calculations with DP4+ analyses. The unprecedented ethyl group of **1** was hypothesized to be derived from methyl migration and ring reduction rearrangement of ring A-*seco* limonoid **4**. Compounds **2** and **4** showed significant multidrug resistance (MDR) reversal activities in MCF-7/DOX cells with reversal fold (RF) values of 13.1 and 8.0, respectively.

Limonoids are a class of natural products with diverse skeleton types and a wide spectrum of biological activities, widely distributed in the plants of Meliaceae and Rutaceae. Simple ring-intact limonoids with 17β-furan ring are formed by loss of four-terminal carbons of the side chain in the apotirucallane or apoeuphane skeleton [1]. Under the action of the enzyme, the ring-intact limonoids with tetracyclic skeleton can undergo oxidation and ring opening to form a variety of ring-*seco* limonoids [2]. Further oxidative rearrangement allows diverse structure types of limonoids to be possible [3]. In previous studies, we reported several kinds of novel limonoid skeletons with ring cleavage and carbon skeleton rearrangement [4]. To our knowledge, the formation of a novel limonoid skeleton *via* methyl migration is very rare, only one 18(13→14)-*abeo*-limonoid skeleton has been reported [5].

The plants from *Munronia* genus (Meliaceae) are dwarf shrubs or semi shrubs, and some of them have been used as traditional Chinese medicine ("Aituotuo" in Chinese) for treatment of bruises, rheumatic joint pains, coughs, stomach-aches, tuberculosis and sores [6]. In recent years, more than 60 ring-intact and ring-*seco* limonoids with significant anti-inflammatory, antimicrobial and anti-tobacco mosaic virus (TMV) biological activities have been isolated from "Aituotuo" [7]. *M. unifoliolata*, one of the resources of "Aituotuo", is widely distributed in the southwest of China and also contains abundant limonoids [8]. In this research, an A-ring rearranged limonoid with an unprecedented ethyl at C-10 and novel 6/6/6/5 fused-ring skeleton, mufolinin A (**1**), together with three new potential precursors (ring A-*seco* limonoids, **2**–**4**) (Fig. 1) were isolated from the whole plant of *M. unifoliolata*. Their structures were elucidated on the basis of extensive one dimension (1D) and two dimension nuclear magnetic resonance (2D NMR) experiments, X-ray crystallographic method, electronic circular dichroism (ECD) calculations and NMR calculations with DP4+ analyses. Based on the traditional medicinal efficacy of *M. unifoliolata*, bioactivities including anti-inflammatory and anti-MDR activities of compounds **1**–**4** were also tested. Herein, the details of the isolation, structure elucidation, biosynthetic relationship and bioactivities of mufolinins A–D (**1**–**4**) were described.

Mufolinin A (**1**) was obtained as white amorphous powder. Its high resolution electrospray ionization mass spectroscopy (HRESIMS) displayed a quasi-molecular ion peak at *m/z* 549.2457 $[M + Na]^+$, corresponding to the molecular formula $C_{30}H_{38}O_8$ with 12 degrees of unsaturation. The ^1H and ^{13}C NMR spectra (Table S1.1 in Supporting information) indicated that **1** was a ring A-*seco* limonoid, and its B, C and D rings with $\Delta^{9,11}$ and $\Delta^{14,15}$ double bonds were readily established by comparison with those of several known limonoids [8] and key heteronuclear multiple bond correlations (HMBCs) (Fig. 2). Surprisingly, the ^1H NMR spectrum (Fig. S4.1.1 in Supporting information) exhibited a secondary methyl

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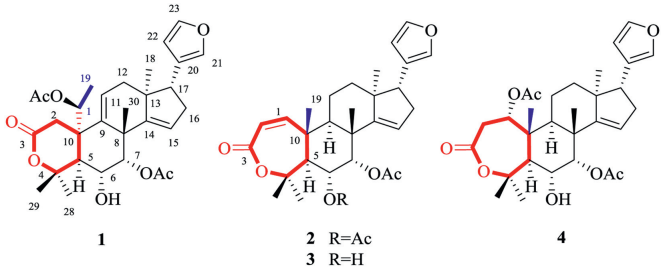


Fig. 1. Structures of compounds **1**–**4**.

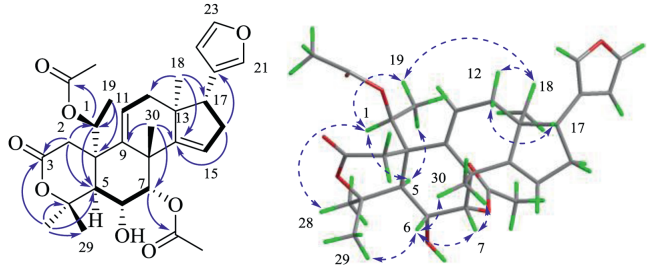


Fig. 2. Key ^{1}H–^{1}H COSY (▬), HMBC (⌒) and ROESY (⌒) correlations of **1**.

group (δ_H 1.35), which showed ^{1}H–^{1}H coupling correlation spectroscopy (COSY) correlation to H-1 (δ_H 4.97) and the HMBC cross-peaks to C-1 (δ_C 76.0) and C-10 (δ_C 46.1). These data indicated that an acetyl-substituted ethyl group was connected to C-10, which was also confirmed by the HMBC correlations from H-1 to C-9 (δ_C 138.9), C-10 (δ_C 46.1), C-2 (δ_C 42.3), C-5 (δ_C 47.8) and a carbonyl carbon (δ_C 170.1). Meanwhile, the heptonolactone ring in the general ring A-*seco* limonoids was reduced into a hexonolactone ring in **1**, which was further confirmed by the HMBC correlations from H-2 (δ_H 2.79 and 2.64) to C-3 (δ_C 171.0), C-5, and from H_3-28 (δ_H 1.62) to C-3. Thus, the unprecedented C-10 ethyl and novel 6/6/6/5 fused-ring skeleton of **1** were assigned.

The relative configuration of **1** was established by analysis of its rotating-frame overhauser spectroscopy (ROESY) spectrum (Fig. 2). The ROESY correlations of H_3-19 and H-1 to H-5 suggested a same orientation of these protons which were assigned to be α-oriented. Meanwhile, the correlations of H_3-19/H_3-18/H-12 (δ_H 2.40) and H-1/H_3-28 determined the spatial orientation of the ethyl group. The β-orientation of H-6, H-7, H-17, H_3-29 and H_3-30 were confirmed by the ROESY correlations of H_3-30/H-7/H-6/H_3-29, and H-17/H-12 (δ_H 2.11) (Fig. S4.1.6 in Supporting information). To determine the configuration of C-1, the ^{13}C chemical shifts of two tentative C-1 epimers (1*R*)-**1** and (1*S*)-**1** were calculated by the density functional theory (DFT) gage independent atomic orbital (GIAO) method at the mPW1PW91/6–311+G(d,p) theory level (Fig. S1.1 in Supporting information) [9]. Further DP4+ analyses based on both ^{1}H and ^{13}C NMR data indicated (1*S*)-1 as the correct structure with 100% probability [10]. Finally, the absolute configuration of **1** (1*S*,5*S*,6*R*,7*S*,8*S*,10*S*,17*R*) was confirmed by comparing the experimental and calculated ECD data with time-dependent density functional theory (TD-DFT) (Fig. S1.3 in Supporting information). Hence, the novel structure of **1** was depicted as shown in Fig. 1.

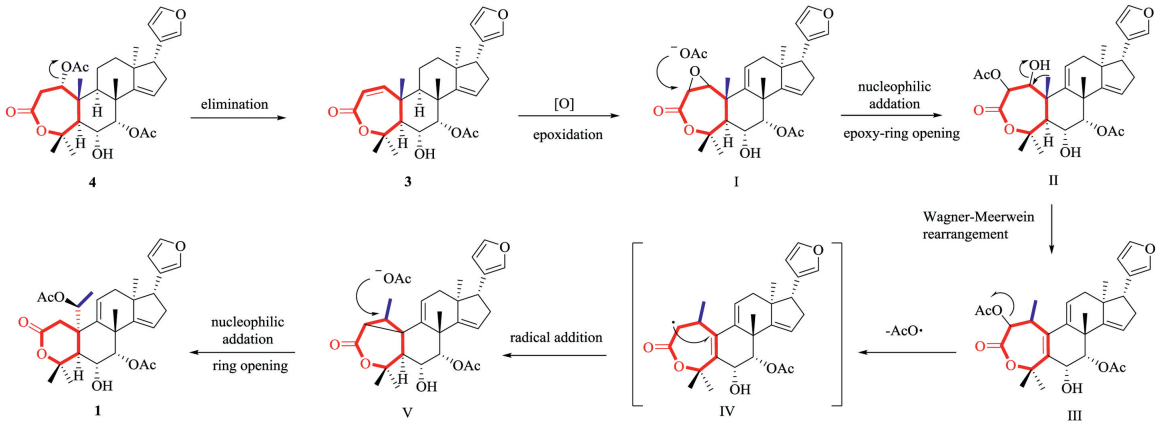
The 1D NMR data (Table S1.1 in Supporting information) of mufolinins B (**2**) and C (**3**) showed characteristic signals of a β-substituted furan ring, an α,β-unsaturated lactone group, and at least one acetoxy substituent in each compound, making them structurally similar to munronoid D [8a], a ring A-*seco* limonoid with a 3,4-lactone. Compared **2** with munronoid D, the only difference was a β-substituted furan ring group (δ_H 7.37, 7.23, 6.26; δ_C 142.8, 139.9, 111.1, 124.4) at C-17 in **2** instead of a γ-lactone ring. The relative configuration of **2** was corroborated by the ROESY spectrum (Fig. S4.2.6 in Supporting information), in which the correlations of H-6/H-7/H_3-30 and H-17/H-12 (δ_H 1.77) revealed that these protons were on the same side as β-orientation. The ROESY cross-peaks of H-5/H-9/H_3-18/H-12 (δ_H 1.85) indicated that H-5, H-9, H_3-18 and H-12 (δ_H 1.85) were α-oriented (Fig. S1.4 in Supporting information). Mufolinin C (**3**) gave a molecular formula of $C_{28}H_{36}O_6$, as determined from its HRESIMS ion at *m/z* 491.2406 $[M + Na]^+$ (calcd. 491.2404), which exhibited 42 daltons less than that of **2**. In NMR spectroscopic data of **3** and **2**, the observed significant upfield shifts of H-6 (δ_H 4.28, $\Delta\delta$ −1.03) and C-6 (δ_C 67.6, $\Delta\delta$ −2.4) indicated replacement of the acetoxy group at C-6 in **2** by a hydroxy group in **3**. Based on 1D NMR data and ROESY correlations, the relative configuration of **3** was assigned to be the same as that of **2** (Fig. S1.4 in Supporting information). Therefore, the structures of **2** and **3** were established as shown in Fig. 1.

Mufolinin D (**4**) was obtained as colorless crystals. It was assigned the molecular formula of $C_{30}H_{40}O_8$, as established by HRESIMS. The 1D NMR data (Table S1.1 in Supporting information) of **4** showed many similarities to those of **3**, with the main differences in the absence of a $\Delta^{1,2}$ double bond and the presence of an acetoxy group in **4**. The above changes were established by analysis of HMBC correlations from H-1 (δ_H 4.86) to C-5 (δ_C 48.3), Me-19 (δ_C 17.4) and the acetoxy carbonyl (δ_C 169.1), and the ^{1}H–^{1}H COSY cross-peak of H-1/H-2 (δ_H 2.64, 2.96) in **4** (Fig. S1.4 in Supporting information). The relative configuration of 1-OAc was assigned as α-oriented by the ROESY correlations of H-1/H_3-19 and H-1/H_3-30. Moreover, a single-crystal X-ray diffraction experiment (Fig. S1.5 in Supporting information) allowed the full assignment of the absolute configuration of **4** (1*S*,5*S*,6*R*,7*S*,8*R*,9*R*,10*R*,13*S*,17*R*) [Flack parameter = 0.05(6); CCDC 2013523].

The hypothetical biosynthetic pathway of **1** was illustrated in Scheme 1. Abundant ring A-*seco* limonoids, such as mufolinin D (**4**) isolated, were considered to be the precursor. Compound **4** was deacetylated to give **3** with $\Delta^{1,2}$ double bond and then oxidized continuously to obtain 1,2 epoxide I with double bond C-9/11 under the action of the enzyme. Subsequently, nucleophilic attack from AcO^- to 1,2-epoxy formed the intermediate II. The hydroxy group left under acidic conditions to form a carbocation, triggering Wagner-Meerwein rearrangement. As a result, Me-19 migrated to C-1, and a rearranged carbocation (C-10) was generated. Subsequently, the loss of a proton delivered the double bond C-5/10 [5a]. The acetoxy group at the *ortho* position of the ester carbonyl group led to the formation of a free radical [11], which then reacted with the double bond in the ring and produced a three-membered ring. Finally, under the nucleophilic attack of AcO^-, the three-membered ring with high tension underwent ring opening process to afford compound **1**.

Mufolinins A–D (**1**–**4**) were tested for their inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells [12] and the reversal activities of multidrug resistance (MDR) in MCF-7/DOX cells. Compound **2** exhibited moderate anti-inflammatory activity with the half maximal inhibitory concentration (IC_{50}) value of 31.2 μmol/L (NG-monomethyl-L-arginine monoacetate was used as a positive control with the IC_{50} value of 44.4 μmol/L). Moreover, **2** and **4** at the concentration of 10 μmol/L showed significant MDR reversal effects on MCF-7/DOX cells subjected to doxorubicin (DOX), of which compound **2** was even better than the positive drug verapamil (Table 1).

In conclusion, mufolinin A (**1**), the first example of ring A rearranged limonoid with unprecedented ethyl at C-10 and novel 6/6/6/5 fused-ring skeleton, was isolated from *M. unifoliolata*, together with three possible biosynthetic precursors mufolinins B–D (**2**–**4**). This is the first report of limonoid undergoing methyl migration and ring reduction rearrangement. In addition, compounds **2** and **4** showed significant multidrug resistance (MDR) reversal ac-



Scheme 1. Hypothetical biosynthetic pathway of mufolinin A (**1**).

Table 1
In vitro MDR reversal activity and cytotoxicity of compounds **2** and **4**.

Compound	Dosage (μmol/L)	MDR reversal effects on MCF-7/DOX cells	
		IC_{50} (μmol/L)[a]	RF[b]
DOX	–	60.9 ± 0.65	1.0
2	10	4.64 ± 0.44	13.1
	30	4.13 ± 0.32	14.8
4	10	7.57 ± 1.17	8.0
	30	4.16 ± 0.78	14.7
Verapamil[c]	10	6.61 ± 0.55	9.21

[a] IC_{50}: The half maximal inhibitory concentration of DOX in the presence of **2** and **4**.
[b] The reversal fold (RF) value was calculated as follows: IC_{50} (MCF-7/DOX cells with DOX alone)/IC_{50} (MCF-7/DOX cells with DOX and isolates).
[c] Verapamil was used as a positive control.

tivities in MCF-7/DOX cells, which was similar to or even better than the positive control verapamil at the same dosage. This research provided a reference for the study of novel limonoids and their pharmacological activities from *M. unifoliolata*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2021.06.050.

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