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Diterpenoids from *Isodon rubescens* and their nitric oxide production inhibitory activity†

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Six new *ent*-kaurane diterpenoids, isodonrubescins A–F (**1–6**), together with twenty-five known *ent*-kaurane diterpenoids (**7–31**), a known *ent*-atisane diterpenoid (**32**), and two known *ent*-abietane diterpenoids (**33–34**), were isolated from *Isodon rubescens*. Their structures were established by means of extensive MS and NMR data analysis. Among the all isolates, compound **7** was found in a natural product for the first time, and *ent*-atisane diterpenoid was discovered from *I. rubescens* in Hubei Province, P. R. China for the first time. Furthermore, all the isolated compounds were tested for their NO production inhibitory activity in LPS stimulated RAW264.7 cells. Compounds **7–9**, **12**, **13**, **16**, and **17** displayed NO production inhibitory activities with IC_{50} values ranging from 1.36 to 18.25 μM, respectively.

1. Introduction

The genus *Isodon*, comprising about 150 different species of under-shrubs, sub-undershrubs, or perennial herbs, is a cosmopolitan and important genus of the Lamiaceae family. It is widely distributed in tropical and subtropical Asia. Previous studies have shown that they are rich sources of diterpenoids with diverse structural scaffolds, such as *ent*-kauranes, *ent*-abietanes, *ent*-atisanes, and have a range of biological activities.[1–3]

Isodon rubescens is a perennial herb distributed widely in Henan, Guizhou, Hebei, Jiangxi, Hubei, and some other provinces of P. R. China.[4] It has attracted great attention due to the traditional uses in folk medicine for the treatment of respiratory and gastrointestinal bacterial infections, inflammation, and cancer.[5–8] Oridonin, an important *ent*-kaurane from *I. rubescens* showed the anti-tumor and anti-inflammatory activities. Previous studies have demonstrated that it exhibits anti-tumor effects on human cancer cells, such as HepG2, SGC-7901, MCF-7, mainly by blocking the cell cycle, inducing apoptosis and autophagy of tumor cells, and shows anti-inflammatory effects by inhibiting the expression of inflammatory factors through nuclear factor-kappa B (NF-κB) signal pathway.[9,10] In addition, previous investigations on the chemical constituents of *I. rubescens* collected from different provinces, P. R. China revealed that they contained different structure types of diterpenoids. For example, the chemical constituents of *I. rubescens* collected from Guizhou Province were mainly 6,7-*seco*-*ent*-kaurane diterpenoids, however, 7,20-epoxy-*ent*-kaurane diterpenoids were main chemical constituents of *I. rubescens* collected from Henan Province.[11,12] Furthermore, the chemical constituents of *I. rubescens* collected from Hubei Province have not been extensively investigated, only 16 new diterpenoids have been reported, including diterpene alkaloids with an aza-*ent*-kaurane skeleton.[13–17] Therefore, in order to fully understand the active constituents of *I. rubescens* from different regions, a reinvestigation of *I. rubescens* collected from Badong county, Hubei Province was undertaken in the hope of discovering diterpenoids with interesting structures and biological activities. As a result, six new diterpenoids (**1–6**), together with twenty-five known *ent*-kaurane diterpenoids (**7–31**), a known *ent*-atisane diterpenoid (**32**) and two known *ent*-abietane diterpenoids (**33–34**) were isolated from this plant, and it should be noted that compounds **10**, and **13–17** have been reported in our previous work.[18] Herein we reported the isolation, structural elucidation of six new diterpenoids and biological activities of all isolated compounds (Fig. 1).

2. Results and discussion

Compound **1** was obtained as colorless needle crystals, and its molecular formula was determined to be $C_{22}H_{32}O_7$ based on the HR-ESI-MS at *m*/*z* 431.20383 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.20402), indicating of seven degrees of unsaturation. The ^1H NMR spectra (Table 1) of **1** revealed the presence of two singlet methyls [δ_H 0.96 (s), 0.93 (s)] and two methoxy groups [δ_H 3.19

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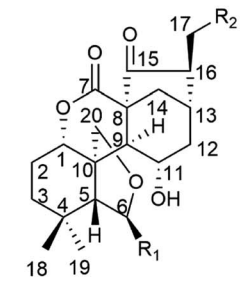
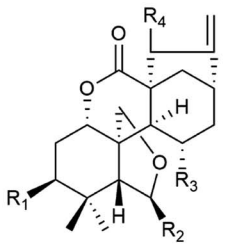
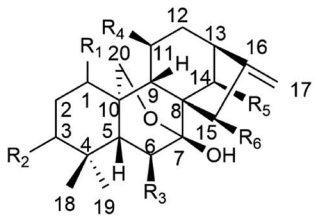


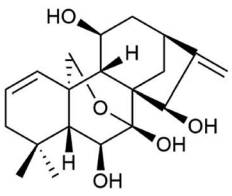
	R_1	R_2
1	OCH_3	OCH_3
2	OH	OCH_2CH_3



	R_1	R_2	R_3	R_4
7	OH	OCH_3	H	=O
8	OH	OH	H	=O
9	H	OCH_3	OH	=O
10	H	OH	OH	α-OH
11	H	OCH_3	OH	α-OH
12	H	OH	OH	=O



	R_1	R_2	R_3	R_4	R_5	R_6
3	α-OH	H	OH	OH	H	OAc
4	α-OH	H	OAc	H	OH	OH
5	H	β-OH	OH	H	OH	OAc
13	α-OH	H	OH	H	OH	=O
14	H	α-OH	OAc	H	OH	OAc
15	α-OH	H	OH	H	OH	OAc
16	H	β-OAc	OAc	H	OH	OAc
17	α-OAc	H	OH	H	OH	=O
18	α-OH	H	OH	OH	H	OH
19	α-OH	H	OAc	OH	H	OH
20	H	H	OAc	OH	OH	OH
21	α-OH	H	OH	OH	H	=O
22	β-OH	H	OH	H	H	OH
23	H	α-OH	OH	H	OH	OH
24	H	α-OH	OAc	H	OH	OH
25	H	β-OH	OAc	H	OH	OAc



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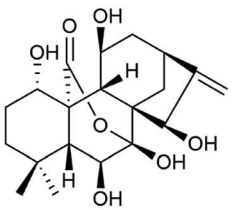

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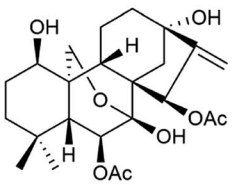

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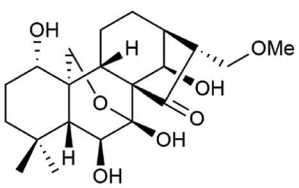

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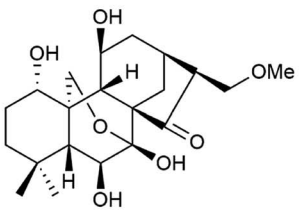

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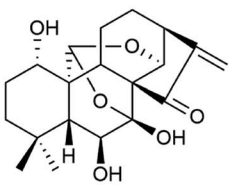

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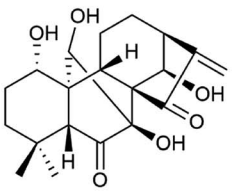

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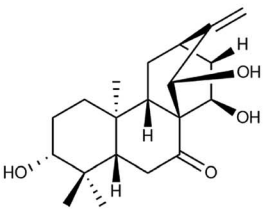

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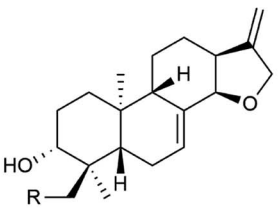

	R
33	H
34	OH

Fig. 1 Structures of compounds **1–34**.

(s), 3.15 (s)], one oxygenated methylene [δ_H 4.32 (d, J = 9.6 Hz), 4.28 (d, J = 9.6 Hz)], three oxygenated methines [δ_H 4.89 (m), 5.03 (s), 4.46 (m)]. Its ^{13}C NMR and DEPT spectra (Table 2) exhibited 22 carbon signals, including two methoxy groups at δ_C 54.8 and δ_C 59.0, two oxygenated methylenes at δ_C 74.5 and δ_C 71.8, two oxygenated methines at δ_C 76.9 and δ_C 64.8, one hemiacetal group at δ_C 109.7, one δ-lactone carbonyl group at δ_C 171.3, one carbonyl group at δ_C 212.9 and three quaternary carbons at δ_C 31.7, δ_C 57.7 and δ_C 51.2, which implied a 6,7-*seco*-*ent*-kaurane skeleton. Detailed analysis of the NMR data of **1**

Table 1 ^{1}H-NMR data of compounds **1–6** in C_5D_5N (600 MHz, δ in ppm, J in Hz)

No.	**1**	**2**	**3**	**4**	**5**	**6**
1	4.89 (m)	4.92 (dd, 6.9, 10.5)	4.35 (m)	3.74 (m)	1.74–1.86 (m)	6.38 (dd, 2.4, 10.8)
2	1.87 (m)	1.91 (m)	1.96 (m)	1.86 (m)	2.07 (m); 1.09 (d, 12.6)	5.73 (ddd, 1.8, 6.0, 10.2)
3	1.31 (m)	1.37 (m); 1.32 (m)	1.51 (m); 1.42 (m)	1.34 (m)	3.75 (s)	1.94 (overlap); 1.77 (dd, 6.0, 16.8)
4						
5	3.48 (s)	3.27 (s)	1.74 (d, 5.4)	1.77 (d, 6.6)	2.44 (d, 6.6)	2.16 (d, 6.6)
6	5.03 (s)	5.76 (s)	4.24 (dd, 5.4, 3.6)	5.86 (d, 6.6)	4.33 (dd, 2.4, 6.6)	4.31 (dd, 4.8, 6.6)
7						
8						
9	2.65 (m)	2.93 (d, 10.8)	2.82 (d, 9.6)	2.92 (dd, 6.0, 12.9)	2.84 (overlap)	2.88 (dd, 1.2, 9.6)
10						
11	4.46 (m)	4.55 (dd, 8.7, 18.9)	4.73 (m)	2.31 (m); 1.91 (m)	1.54 (m); 1.23 (m)	4.58 (m)
12	2.91 (m); 1.60 (dd, 9.0, 13.8)	2.95 (m); 1.58 (dd, 9.0, 14.4)	2.86 (m); 1.91 (m)	2.42 (m); 1.73 (m)	2.32 (m); 1.61 (m)	2.96 (m); 1.91 (overlap)
13	2.61 (dd, 4.2, 9.6)	2.68 (dd, 3.6, 9.6)	2.72 (dd, 9.6, 3.6)	2.87 (d, 9.6)	2.84 (overlap)	2.76 (dd, 5.4, 10.4)
14	2.68 (m); 2.33 (dd, 4.2, 12.0)	2.72 (d, 12.0); 2.39 (dd, 3.9, 12.3)	2.22 (d, 12.6); 2.17 (dd, 12.6, 4.2)	5.15 (overlap)	5.08 (s)	2.19 (dd, 4.8, 12.6); 2.01 (d, 12.6)
15			6.55 (s)	5.57 (d, 2.4)	6.93 (s)	5.20 (overlap)
16	2.67 (m)	2.63 (br t, 5.4)				
17	3.60 (m); 3.52 (dd, 4.2, 9.0)	3.61 (m)	5.29 (s); 5.12 (s)	5.69 (s); 5.40 (s)	5.41 (s); 5.28 (s)	5.51 (s); 5.22 (overlap)
18	0.96 (s)	0.98 (s)	1.24 (s)	0.92 (s)	1.57 (s)	1.20 (s)
19	0.93 (s)	0.98 (s)	1.21 (s)	1.22 (s)	1.21 (s)	1.12 (s)
20	4.32 (d, 9.6); 4.28 (d, 9.6)	4.42 (d, 9.0); 4.30 (d, 9.0)	4.81 (d, 9.6); 4.50 (d, 9.6)	4.86 (d, 9.6); 4.46 (d, 9.6)	4.36 (d, 9.6); 4.05 (d, 9.6)	4.36 (d, 9.6); 4.16 (dd, 1.2, 9.6)
OAc			2.20 (s)	2.21 (s)	2.28 (s)	
OMe	3.19 (s)					
OMe	3.15 (s)					
OCH_2CH_3		3.32 (m); 1.07 (t, 7.2)				
HO-1			6.75 (d, 4.2)	5.97 (d, 4.2)		
HO-3					6.00 (s)	
HO-6		9.11 (s)	6.25 (d, 3.0)		5.91 (s)	8.17 (d, 4.2)
HO-7			7.91 (s)	8.31 (s)	8.01 (s)	8.07 (s)
HO-11	5.75 (s)	7.24 (overlap)	7.10 (d, 6.0)			5.77 (br s)
HO-14				8.01 (s)	8.06 (s)	
HO-15				4.40 (d, 3.0)		6.84 (d, 2.4)

Table 2 ^{13}C NMR data of compounds **1–6** in C_5D_5N (150 MHz, δ in ppm)

No.	1	2	3	4	5	6
1	76.9(d)	77.1(d)	74.2(d)	73.5(d)	26.4(t)	130.2(d)
2	24.2(t)	24.4(t)	28.8(t)	30.7(t)	24.6(t)	125.3(d)
3	37.1(t)	37.3(t)	40.5(t)	39.1(t)	74.8(d)	41.4(t)
4	31.7(s)	31.9(s)	34.5(s)	34.2(s)	38.7(s)	32.7(s)
5	53.1(d)	54.2(d)	57.3(d)	55.5(d)	50.3(d)	57.5(d)
6	109.7(d)	102.5(d)	74.5(d)	75.3(d)	73.5(d)	74.3(d)
7	171.3(s)	171.4(s)	97.2(s)	98.8(s)	100.1(s)	97.6(s)
8	57.7(s)	57.4(s)	53.1(s)	53.8(s)	53.0(s)	53.8(s)
9	52.6(d)	52.7(d)	52.1(d)	45.7(d)	46.5(d)	50.8(d)
10	51.2(s)	51.3(s)	42.9(s)	41.7(s)	35.9(s)	39.4(s)
11	64.8(d)	63.9(d)	63.6(d)	18.8(t)	15.4(t)	62.8(d)
12	42.1(t)	41.9(t)	41.4(t)	33.0(t)	32.4(t)	45.7(t)
13	32.2(d)	32.1(d)	37.7(d)	46.2(d)	46.1(d)	37.2(d)
14	34.3(t)	34.1(t)	28.9(t)	76.4(d)	76.4(d)	28.0(t)
15	212.9(s)	213.1(s)	75.3(d)	73.4(d)	74.2(d)	75.9(d)
16	58.5(d)	58.8(d)	160.7(s)	161.5(s)	160.0(s)	161.7(s)
17	71.8(t)	69.5(t)	107.7(t)	110.2(t)	110.6(t)	107.9(t)
18	33.1(q)	33.3(q)	33.8(q)	32.0(q)	29.8(q)	31.4(q)
19	23.6(q)	23.4(q)	23.3(q)	22.2(q)	23.8(q)	22.7(q)
20	74.5(t)	74.0(t)	64.7(t)	64.1(t)	67.0(t)	66.8(t)
OAc			171.4(s)	169.6(s)	171.6(s)	
			22.3(q)	21.7(q)	22.5(q)	
OMe	54.8(q)					
OMe	59.0(q)					
OCH_2CH_3		66.9(t)				
		15.5(q)				

indicated that **1** is structurally related to dayecrystal D.[19] The significant difference between them was the change of the chemical shift of C-12 from δ_C 42.1 in **1** to δ_C 33.1 in the latter, which was caused by a γ-gauche shielding effect between 16-methoxymethyl group and H-12α. Therefore, it can be deduced that the methoxymethyl group at C-16 in **1** was β-oriented. The location of the methoxymethyl group at C-16 was revealed by the HMBC correlation of OMe (δ_H 3.15) with C-17 (δ_C 71.8) and the ^{1}H–^{1}H COSY correlations (Fig. 2) of H_2-17 (δ_H 3.60, δ_H 3.52) with H-16 (δ_H 2.67). The ROESY (Fig. 3) correlations of H-16 with H-12α (δ_H 1.60), of H-12β (δ_H 2.91) with H-13β and H-11β confirmed the β-orientation of the methoxymethyl group. Consequently, the structure of **1** was assigned as 11α-hydroxy-6β-methoxy-16β-methoxymethyl-6,7-*seco*-6,20-exoxy-1α,7-olide-*ent*-kaur-15-one, and named as isodonrubescin A.

Compound **2** was isolated as colorless crystals (MeOH), and its molecular formula was the same as **1**, as established to be $C_{22}H_{32}O_7$ by HR-ESI-MS at *m*/*z* 431.20407 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.20402) and ^{13}C NMR data. A comparison of the NMR data of **2** (Tables 1 and 2) with those of **1** suggested that compound **2** had a 6,7-*seco*-*ent*-kaurane skeleton as **1**, except for the disappearance of two methoxyl signal and the presence of an additional ethoxyl signal. The location of the ethoxyl group at C-17 was revealed by the HMBC correlations of H_2-17 (δ_H 3.61) with the carbon (δ_C 66.9) of the ethoxyl group. In addition, the chemical shift of C-6 was shifted upfield from δ_C 109.7 in **1** to δ_C 102.5 in **2** due to the change of the substituent at C-6 from a methoxyl group in **1** to a hydroxyl group in **2**. The relative stereochemistry of **2** was consistent with those of **1** and was ensured by the ROESY correlations (Fig. 3). Accordingly, the structure of compound **2** was established as 6β,11α-dihydroxy-16β-ethoxymethyl-6,7-*seco*-6,20-exoxy-1α,7-olide-*ent*-kaur-15-one and given the name isodonrubescin B.

Compound **3** was exhibited to have the molecular formula $C_{22}H_{32}O_7$ by HR-ESI-MS (*m*/*z* 431.20288 $[M + Na]^+$, calcd 431.20402). The ^{1}H-NMR spectra (Table 1) of **3** established the existence of three single methyls [δ_H 1.24 (s), 1.21 (s), 2.20 (s)], one olefinic methylene [δ_H 5.29 (s), 5.12 (s)], one oxygenated methylene [δ_H 4.81 (d, J = 9.6 Hz), 4.50 (d, J = 9.6 Hz)], four oxygenated methines [δ_H 4.35 (m), 4.24 (dd, J = 5.4, 3.6 Hz), 4.73 (m), 6.55 (s)]. The methyl at δ_H 2.20 (3H, s) and the carbonyl group at δ_C 171.4 in the NMR spectrum suggested the presence of an acetoxyl group in **3**. Apart from the acetoxyl group, there were 20 carbon resonances, consisting of two methyls, six methylenes (one oxygenated carbon at δ_C 64.7 and one olefinic carbon at δ_C 107.7), seven methines (four oxygenated carbons at δ_C 74.2, δ_C 74.5, δ_C 63.6 and δ_C 75.3, respectively), and five quaternary carbons (one hemiacetal group at δ_C 97.2 and one olefinic carbon at δ_C 160.7). The above-mentioned data suggested compound **3** to be a 7,20-epoxy-*ent*-kaurane diterpenoid. Comparison of the NMR date of **3** with those of hebeirubescensin K[20] indicated that their structures were closely related. The only structural difference between them was that the hydroxyl group at C-15 in the latter was replaced by an acetoxyl group in **3**, which can be deduced by the change of the chemical shift of H-15 from δ_H 5.06 in the latter to δ_H 6.55 in **3** and was further confirmed by the HMBC correlations (Fig. 2) from H-15 to C-16 (δ_C 160.7) and OAc (δ_C 171.4). The remaining structure was corroborated by the HMBC experiment.

The relative configuration of **3** was revealed by analysis of the ROESY spectrum (Fig. 3), in which the correlations of H-6/H_3-19α (δ_H 1.21), H-11/H-12α (δ_H 2.86)/H-13α (δ_H 2.72), H-15/H-14β (δ_H 2.17)/H-13α were clearly observed, indicating that HO-6, HO-11, and AcO-15 were β-orientation. Correlations of H-1/H-5β assigned HO-1 to be α-oriented. Thus. Compound **3** was determined as 1α,6β,11β-trihydroxy-15β-acetoxy-7,20-exoxy-*ent*-kaur-16-ene, and named as isodonrubescin C.

Compound **4** had the same molecular formula $C_{22}H_{32}O_7$ as that of **3**, which was established by HR-ESI-MS at *m*/*z* 431.20404 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.20402). Its ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) showed that compound **4** possessed the same 7,20-epoxy-*ent*-kaurane skeleton as that of **3**. A comparision of the NMR data of **4** (Tables 1 and 2) with those of enmenol[21] disclosed that **4** was a 6-acetyl derivative of enmenol. The key HMBC correlation (Fig. 2) from H-6 (δ_H 5.86) to OAc (δ_C 169.6) in **4** confirmed this conclusion. Furthermore, the ^{1}H–^{1}H COSY correlations (Fig. 2) of H-1 (δ_H 3.74) with H_2-2 (δ_H 1.86), of H-14 (δ_H 5.15) with H-13 (δ_H 2.87) and the HMBC correlations of H-15 (δ_H 5.57) with C-16 (δ_C 161.5) and C-17 (δ_C 110.2) indicated that three hydroxyl groups were located at C-1, C-14 and C-15 respectively. The relative configuration of **4** was assigned by the ROESY correlations (Fig. 3) of H-1/H-9β (δ_H 2.92), H-6/H_3-19α (δ_H 1.22), HO-15 (4.40)/H-9β (2.92), which revealed the α-orientation of HO-1 and the β-orientation of AcO-6, HO-14, HO-15. Therefore, the structure of **4** was elucidated as

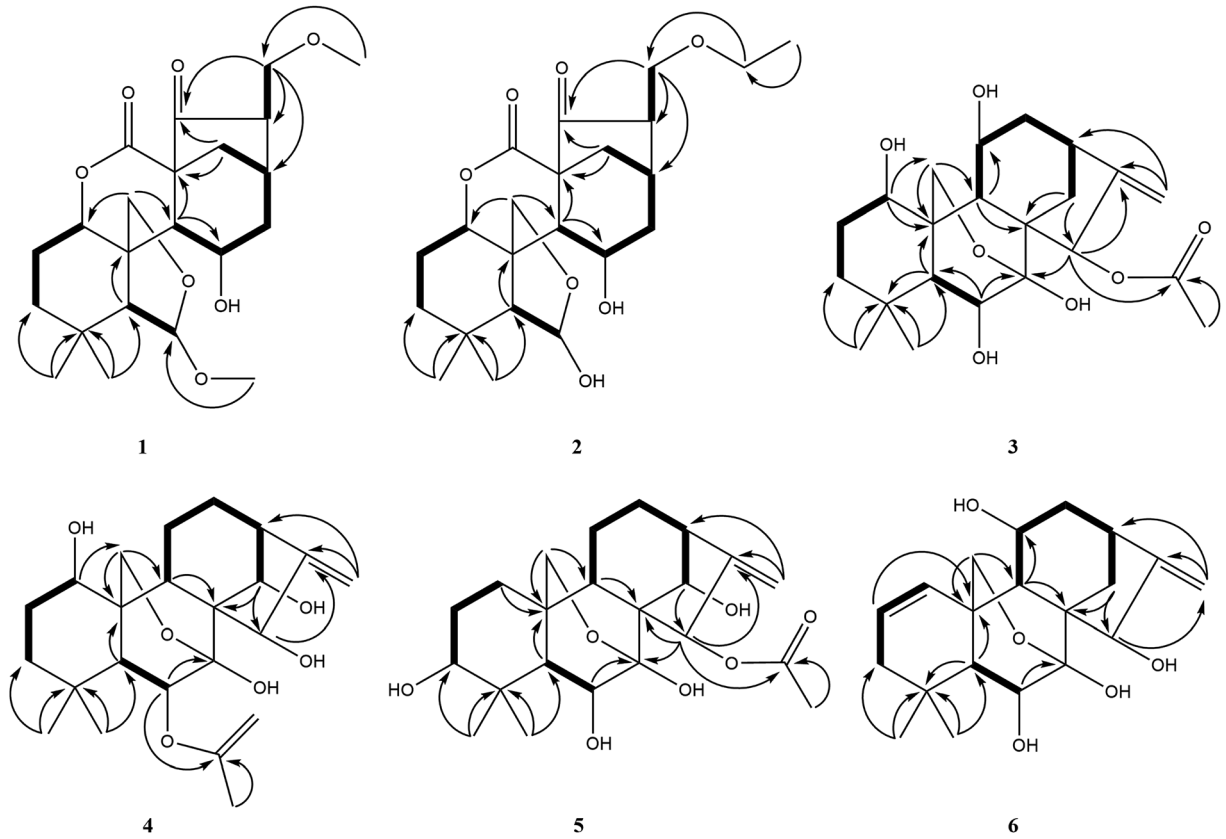

Fig. 2 Key HMBC and $^{1}H–^{1}H$ COSY correlations of compounds **1–6**.

1α,14β,15β-trihydroxy-6β-acetoxy-7,20-exoxy-*ent*-kaur-16-ene, and given the name isodonrubescin D.

Compound **5** was obtained as a white amorphous powder with a molecular formula of $C_{22}H_{32}O_7$ as assigned by HR-ESI-MS (*m*/*z* 431.20380 $[M + Na]^+$, calcd 431.20402). Its ^{1}H and ^{13}C NMR data (Tables 1 and 2) resembled those of hikiokoshins G,[22] suggesting that **5** had the same carbon skeleton as that of hikiokoshins G. The difference between them was that hikiokoshins G had two acetoxyl groups while compound **5** only possessed one acetoxyl group, and in the HMBC spectrum of **5** (Fig. 2), the cross-peak of H-15 with OAc (δ_C 171.6) indicated that the acetoxyl group was located at C-15. Thus, **5** was a 6-deacetyl derivative of hikiokoshins G, this conclusion was further supported by the change of the chemical shift of H-6 from δ_H 5.98 in hikiokoshins G to δ_H 4.33 in **5**. The relative stereochemistry of **5** was consistent with those of hikiokoshins G, and was confirmed by the ROESY analysis (Fig. 3). Accordingly, compound **5** was established as 3β,6β,14β-trihydroxy-15β-acetoxy-7,20-exoxy-*ent*-kaur-16-ene, and named as isodonrubescin E.

Compound **6** had the molecular formula of $C_{20}H_{28}O_5$ as determined by its HR-ESI-MS (*m*/*z* 349.20029 $[M + H]^+$, calcd 349.20095) and ^{13}C NMR data, indicating seven degrees of unsaturation. The ^{1}H NMR and ^{13}C NMR spectra (Tables 1 and Table 2) of **6** implied that compound **6** was a 7,20-epoxy-*ent*-kaurane diterpenoid. However, unlike the normal type of 7,20-epoxy-*ent*-kaurane diterpenoids, such as compound **3–5**, a *cis* double bond signal [δ_H 6.38 (dd, J = 2.4, 10.8 Hz), 5.73 (ddd, J = 1.8, 6.0, 10.2 Hz); δ_C 130.2, 125.3] was presented in the NMR spectra of **6**, and the double bond was assigned to C-1 and C-2 by the key $^{1}H–^{1}H$ COSY correlations of **6** (Fig. 2) from H-1 (δ_H 6.38) to H-2 (δ_H 5.73), from H-2 to H-3 (δ_H 1.94) and the key HMBC correlations (Fig. 2) from H_3-18 (δ_H 1.20) to C-3 (δ_C 41.4), from H-2 to C-10 (δ_C 39.4). The remaining three hydroxyl groups were respectively assigned to C-6, C-11 and C-15 by interpretation of the $^{1}H–^{1}H$ COSY and HMBC correlations. The relative configuration of **6** was determined by the ROESY correlations (Fig. 3) of H-6 (δ_H 4.31)/H_3-19α (δ_H 1.12), H-11 (δ_H 4.58)/H-20 (δ_H 4.36) and H-15 (δ_H 5.20)/H-13α (δ_H 2.76), which suggested the β-orientation of HO-6, HO-11 and HO-15. Consequently, the structure of **6** was assigned as 6β,11β,15β-trihydroxy-7,20-exoxy-*ent*-kaur-1,16-diene, and given the name isodonrubescin F.

The other twenty-eight known diterpenoids (**7–34**) were identified by comparison of their NMR data with those reported in the literature. As a result, they were identified to be 3β-hydroxy-6β-methoxy-6,7-*seco*-6,20-epoxy-1α,7-olide-*ent*-kaur-16-en-15-one (**7**),[23] enmein (**8**),[20] rabdosin A (**9**),[19] epinodosinol (**10**),[18] isojaponin A (**11**),[24] epinodosin (**12**),[25] oridonin (**13**),[18] hubeirubesin K (**14**),[18] neolaxiflorin U (**15**),[18] hubeirubesin I (**16**),[18] lasiokaurin (**17**),[18] hebeirubescensin K (**18**),[20] maoyecrystal F (**19**),[24] rabdoternin D (**20**),[26] lasiodonin (**21**),[27] enmelol (**22**),[28] rabdonervosin G (**23**),[29] rabdonervosin D (**24**),[29] hikiokoshins G (**25**),[22] isodonhenrin E (**26**),[30] maoyecrystal L (**27**),[20] dayecrystal B (**28**),[31] lushanrubescensin F (**29**),[32] ponicidin (**30**),[33]

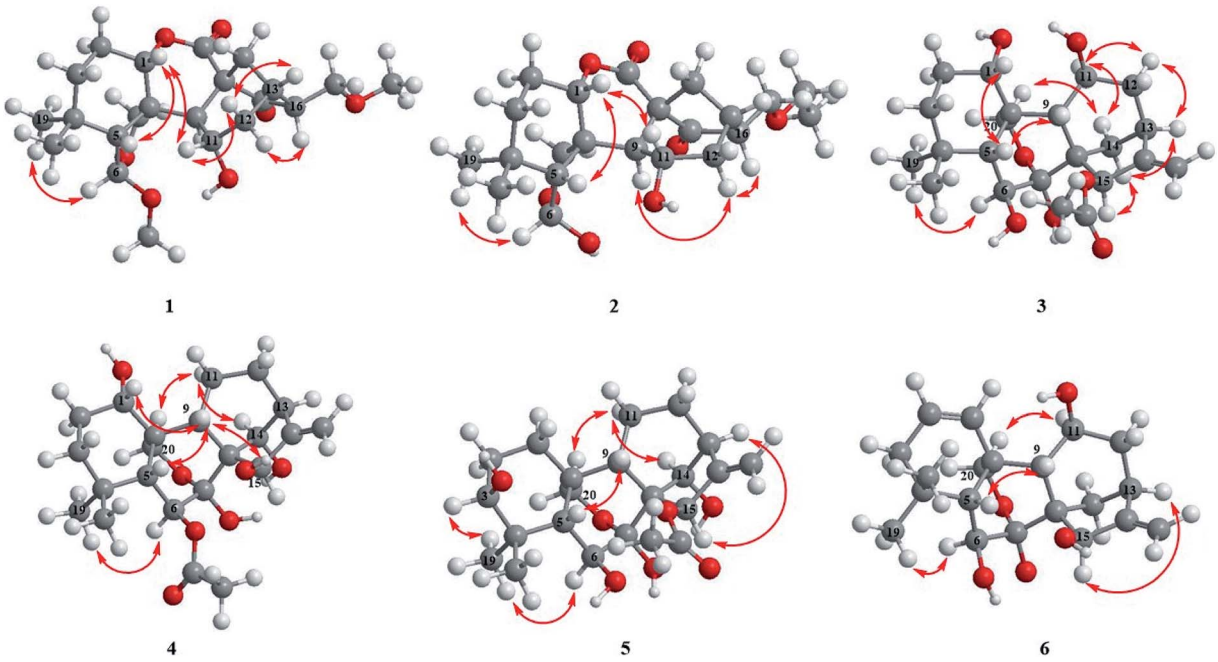

Fig. 3 Key ROESY correlations of compounds **1–6**.

rubescensin D (**31**),[34] isorosthornin D (**32**),[35] isoadenolin M (**33**),[36] rubescensin J (**34**).[37]

In addition, all the isolated compounds were assessed for their inhibitory activity against NO production in LPS stimulated RAW264.7 cells with dexamethasone as a positive control ($IC_{50} = 9.58$ μM). The cell viability of the tested compounds was firstly measured using CCK-8 assay to determine whether the NO production inhibitory activities were induced by the cytotoxicity. As a result, compounds **7**, **9**, **13**, **16**, and **17** exhibited obvious NO production inhibitory effects with IC_{50} values of 3.97, 2.25, 6.51, 1.48 and 1.36 μM, respectively. Compounds **8** and **12** displayed mild NO production inhibitory effects with IC_{50} values of 17.43 and 18.25 μM, respectively, while the rest of the tested compounds had no obvious NO production inhibitory activity ($IC_{50} > 20$ μM). In the present study, the 6,7-*seco*-*ent*-kaurane diterpenoids, such as **7–9** and **12** which possessed an α,β-unsaturated ketone moiety, exhibited NO production inhibitory effects, the result indicated that α,β-unsaturated ketone moiety was an essential pharmacophore. However, this conclusion did not fully be applied to 7,20-epoxy-*ent*-kaurane diterpenoids. For compounds **13**, **17**, **21** and **30**, they shared an α,β-unsaturated ketone moiety, but compound **21** and **30** did not show the activity. This could be caused by the lack of HO-14β in **21** and **30**. Additionally, compound **16** without an α,β-unsaturated ketone moiety also exhibited obvious NO production inhibitory effects. This result further demonstrated that the α,β-unsaturated ketone moiety was not absolutely essential active center for the activity. Besides, it was interesting that compound **25** was a 3-deacetyl derivative of **16**, but it did not show NO production inhibitory effect, the result suggested that 3β-OAc might played an important role in the NO production inhibitory activity.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with an Autopol IV polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). UV spectra were recorded on a UH5300 UV-VIS Double Beam spectrophotometer (Hitachi Co., Tokyo, Japan). NMR spectra were obtained on a Bruker AVANCE IIITM 600 MHz spectrometer (Bruker, Ettlingen, Germany) in C_5D_5N with tetramethylsilane (TMS) as an internal reference standard. Chemical shifts (δ) have been given in ppm and the coupling constants (J) have been expressed in Hz. High-resolution electrospray mass spectroscopy was conducted on a Thermo Scientific Q Exactive Orbitrap LC-MS/MS System (HR-ESI-MS) (Thermo Scientific, Waltham, MA, USA). High-performance liquid chromatography (HPLC) was performed on an Ultimate 3000 HPLC system (Dionex Co., Sunnyvale, CA, USA) equipped with an Ultimate 3000 pump and Ultimate 3000 Variable Wavelength detector, as well as a semi-preparative YMC-Pack ODS-A column (250 × 10 mm, 5 μm), column chromatography (CC) was conducted with silica gel (200–300 mesh and 300–400 mesh, Qingdao Haiyang Chemical Industry Co., Ltd., Qingdao, China). Chromatographic grade acetonitrile was purchased from Chang Tech Enterprise Co., Ltd (Taiwan, China). RAW264.7 murine macrophages were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). Dexamethasone and lipopolysaccharides (LPS) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Cell Counting Kit (CCK-8) was purchased from Beyotime Biotechnology (Shanghai, China). Dulbecco modified Eagle medium (DMEM) and penicillin-streptomycin solution were purchased from GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) was

purchased from Gibco, Life Technologies (Grand Island, NY, USA). Reagent grade dimethyl sulfoxide (DMSO) was purchased from Vetec, Sigma Chemical Co. (St. Louis, MO, USA). The absorbance was read on a Multiskan GO microplate reader (Thermo Fisher Scientific Inc. Waltham, MA, USA).

3.2 Plant material

Isodon rubescens were collected from Badong county, Hubei Province and identified by Prof. Fajun Song, College of Life Science, South Central University for Nationalities. The voucher specimen (2016101201) was deposited in the herbarium of School of Pharmaceutical Sciences, South Central University for Nationalities.

3.3 Extraction and isolation

The air-dried and powdered parts of *I. rubescens* (11.2 kg) were extracted with 95% EtOH (25 L × 3, each 24 h) at room temperature. The extract was filtered and evaporated to afford a crude extract (1.1 kg), which was partitioned successively with petroleum ether (P. E.) and EtOAc. The EtOAc extract (556 g) was subjected to column chromatography on a silica gel column eluting with the gradient of $CHCl_3$–acetone (10 : 0, 9 : 1, 8 : 2, 7 : 3, 6 : 4, 1 : 1, 3 : 7, 0 : 10) to yield eight fractions (Fr. A–Fr. H). Fr. D (123.7 g) was separated by silica gel CC (CH_2Cl_2–EtOAc, 10 : 1, 8 : 2, 6 : 4, 1 : 1) into fractions D1–D10. Fr. D6 was subjected to RP-18 CC (MeOH–H_2O, 3 : 7, 5 : 5, 7 : 3, 0 : 10) to obtain eleven fractions (Fr. D6A–Fr. D6K). Fr. D6D was purified by semi-preparative HPLC (MeOH–H_2O 33 : 67) to afford compounds **2** (15 mg, t_R 20.8 min) and **25** (25 mg, t_R 15.1 min).

Fr. E (56.3 g) was separated on RP-18 CC into six fractions (Fr. E1–Fr. E6) by eluting with MeOH–H_2O (3 : 7, 5 : 5, 7 : 3, 0 : 10). Fr. E2 and Fr. E4 was purified by recrystallizing in MeOH to afford compounds **8** (735 mg). Fr. E3 was firstly purified by a silica gel column (eluted with CH_2Cl_2–MeOH, 100 : 1, 50 : 1, 25 : 1, 15 : 1, 12 : 1 gradient) to yield nine fractions Fr. E3A–Fr. E3I. Fr. E3C was purified by recrystallizing in MeOH to afford compound **12** (8 mg), then Fr. E3A was subjected to silica gel CC (petroleum ether–EtOAc, 9 : 1, 8 : 2, 7 : 3 gradient) to obtain fractions E3A1–E3A8. Fr. E3A8 was finally purified by semi-preparative HPLC (MeOH–H_2O 43 : 57) to afford compounds **1** (10 mg, t_R 16.3 min) and **9** (18 mg, t_R 17.6 min). Similarly, compound **7** (5 mg, t_R 15.8 min) was obtained from Fr. E3B by semi-preparative HPLC (MeOH–H_2O, 40 : 60). Fr. E3F was successively chromatographed over silica gel CC (CH_2Cl_2–MeOH, 50 : 1, 25 : 1, 12 : 1) and semi-preparative HPLC to yield compounds **6** (1 mg, MeOH–H_2O, 36 : 64, t_R 41.8 min), **26** (9 mg, MeOH–H_2O, 36 : 64, t_R 43.3 min), **18** (25 mg, MeOH–H_2O, 45 : 55, t_R 22.4 min), **19** (19 mg, MeOH–H_2O, 47 : 53, t_R 22.3 min) and **32** (5 mg, MeOH–H_2O, 36 : 64, t_R 46.3 min). Fr. E6 was similarly purified with semi-preparative HPLC to yield compounds **33** (1.5 mg, CH_3CN–H_2O, 77 : 23, t_R 14.2 min) and **34** (7 mg, MeOH–H_2O, 79 : 21, t_R 15.5 min).

Fr. F (51.3 g) was separated over RP-18 CC (MeOH–H_2O, 3 : 7, 5 : 5, 7 : 3, 0 : 10 gradient) into five fractions (Fr. F1–Fr. F5), Fr. F2 and Fr. F3 was separated over repeatedly chromatographed by silica gel column, and then further purified by semi-preparative HPLC to afford compounds **3** (46 mg, MeOH–H_2O, 49 : 51, t_R 23.9 min), **4** (5.0 mg, MeOH–H_2O, 35 : 65, t_R 12.2 min), **5** (8.0 mg, MeOH–H_2O, 25 : 75, t_R 19.5 min), **11** (3.0 mg, MeOH–H_2O, 60 : 40, t_R 12.9 min), **20** (13 mg, CH_3CN–H_2O, 35 : 65, t_R 11.7 min), **21** (23 mg, CH_3CN–H_2O, t_R 7.7 min), **22** (2.5 mg, CH_3CN–H_2O, 25 : 75, t_R 11.7 min), **23** (9 mg, MeOH–H_2O, 45 : 55, t_R 11.4 min), **24** (12 mg, MeOH–H_2O, 35 : 65, t_R 34.7 min), **27** (6.0 mg, MeOH–H_2O, 40 : 60, t_R 17.0 min), **28** (12 mg, CH_3CN–H_2O, 35 : 65, t_R 8.8 min), **29** (23 mg, CH_3CN–H_2O, 35 : 65, t_R 6.8 min), **30** (1.7 mg, MeOH–H_2O, 35 : 65, t_R 26.9 min), **31** (3 mg, CH_3CN–H_2O, 35 : 65, t_R 10.8 min).

3.4 Spectroscopic data

Isodonrubescin A (**1**): colorless needle crystals (MeOH); $[\alpha]_D$ = −107.8° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 215 (2.48), 295 (1.58) nm; ^{1}H and ^{13}C NMR data see Tables 1 and 2; HR-ESI-MS *m*/*z* 431.20383 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7$ Na, 431.20402).

Isodonrubescin B (**2**): colorless crystals (MeOH); $[\alpha]_D$ = −47.6° (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε): 205 (2.92) nm; ^{1}H and ^{13}C NMR data see Tables 1 and 2; HR-ESI-MS *m*/*z* 431.20407 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.20402).

Isodonrubescin C (**3**): colorless crystals (MeOH); $[\alpha]_D$ = −101.0° (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε): 205 (3.21), 250 (2.43) nm; ^{1}H and ^{13}C NMR data see Tables 1 and 2; HR-ESI-MS *m*/*z* 431.20288 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.20402).

Isodonrubescin D (**4**): white amorphous powder; $[\alpha]_D$ = +6.2° (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε): 210 (3.24), 250 (2.29) nm; ^{1}H and ^{13}C NMR data see Tables 1 and 2; HR-ESI-MS *m*/*z* 431.20404 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.20402).

Isodonrubescin E (**5**): white amorphous powder; $[\alpha]_D$ = +9.1° (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε): 205 (3.29), 250 (2.86) nm; ^{1}H and ^{13}C NMR data see Tables 1 and 2; HR-ESI-MS *m*/*z* 431.20380 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.20402).

Isodonrubescin F (**6**): white amorphous powder; $[\alpha]_D$ = +12.2° (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε): 210 (3.38) nm; ^{1}H and ^{13}C NMR data see Tables 1 and 2; HR-ESI-MS *m*/*z* 349.20029 $[M + H]^+$ (calcd for $C_{20}H_{29}O_5$, 349.20095).

3.5 NO production measurement and cell viability assay

The NO production and cell viability were determined by the Griess reaction and CCK-8 method respectively, which have been described in our previous paper.[38]

4. Conclusions

In this study, six previously undescribed *ent*-kaurane diterpenoids, including two 6,7-*seco*-*ent*-kaurane diterpenoids (**1–2**), four 7,20-epoxy-*ent*-kaurane diterpenoids (**3–6**), together with twenty-five known *ent*-kaurane diterpenoids (**7–31**), a known *ent*-atisane diterpenoids (**32**), and two known *ent*-abietane diterpenoids (**33–34**) were isolated from *I. rubescens* collected from Badong county of Hubei Province, P. R. China. It was noteworthy that compounds **7** was isolated as a natural product for the first time, and *ent*-atisane diterpenoid was found from *I. rubescens* in Hubei Province for the first time. Among all the isolated compounds, 7,20-epoxy-*ent*-kaurane diterpenoids and 6,7-*seco*-*ent*-kaurane diterpenoids were the main chemical

constituents of *I. rubescens* collected from Hubei Province, which contained two types of diterpenoids isolated from *I. rubescens* collected from Henan and Guizhou Province, this may be related to the geographical location of Hubei Province lying between Henan and Guizhou Province. Moreover, all the isolated compounds were evaluated for their inhibitory effect against LPS induced nitric oxide production in RAW 264.7 macrophages. Compounds **7–9**, **12**, **13**, **16**, and **17** displayed obvious NO production inhibitory effects. In conclusion, those results have further facilitated our understanding of the active constituents of *I. rubescens* from Badong region and the potential bioactive constituents of *I. rubescens* accounting for the application as anti-inflammatory agents.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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