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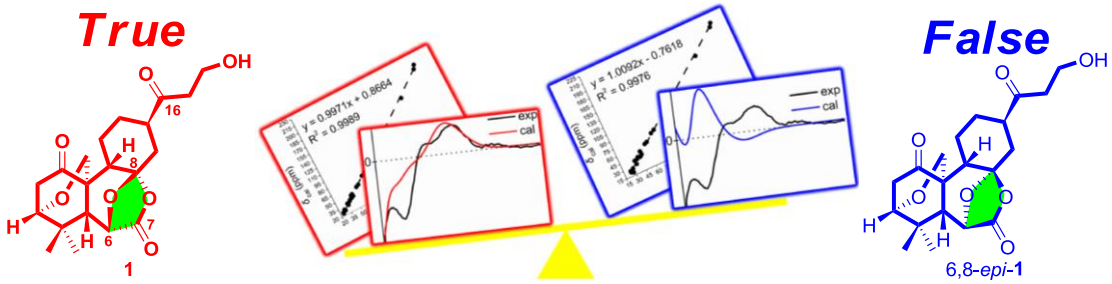

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Laxiflorol A (1), an unprecedented 7,8:15,16-di-*seco*-15-nor-21-homo-*ent*-kauranoid and its precursor analogue, laxiflorol B (2), were isolated from the leaves of *Isodon eriocalyx* var. *laxiflora*. The absolute configuration of 1 was determined by spectral methods and quantum chemical calculations. Compound 2 exhibited weak cytotoxicity.

1. Introduction

The Natural Products Library provided a diverse and unique source of bioactive lead compounds for drug discovery.[1] The *ent*-kaurane diterpenoids library was constructed by our group since 1976 for the development of therapeutic agents to treat cancer, and more than 1000 pure *ent*-kauranoids including more than 700 novel ones have been identified from *Isodon* genus.[2]

Ent-kaurane-type diterpenoids represent one of the most excellent examples of natural products with diverse structural scaffolds and important pharmaceutical activities.[2, 3] Among these compounds in our library, the unique structures including ternifolide A,[4] neoadenoloside A,[4] neolaxiflorin A,[5] nervonin A,[6] maoecrystal Z,[7] maoecrystal V,[8] xindongnin M,[9] neoangustifolin[10] and epinodosino[10] have been reported by *Natural Product Reports* as *hot off the press*. Some compounds such as oridonin,[11] eriocalyxin B,[12] adenanthin,[3a] pharicin B,[3b] and other compounds have brought great attention to their potential application in antitumor. In order to enrich and improve our *ent*-kaurane diterpenoids library, chemical constituents of the leaves of *Isodon eriocalyx* var. *laxiflora* was further investigated.[2, 13] A trace amount of an *ent*-kauranoid (0.00003 %) with a novel 7,8:15,16-di-*seco*-15-nor-21-homo-*ent*-kaurane skeleton, designated as laxiflorol A (**1**) (3.0 mg), together with its biogenetic precursor analogue, laxiflorol B (**2**), were obtained from this plant (Figure 1).

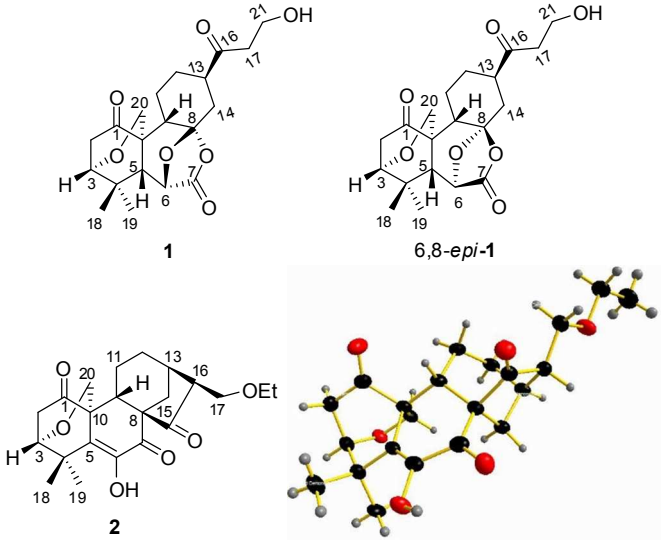


Figure 1. Chemical structures of compounds **1**, **2**, and 6,8-*epi*-**1**, and X-ray crystallographic structure of **2**.

Laxiflorol A (**1**) was the first example of 7,8:15,16-di-*seco*-*ent*-kauranoid, which was biosynthetically formed from multiple ring cleavage, adding (C-21) and losing (C-15) carbons, and a sequential reactions on an *ent*-kauranoid (Scheme 1). Nevertheless, the 1D and 2D NMR experiments showed that compound **1** have two possible structures, **1** and 6,8-*epi*-**1** (Figure 1). After multiple attempts made by us, the

definite structure of **1** was established by NMR data coupled with quantum chemical calculations including ^{13}C NMR chemical shifts and ECD spectra. Herein, we report the isolation, structure elucidation including absolute stereochemistry, and cytotoxic activities of compounds **1** and **2**.

2. Results and discussion

As the precursor analogue of laxiflorol A (**1**), laxiflorol B (**2**) was obtained as colorless needles. On the basis of careful analysis of 1D NMR, 2D NMR (Table S1), and single-crystal X-ray diffraction using anomalous scattering of CuK_α radiation (CCDC 970056) data (Figure 1),[14] the absolute configuration of compound **2** was assigned and could be described according to the following nomenclature: (3*R*,8*S*,9*S*,10*S*,13*R*,16*S*)-3,20-epoxy-6-hydroxy-17-ethoxy-5(6)-en-*ent*-kaur-1,7,15-trione.

Table 1. NMR spectroscopic data of compound **1** and its calculated ^{13}C NMR data (δ in ppm, *J* in Hz)

no.	δ_H	δ_C		
	exp[a]	exp[a]	cal[b]	cal[c]
1	-	207.3 s	206.9 s	207.3 s
2	2.83 br d (2.6)	41.4 t	41.8 t	43.5 t
3	3.68 br t (2.6)	77.2 d	75.6 d	77.2 d
4	-	35.3 s	37.3 s	39.3 s
5	2.28 br s	45.2 d	48.1 d	42.6 d
6	5.07 d (3.6)	71.9 d	72.0 d	73.6 d
7	-	172.5 s	168.9 s	169.7 s
8	-	111.4 s	110.8 s	112.3 s
9	2.65 dd (13.1, 3.8)	39.3 d	39.3 d	40.6 d
10	-	47.4 s	49.5 s	51.4 s
11	1.70 m; 1.17 m	24.4 t	25.4 t	22.5 t
12	2.03 m; 1.41 m	27.7 t	29.7 t	20.6 t
13	2.90 overlap	47.8 d	45.4 d	45.2 d
14	2.27 br d (13.2); 2.14 br d (13.2)	35.9 t	34.3 t	32.4 t
16	-	210.3 s	214.3 s	213.9 s
17	2.90 overlap	44.5 t	43.5 t	42.0 t
18	1.00 s	28.8 q	27.6 q	27.8 q
19	1.54 s	24.4 q	23.6 q	25.0 q
20	4.51 d (10.8); 3.88 d (10.8)	61.4 t	60.2 t	62.2 t
21	4.20 t (6.0)	57.6 t	57.8 t	60.1 t

[a] Data were recorded in C_5D_5N on 600 MHz spectrometer. [b]Calculated ^{13}C NMR data of **1**. [c]Calculated ^{13}C NMR data of 6,8-*epi*-**1**.

Laxiflorol A (**1**) was obtained as white, amorphous powder. The molecular formula $C_{20}H_{26}O_7$, with eight degrees of unsaturation, was established based on HRESIMS ($[M + Na]^+$, 401.1571; calcd for $C_{20}H_{26}O_7Na$, 401.1576) and NMR spectroscopy (Table 1). The analysis of the ^{13}C NMR and DEPT spectra revealed the presence of 20 carbons, which were assigned as two methyl, seven methylene (two oxygenated), five methine (two oxygenated), and six quaternary carbons (one oxygenated, one ester and two carbonyls), which suggested that **1** is a highly oxygenated diterpenoid with a C_{20} skeleton quite different from the *ent*-kaurane skeleton reported previously.[2]

The HMBC spectrum of **1** showed correlations from the geminal methyls Me-18 (δ_H 1.00) and Me-19 (δ_H 1.54) to C-3, C-4, and C-5. Furthermore, the AB spin system of methylene H_2-20 showed HMBC correlations with C-1, C-3, C-5, C-9, and C-10. Other HMBC correlations were noted between methylene H_2-2 (δ_H 2.83) and C-1, C-3, C-4, and C-10, between oxygenated methine H-3 (δ_H 3.68) and C-1, C-5, and C-20, between methine H-5 (δ_H 2.28, 1H, br s) and C-1, C-4, C-6, C-7, C-9, C-10, C-18, C-19, and C-20, and between oxygenated methine H-6 (δ_H 5.07) and C-7 and C-10. These observed HMBC correlations, coupled with two spin systems (CH_2CH, H_2-2/H-3 and CHCH, H-5/H-6) established by ^1H-^1H COSY correlations and the HSQC spectra, gave rise to partial structure part a (Figure 2).

The HMBC spectrum showed that oxygenated methylene group H_2-21 (δ_H 4.20) correlated with C-16 (δ_C 210.3, s) and C-17, H-13 correlated with C-16, and that H-17 correlated with C-21 (δ_C 57.6, t). This evidence, along with two proton spin systems deduced from the ^1H-^1H COSY correlations, H_2-17/H_2-21, and H-9/H_2-11/H_2-12/H-13/H_2-14 suggested the partial structure part b (Figure 2).

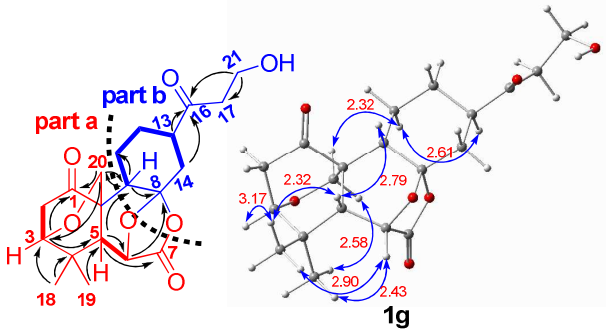


Figure 2. ^1H–^1H COSY(bold), selected HMBC(arrow) correlations of compound **1**, and key ROESY (full arrow) correlations of conformer **1g**.

Moreover, the key HMBC correlations of H-6 with C-8 (δ_C 111.4, s) and of H-9 (δ_H 2.65, 1H, dd, 13.1, 3.8 Hz) with C-1, C-5, C-8, C-10, C-11, and C-20 permitted the partial structures part a and part b to be connected through a carbon-carbon connection between C-9 and C-10 and an oxo bridge between C-6 and C-8. The molecular formula $C_{20}H_{26}O_7$ of **1** demonstrated eight degrees of unsaturation, indicating the existence of a lactone group between C-7 and C-8, and this assignment was also supported by the similar chemical shift of C-7 (δ_C 172.5, s) and C-8 (δ_C 111.4, s) compared to those of norstaminolactone A[15] (a norstaminane-type diterpenoid bearing similarly structural unit with its C-14 at δ_C 172.6 and C-8 at δ_C 107.0). In the ROESY spectrum of **1**, the NOE correlations of Me-19/H_2-20, H-11α/H_2-20/H-13 suggested that H-13, Me-19, and C-20 all adopted an α-orientation. The cross-peaks between H-3/H-5, H-5/H-9, and H-5/Me-18 in

the ROESY spectrum demonstrated that H-3, H-5, H-9, and Me-18 were *β*-oriented (Figure 2).

The Nuclear Overhauser Effect (NOE) is commonly recognized as one of the best approaches in structural and conformational analysis. However, if the internuclear distance was less than 3 Å, even the two spin systems bearing opposite orientations, the NOE correlations could also be observed. Then, the NOE experiment often fails to predict the structures of these types of compounds. For example, the structure of rubescensin S, an *ent*-kauranoid, was revised to account for an error in assigning the configuration at C-13 by using NOE.[16] Therefore, more evidence should be provided when dertemining the configuration of structures especially assining their stereochemistry.

Certainly, the H-6 was only one possiable orientation, *α* or *β*. However, the both NOE correlations between H-6 and Me-18*β* and between H-6 and Me-19*α* could be observed definitely, which suggested that H-6 of **1** might has two possiable orientations. This evidence implied that compound **1** had two possible structures, **1** and 6,8-*epi*-**1** (Figure 1). X-ray diffraction or chemical transformation of **1** was scarcely finished. Under the circumstances, calculation of ^{13}C NMR chemical shifts of **1** and 6,8-*epi*-**1** were performed in order to confirm the definite structure. After all the conformers of **1** and 6,8-*epi*-**1** were optimized at B3LYP/6-31G(d,p), the ^{13}C NMR chemical shifts of all the conformers were calculated with GIAO method at mPW1PW91/6-31G(d,p) level, which has been reported to be applicable for highly oxygenated diterpenoids.[17]

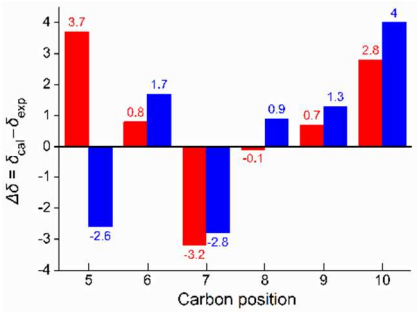


Figure 3. Partial comparison of calculated chemical shifts for two possible structrures, **1** (red) and 6,8-*epi*-**1** (blue), with experimentally observed shifts.

As shown in Table 1, the largest error and the mean average error of the Boltzmann-averaged ^{13}C NMR chemical shifts were 7.1 (C-12) and 1.5 ppm, respectively. Overall, the calculated ^{13}C NMR chemical shifts of **1** and 6,8-*epi*-**1** were both in good agreement with the structure elucidated by the NMR data (Table 1). Since the 7,8:15,16-di-*seco*-15-nor-21-homo-*ent*-kaurane skeleton of **1** was definite, the orientation of H-6 was still not assigned for the weak errors of the calculated ^{13}C NMR data between **1** and 6,8-*epi*-**1** in C-5, C-6, C-7, C-8, C-9, and C-10 (Figure 3). In addition, from the calculations given in the Supporting Information and Figure 2, the distance between H-6 to H3-18 and H3-19 in the most stable conformer 1g are 2.90 and 2.43 Å, respectively. These distances are 3.00 and 2.14 Å in the most stable conformer 6,8-*epi*-1d, respectively. These evidence interpreted that why the NOE could not be used to analyse the relative configuration of C-6 of **1**.

Therefore, the calculated electronic circular dichroism (ECD) spectra for (3*R*, 5*R*, 6*S*, 8*S*, 9*S*, 10*R*, and 13*R*)-**1** and (3*R*, 5*R*, 6*R*, 8*R*, 9*S*, 10*R*, and 13*R*)-6,8-*epi*-**1** were performed using time-dependent density-functional theory (TDDFD) method20 at B3LYP-SCRF/6-31G++(d,p) level with PCM in methanol,[18] in order to determine its absolute configuration.

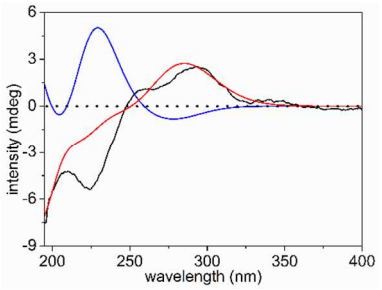


Figure 4. Experimental ECD of **1** (black), calculated ECD of **1** in methanol (red), and calculated ECD of 6,8-*epi*-**1** in methanol (blue).

The calculated ECD spectra of (3*R*, 5*R*, 6*S*, 8*S*, 9*S*, 10*R*, and 13*R*)-**1** were compatible with the experimental ECD curve (Figure 4). Molecular orbital (MO) analysis of the predominant conformer 1g at B3LYP/6-31G++(d,p) level with PCM in methanol, gave us comprehension of the experimental ECD spectrum (Figure 5). The curve peak at 293 nm correlated to two positive rotatory strengths at 289.9 and 280.8 nm, which were resulted from the electronic transitions from MO101 to MO102 involving an n → π* transition in the carbonyl group of ring A; and from MO100 to MO103 involving an n → π* transition in the side chain, respectively. The curve trough at 195 nm might arise from the electronic transitions from MO97 to MO104 involving an n → π* transition in the *γ*-lactone group of ring D, which gave rise to the negative rotatory strengths at 194.8 nm. As a result, the absolute configuration of **1** was established as shown.

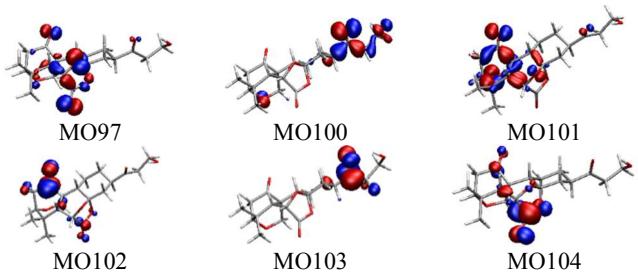
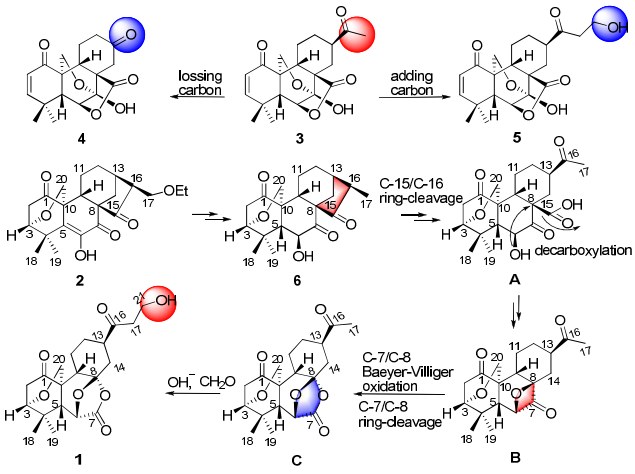


Figure 5. Important MOs in ECD of the optimized conformer **1g**.

Three 15,16-seco-ent-kauranoid, compounds **3**–**5**, have been isolated from the title plant, and their possible biogenetic route had been discussed in the literature.[13f] The hypothetical biogenetic pathway of **1** was biogenesis inspired from **3**–**5**, and could be plausibly traced back to laxiflorol B (**2**) and

rabdonervosin J (**6**).[19] The formation of intermediate C from intermediate A and intermediate B by rearrangement involving the ring-cleavage reactions, decarboxylation, aldol condensation route is the key step to form compound **1**.



Scheme 1. Hypothetical Biogenetic Pathway of **1**.

Compounds **1** and **2** were tested for in vitro cytotoxicity against A-549, MCF-7, SMMC-7721, SW-480 and HL-60 human cancer cell lines using the MTT method;[20] cis-Platin was used as the positive control. Only compound **2** showed weak cytotoxic activity against the above cell lines with IC50 values of 26.06, 16.63, 35.64, 24.29, and 17.92 μM, respectively.

3. Conclusions

In summary, this paper describes the isolation and structure elucidation of laxiflorol A (**1**), the first example of 7,8:15,16-di-*seco*-*ent*-kauranoid, together with its biogenectic precursor analogue, laxiflorol B (**2**) obtained from the *I. eriocalyx* var. *laxiflora*. The structure of laxiflorol A (**1**) might be biosynthetically formed from multiple ring cleavage and adding (C-21) and losing carbons (C-15) on an *ent*-kauranoid. Although more than 1000 natural *ent*-kauranoids have been reported in the literature to date, laxiflorol A (**1**) was a novel compound bearing a 7,8:15,16-di-seco-15-nor-21-homo-*ent*-kaurane skeleton.

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Notes and references

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‡ X-ray crystallographic data of laxiflorol B (**2**): $C_{22}H_{28}O_6$, M = 388.44, orthorhombic, a = 9.4749(3) Å b = 11.6421(3) Å c = 17.1244(5) Å α = 90.00°, β = 90.00°, γ = 90.00°, V = 1888.95(9) Å^3, T = 100(2) K, space group $P212121$, Z = 4, 9900 reflections measured, 3262 independent reflections (R_{int} = 0.0350). The final R_1 values were 0.0354 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0987 ($I > 2\sigma(I)$). The final R_1 values were 0.0354 (all data). The final $wR(F^2)$ values were 0.0988 (all data). Flack parameter = -0.03(15).

Electronic Supplementary Information (ESI) available: Detailed experimental procedures, method of cytotoxicity test, physico-chemical properties, 1D and 2D NMR, MS, UV, ORD spectra of compounds **1** and **2**, ECD spectra of compound **1**, and X-ray crystal structure of **2**. See DOI: 10.1039/c000000x/

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