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Cytotoxic *ent*-kauranoid derivatives from *Isodon rubescens*

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Abstract—An extensive study of the diterpenoids produced by the species of *Isodon rubescens*, has led to the isolation of 12 new *ent*-kaurane diterpenoids, hebeirubescensins A–L (**1**–**12**), and 19 known analogues. Their structures were determined on the basis of spectroscopic analysis. Selected compounds were assayed for their inhibitory ability against human A549, HT-29, and K562 cells. Among them, hebeirubescensins B and C exhibited significant cytotoxicity with IC_{50} values of <2.0 μM. The structure–activity relationships were discussed.

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1. Introduction

Herbal drugs have been widely used for thousands of years in traditional Chinese medicine for the treatment of human disease. *Isodon* species are claimed to exhibit antitumor and anti-inflammatory activities, diterpenoids with a diversity of highly oxygenated structures are the major bioactive constituents of this genus.[1] Given the important bioactivities, structural complexity, and interesting chemical diversity of the composition of this genus, since 1976, more than 50 *Isodon* species in China have been investigated systematically by our group. About 500 new diterpenoids including kauranoids, abietanoids, labdanoids, pimaranoids, isopimaranoids, gibberellanoids, and clerodanoids have been isolated and characterized.[2,3] Among them, some *ent*-kauranoids have potent anti-tumor activities with very low toxicity, for instance: maoecrystal P,[4] eriocalyxin B,[5] oridonin, and ponicidin.[6]

Isodon rubescens belongs to the genus *Isodon* and is commonly used as an antitumor and anti-inflammatory herb in China. It has been stated that this herb is useful for the treatment of cancers of liver, pancreas, esophagus, breast, and rectum. Previous phytochemical studies showed that this species was rich in *ent*-kauranoids. Oridonin and ponicidin, two 7,20-epoxy-*ent*-kauranoids, are the major bioactive constituents of this plant.[7] Recently, pharmaceutical study showed that oridonin and ponicidin had significant antiangiogenic activity.[8] More recently, these two compounds were found to be potent inhibitors of NF-κB transcription activity and the expression of its downstream targets, COX-2 and inducible nitric-oxide synthase.[9] With an aim to isolate more potent and selective NF-κB inhibitors, we further systematically reinvestigated the chemical constituents of *I. rubescens*, and 31 7,20-epoxy-*ent*-kauranoids, including 12 new ones, hebeirubescensins A–L (**1**–**12**), were isolated. In this paper, the isolation, structure elucidation, and cytotoxic properties of those new *ent*-kauranoids are reported below.

2. Results and discussion

Hebeirubescensin A (**1**) was obtained as an amorphous powder. It exhibited an even pseudomolecular ion peak at *m/z* 514 $[M+Na]^+$ in the ESIMS spectrum, suggesting that it might be a N-containing compound. The HRESIMS data (*m/z* 514.2430 $[M+Na]^+$, calcd for 514.2416) further confirmed this assumption, giving rise to the molecular formula $C_{26}H_{37}NO_8$. Its strong IR absorptions at 3387 and 1711 cm^{-1} suggested the presence of hydroxyl and carbonyl groups. The ^{13}C NMR data (Table 1) in combination with analysis of the DEPT and HSQC spectra revealed 26 carbon

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2006.02.079.

Keywords: *Isodon rubescens*; *ent*-Kaurane; Hebeirubescensin; Cytotoxicity.

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Table 1. NMR data for hebeirubescensin A (**1**) in C_5D_5N, δ (ppm)

No.	^{1}H	^{13}C	HMBC	ROESY
1	3.78 (dd, J=12.5, 5.7 Hz)	76.7 d	C-2, 3, 9, 20, 1′	H-3, 5, 3′
2α	1.92–2.04 (m)	22.7 t	C-1, 4	H-20, 1′, and H_3-19
2β	1.50–1.57 (m)		C-1, 4	H-1, 3β
3α	1.26 (overlapped)	38.3 t	C-1, 2, 4	
3β	1.33–1.42 (m)		C-1, 2, 4	H-1
4		38.6 s		
5	1.44 (d, J=8.6 Hz)	57.5 d	C-1, 4, 6, 10, 18, 19	H-1, 9, H_3-18, and OH-6
6	4.23 (dd, J=11.4, 8.6 Hz)	75.5 d	C-5, 7, 8, 10	H-20 and H_3-19
7		100.5 s		
8		61.8 s		
9	1.89 (overlapped)	53.6 d	C-5, 11, 12, 14, 15, 20	H-5
10		33.2 s		
11α	2.78–2.87 (m)	23.6 t	C-9, 12	H-14, 12α
11β	1.87 (overlapped)		C-8, 9, 12, 13	
12α	2.27–2.36 (m)	31.4 t	C-9, 11, 13, 16	H-11α, 13
12β	1.43 (overlapped)		C-9, 11, 13, 14, 16	
13	3.16 (br d, J=9.8 Hz)	43.7 d	C-8, 11, 12, 15, 16, 17	H-12α, 14
14	5.49 (br s)	72.9 d	C-7, 8, 12, 13, 16	H-11α, 13
15		208.9 s		
16		153.2 s		
17	5.50 (br s), 6.26 (br s)	119.8 t	C-12, 13, 15, 16	
18	1.27 (s)	33.8 q	C-3, 4, 5, 19	H-5
19	1.00 (s)	23.3 q	C-3, 4, 5, 18	H-2α, 6, 20
20	5.75 (br s)	98.4 d	C-1, 7, 9, 10, 1′	H-2α, 6, 1′ and H_3-19
1′	5.09 (br s)	93.6 d	C-1, 20, 3′	H-2α, 20
2′	1.80–1.91 (overlapped)	32.5 t	C-1′, 4′	
3′	1.78–1.90 (overlapped)	25.0 t	C-4′	H-1
4′	3.50 (q, like, J=5.2 Hz)	39.5 t	C-2′, 3′, 5′	NH
5′		170.9 s		
6′	2.05 (s)	23.2 q	C-5′	
NH	8.50 (br s)		C-4′, 5′	H-4′
6-OH	6.55 (d, J=11.4 Hz)		C-6, 7	H-5

signals due to seven quaternary carbons, eight methines, eight methylenes, and three methyls, of which 20 were assigned to the diterpene skeleton, and the remaining six were ascribed to the other moiety. Careful analysis of the NMR data of the diterpene part indicated that it was a 7,20-epoxy-kauranoid due to the characteristic signal of a hemiketal carbon (C-7 at δ_C 100.5). Comparison of the ^{1}H and ^{13}C NMR data of the diterpene part with those of rabdoternins E (**14**) and F (**13**), two known 7,20-epoxy-kauranoids that had been isolated as well, suggested that the diterpene part in **1** was strongly resembling to that of rabdoternin F (**13**).[10] Further analysis of 2D NMR data allowed us to determine the gross structure of the diterpene part as shown in Figure 1. The other moiety contained one N- and six C-atoms, including one methyl (δ_C 23.2), three methylene (δ_C 32.5, 25.0, and 39.5, respectively), a carbonyl group (δ_C 170.9), and one acetal group (δ_C 93.6). The CH_2-4′ group resonating at δ_H 3.50 was linked with an acetamide NH (δ_H 8.50), as deduced from a ^{1}H–^{1}H COSY correlation of H_2-4′ with NH (Fig. 1), as well as from the HMBC correlations of NH with C-4′, and of H_2-4′ with C-2′ and C-5′ (Fig. 1). In the ^{1}H–^{1}H COSY spectrum, the overlapped H_2-2′ and H_2-3′ resonances (δ_H 1.78–1.91) exhibited correlations with H-1′ (δ_H 5.09) and H_2-4′, respectively, suggesting that C-1′ to C-4′ were anchored in a line. The connection of two parts was provided by the HMBC correlations of H-1′ with C-1 (δ_C 76.7, d) and C-20 (δ_C 98.4, d), and of H-20 (δ_H 5.75, br s) with C-1′ (δ_C 93.6, d), giving rise to the connectivities of C-1′ to C-1 and C-20 through an acetal group.

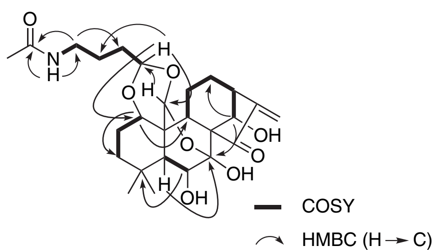


Figure 1. Key correlations of HMBC and ^{1}H–^{1}H COSY for **1**.

The relative stereochemistry of compound **1** was established using information from ROESY spectrum and by comparison of its spectroscopic data to those of rabdoternin F (**13**).[10] The same relative stereochemistry of diterpene part in compound **1** as in **13** was deduced from the similar carbon and proton chemical shifts and ROESY correlations found in **1** (Table 1). Considering that all the kauranoids isolated from the genus *Isodon* possessed an *ent*-configuration, **1** was also presumed to be an *ent*-kauranoid. The *S* configuration for C-20 was suggested from the strong ROESY correlations of H-20 with H_3-19 and H-6 as shown in computer-generated 3D drawing (Fig. 2). The configuration of C-1′ was inferred to be *R*, judging from ROESY cross-peaks of H-1′ with H-20 and H-2α. Thus, the structure of **1**, named as hebeirubescensin A, was unambiguously determined to be (1α,20*S*)-6β,7β,14β-trihydroxy-1,20-{[(1*R*)-4-(acetylamino)butane-1,1-diyl]dioxy}-7α,20-epoxy-*ent*-kaur-16-en-15-one.

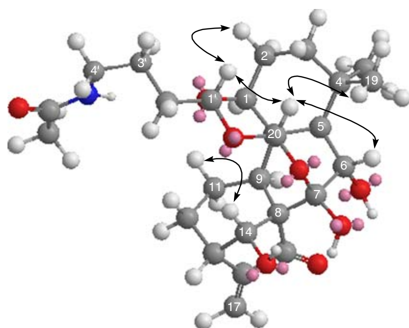


Figure 2. Key ROESY correlations for **1**.

Hebeirubescensins B–L (**2**–**12**) were analogues of hebeirubescensin A. Their stereostructure determination was thus aided by comparison of their spectroscopic data with those of **1** and some known *ent*-kauranoids. However, complete NMR studies on each new metabolite were performed in order to unambiguously determine the structures of the isolated compounds and to assign all the proton and carbon resonances. In particular, COSY and HSQC spectra, in combination with HMBC spectrum, acquired for all new compounds, showed that compounds **2**–**12** contained the same *ent*-kaurene core and similar oxygenation patterns. Interpretation of HMBC spectrum also allowed us to locate the substitution groups on methine carbons, while ROESY spectrum gave the relative stereochemistry information of chiral centers. Some key points for structure elucidations of compounds **2**–**12** were described below.

Hebeirubescensin B (**2**) was obtained as colorless prisms, possessing a molecular formula of $C_{25}H_{38}O_7$ as established by HRESIMS (calcd *m*/*z* 473.2515; found *m*/*z* 473.2511, $[M+Na]^+$). Its IR absorptions at 3395 and 1710 cm^{-1} suggested the presence of hydroxyl and carbonyl groups. Comparison of the ^1H and ^{13}C NMR data of **2** with those of rabdoternin F (**13**),[10] suggested that both compounds were closely similar and sharing the same oxygenation pattern. The only difference was in the signals due to the substitution group at C-20, including the absence of methoxyl carbon with the appearance of an isoamoxyl group on the basis of NMR data at δ_C 22.4 (q), 22.5 (q), 25.4 (d), 38.7 (t), and 67.6 (t) and δ_H 0.80 (6H), 1.68 (1H), 1.54 (2H), 4.09, and 3.55 (each 1H, q like, J=7.8 Hz) (Table 2 and Supplementary data) in **2**. Thus, the gross structure of **2** was determined to be a 7,20-epoxy-*ent*-kauranoid with the substitution of an isoamoxyl group at C-20, which was further confirmed by the HMBC correlations of H-20 with C-1′, and of H-1′ with C-20. The ROESY correlations of H-20 with H-6 and H_3-19 suggested that C-20 possessed an *S* configuration. Therefore, compound **2** was elucidated as (20*S*)-1α,6β,7β,14β-tetrahydroxy-20-isoamoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one.

Hebeirubescensins B and C (**2** and **3**) were obtained initially as a mixture by silica gel column chromatography and then separated by recrystallization and semipreparative HPLC. Both compounds had the same molecular formula, $C_{25}H_{38}O_7$, as determined by HRESIMS. Careful analysis of their ^1H and ^{13}C NMR data indicated that **2** and **3** might be C-20 epimers. Detailed comparison of NMR data of **3** with those of rabdoternin E (**14**),[10] showed that **3** and **14** were closely identical with each other, except for the substitution group at C-20. The stereochemistry at C-20 was further confirmed by the key ROESY correlation between H-20 and H-11α. Thus, hebeirubescensin C was elucidated

Table 2. ^{13}C NMR data for hebeirubescensins B–L (**2**–**12**) in C_5D_5N, δ (ppm)

No.	2	3	4	5	6	7	8	9	10	11	12[a]
1	75.4	74.2	75.3	75.1	31.1	75.2	75.0	75.5	30.4	73.8	31.8
2	31.0	30.9	31.0	29.5	18.9	30.5	31.1	30.9	18.7	28.5	15.5
3	39.5	40.5	39.5	39.7	41.6	39.4	40.9	39.3	41.6	39.9	41.5
4	34.1	33.2	34.1	34.3	34.2	34.1	33.3	33.3	34.2	34.3	33.6
5	60.1	59.0	60.0	60.3	58.6	59.9	59.6	57.8	57.5	57.9	53.7
6	74.7	74.7	74.6	74.5	73.8	74.8	74.7	73.6	73.8	74.6	75.2
7	99.7	100.1	99.7	97.0	101.4	99.3	99.9	101.8	101.6	97.1	96.3
8	62.2	63.0	62.1	59.8	53.8	62.4	63.2	52.8	52.6	53.4	53.3
9	53.7	56.6	53.7	58.8	52.1	54.1	56.7	45.1	44.3	50.0	47.5
10	43.7	45.2	43.7	44.0	40.3	43.3	45.6	43.6	39.4	42.7	35.9
11	23.3	21.8	23.3	66.2	63.5	23.5	21.8	21.6	17.7	63.3	18.9
12	31.3	31.3	31.3	39.1	45.1	31.5	31.4	34.0	33.1	42.7	32.0
13	44.4	43.4	44.4	35.0	47.3	44.4	43.4	47.0	46.8	37.3	43.4
14	73.7	72.9	73.7	27.8	76.6	73.9	73.0	76.1	76.1	27.5	76.9
15	210.2	208.5	210.2	212.0	73.2	210.6	208.8	73.2	73.0	75.3	73.6
16	153.3	153.5	153.3	154.3	160.4	153.7	153.6	161.4	161.5	161.9	157.5
17	118.7	119.6	118.8	115.4	109.3	118.6	119.4	108.8	109.0	106.8	111.5
18	33.5	35.9	33.5	33.3	33.9	33.6	35.9	33.3	33.9	33.1	33.1
19	22.3	23.2	22.2	22.7	22.9	22.2	23.6	22.2	22.6	22.8	22.5
20	102.3	99.6	102.4	101.7	102.4	96.1	93.2	103.7	103.7	64.3	66.8
1′	67.6	67.1	75.7	67.3	67.2						
2′	38.7	38.8	28.6	38.7	39.0						
3′	25.4	25.5	19.5	25.5	25.4						
4′	22.5	22.6	19.4	22.5	22.7						
5′	22.4	22.7		22.5	22.6						
OCH_3								55.3	56.1		

[a] Other signals: 12, 171.1, 171.0, 169.9 (C=O), 22.1, 21.6, 21.3 (CH_3) (OAc).

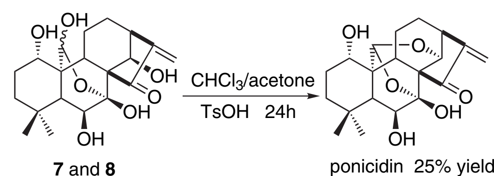
as (20*R*)-1α,6β,7β,14β-tetrahydroxy-20-isoamoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one.

Hebeirubescensin D (**4**) was isolated as colorless needles, and the molecular formula $C_{24}H_{36}O_7$ was deduced from pseudomolecular ion $[M+Na]^+$ at *m/z* 459 in ESIMS and NMR data, and further confirmed by the positive HRESIMS (*m/z* 459.2353 $[M+Na]^+$). The NMR spectroscopic data of **4** were closely identical to those of **2** (Table 2), except for the substitution group at C-20. Besides the signals for diterpene moiety, the ^{13}C NMR and DEPT spectra of **4** (Table 2) displayed four signals for two methyls (δ_C 19.5 and δ_C 19.4), one methine (δ_C 28.6), and one oxymethylene (δ_C 75.7) ascribed for an isobutoxyl residue. Therefore, compound **4** was concluded to be (20*S*)-1α,6β,7β,14β-tetrahydroxy-20-isobutoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one.

Hebeirubescensin E (**5**) was found by HRESIMS to possess the molecular formula, $C_{25}H_{38}O_7$, the same as those of **2** and **3**. Detailed analysis of the NMR spectra of **2** and **5** made it clear that these two compounds were similar except for the presence of a hydroxyl group at C-11 and the absence of a hydroxyl group at C-14 in **5**. The β-orientation for OH-11 was suggested from the intense ROESY correlation of H-11 with H-14α and the *trans* coupling constant between H-9 and H-11 (J=8.5 Hz). Thus, compound **5** was concluded to be (20*S*)-1α,6β,7β,11β-tetrahydroxy-20-isoamoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one.

Hebeirubescensin F (**6**) was obtained as an amorphous powder. It exhibited a quasimolecular ion peak at *m/z* 475.2671 $[M+Na]^+$ in the HRESIMS spectrum, suggesting a molecular formula $C_{25}H_{40}O_7$. Its IR and NMR spectral data suggested **6** to be a 7,20-epoxy-*ent*-kauranoid, with an isoamoxyl group and six oxygenated carbons. A careful analysis of the 2D NMR spectral data and comparison with rubescensin C (**23**),[11] led to the conclusions that the C-6, C-7, C-11, C-14, and C-15 positions were each substituted by a hydroxyl group, and the isoamoxyl group was at C-20, on the basis of the HMBC correlations of H-20 (δ_H 5.55) with C-1′ (δ_C 67.2, t), and of H_2-1′ (δ_H 4.06 and 3.48, each 1H) with C-20. Moreover, because of the ROESY correlations of H-14 with H-11, both hydroxyl groups at C-11 and C-14 were deduced to be sharing the same β-orientation. Therefore, compound **6** was determined to be (20*S*)-6β,7β,11β,14β,15β-pentahydroxy-20-isoamoxy-7α,20-epoxy-*ent*-kaur-16-ene.

Hebeirubescensins G and H (**7** and **8**) were isolated as an inseparable mixture of two isomers. Their HRESIMS spectra gave a pseudomolecular ion peak at *m/z* 403.1738 $[M+Na]^+$, consistent with the molecular formula $C_{20}H_{26}O_7$. Besides the absence of signal for OCH_3 group, most NMR signals of compounds **7** and **8** were nearly identical to those of **13** and **14**, respectively. Thus, **7** and **8** were determined to be a C-20 epimers. Detailed 2D NMR analysis confirmed this structure to be 1α,6β,7β,14β,20-pentahydroxy-7α,20-epoxy-*ent*-kaur-16-en-15-one. Interestingly, we found that this epimer could be converted into ponicidin (**27**) during the separation on silica gel column eluted with cyclohexane–chloroform–acetone (5:5:2). So the conversion of this epimer into ponicidin under mild acid condition was investigated (Scheme 1). It was therefore assumed that **7** and **8** might be the biosynthetic precursor of ponicidin.



Scheme 1.

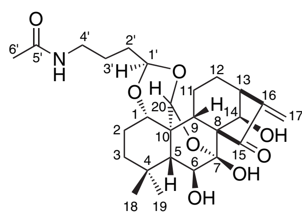
Hebeirubescensin I (**9**) was isolated as amorphous powder, and its molecular formula $C_{21}H_{32}O_7$ was established by HRESIMS. Comparison of the NMR data of **9** with those of rabdoternin F (**13**) led to the deductions that the only difference was the ketone group at C-15 in **13** being replaced by a hydroxyl group in **9**. The β-orientation of hydroxyl group at C-15 was suggested by the absence of any ROE of H-15 and the abnormal upfield shift of C-9 (δ_C 45.1, d) due to the γ-steric compression effect between OH-15 and H-9.[12,13] Thus, compound **9** was concluded to be (20*S*)-1α, 6β,7β,14β,15β-pentahydroxy-20-methoxy-7α,20-epoxy-*ent*-kaur-16-ene.

Hebeirubescensin J (**10**) was assigned to have the molecular formula $C_{21}H_{32}O_6$ from its HRESIMS and NMR data. Comparison of the spectral data of **10** with those of **9** (Table 2) showed similarities except for the substitution of a hydroxyl group at C-1 in **9** being replaced by a methylene group (δ_C 30.4, t) in **10**. The similar consideration allowed us to determine the relative stereochemistry of OH-15 with a β-orientation. Thus, the structure of **10** was established as (20*S*)-6β,7β,14β,15β-tetrahydroxy-20-methoxy-7α,20-epoxy-*ent*-kaur-16-ene.

Hebeirubescensin K (**11**) was obtained as amorphous powder. The only differences between the ^1H NMR spectrum of maoyecrystal F and **11** were that of **11** lacked a methyl signal of acetyl group and the signal for the H-6α was shifted upfield from δ_H 5.27 in mayecrystal F to δ_H 4.26 in **11**.[26] This fact suggested **11** was 1α,6β,7β,11β,15β-pentahydroxy-7α,20-epoxy-*ent*-kaur-16-ene.

The molecular formula of hebeirubescensin L (**12**) was determined to be $C_{26}H_{36}O_8$ from the HRESIMS. The ^1H NMR spectrum almost superimposable with that of rabdoternin C (**15**),[14] the only exception due to the hydroxyl group at C-14 in **15** with an acetyl group in **12**. HMBC correlation of the carbonyl group of this additional acetate with H-14 unambiguously located this residue at C-14. Thus, compound **12** was determined to be 7β-hydroxy-6β,14β,15β-triacetoxy-7α,20-epoxy-*ent*-kaur-16-ene.

The structures of the known compounds **13**–**31** were established to be rabdoternin F (**13**),[10] rabdoternin E (**14**),[10] rabdoternin C (**15**),[14] oridonin (**16**),[15,16] rubescensin O (**17**),[13] rabdoternin B (**18**),[14] lasiokaurin (**19**),[17,18] rabdoternin A (**20**),[14] enmenol (**21**),[19] lasiodonin (**22**),[17,18] rubescensin C (**23**),[11] rabdoternin G (**24**),[10] rabdoternin D (**25**),[10] rubescensin Q (**26**),[20] ponicidin (**27**),[21] macrocalin B (**28**),[22] xerophilusin B (**29**),[23] acetonide of maoyecrystal F (**30**),[20,26] and trichokaurin (**31**),[24,25] by comparison of their spectral data with literature values.



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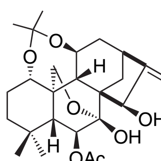

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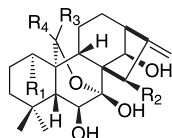
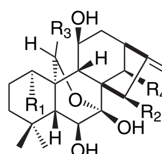
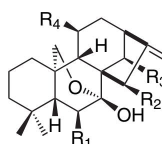


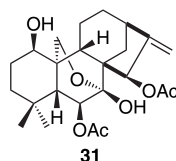
	R_1	R_2	R_3	R_4
2	OH	=O	OC_5H_{11}	H
3	OH	=O	H	OC_5H_{11}
4	OH	=O	OC_4H_9	H
7	OH	=O	OH	H
8	OH	=O	H	OH
9	OH	OH	OCH_3	H
10	H	OH	OCH_3	H
13	OH	=O	OCH_3	H
14	OH	=O	H	OCH_3
16	OH	=O	H	H
17	OH	OH	H	OCH_3
18	OH	OH	=O	
19	OAc	=O	H	H
20	H	OH	=O	
21	OH	OH	H	H



	R_1	R_2	R_3	R_4
5	OH	=O	OC_5H_{11}	H
6	H	OH	OC_5H_{11}	OH
22	OH	=O	H	H
11	OH	OH	H	H
23	H	OH	H	OH
24	OH	=O	OCH_3	H

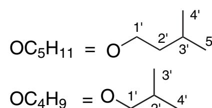


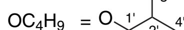
	R_1	R_2	R_3	R_4
12	OAc	OAc	OAc	H
15	OAc	OAc	OH	H
25	OAc	OH	OH	OH
26	OAc	OH	OH	H



31

	R_1	R_2
27	OH	H
28	H	OH
29	H	H





3. Biological activity

The cytotoxicities of compounds **1–14**, **16**, **19**, **22**, and **24** against A549, HT-29, and K562 cells were summarized in Table 3. Compounds **1–5**, **7**, **8**, **13**, **14**, **16**, **19**, **22**, and **24** showed inhibitory effects against those tumor cells, while compounds **6**, and **9–12** were completely inactive, which suggested that the cyclopentanone conjugated with an *exo*-methylene group was the active center for the inhibitory effect.[27] Moreover, careful examination of the results allowed us to determine some other structure–activity relationship. A better activity was observed when the carbon C-20 was an *S* configuration. Additionally, the presence of a hydroxyl group at C-11 would result in a marked decrease in cytotoxicity. Finally, compounds **2** and **3** were more potent than compounds **4**, **7**, **8**, **13**, and **14**, which indicated that the isoamoxyl group at C-20 could greatly improve the cytotoxicity. Further investigations of their cytotoxic

Table 3. Cytotoxicity data of compounds **1–14**, **16**, **19**, **22**, and **24** with IC_{50} values (μM)[a]

	1	**2**	**3**	**4**	**5**	**6**	**7** and **8**	**9**	**10**	**11**	**12**	**13**	**14**	**16**	**19**	**22**	**24**
A549	4.80	0.68	1.17	5.32	1.67	>100	8.99	>100	>100	>100	>100	2.16	2.83	9.54	6.04	46.47	53.21
HT–29	16.57	1.19	1.93	6.04	2.84	>100	18.42	>100	>100	>100	>100	5.81	6.30	12.31	9.61	31.62	15.88
K562	5.93	1.21	1.88	7.31	6.17	>100	8.32	>100	>100	>100	>100	6.84	7.82	7.86	3.08	13.52	42.71

[a] Amrubicin hydrochloride (positive control): IC_{50}=0.82 (A549), 4.36 (HT-29), and 1.26 (K562), respectively.

activities and structure–activities relationships are in progress and will be described later.

4. Conclusion

In conclusion, this was the first report of a N-containing *ent*-kauranoids, hebeirubescensin A (**1**), isolated from genus *Isodon*. Additionally, a series of new *ent*-kauranoids, named hebeirubescensins B–F (**2**–**6**), were the first examples of *ent*-kauranoids having an isoamoxyl or isobutoxyl group in the molecule. This discovery expanded considerably the library for this class of natural products. Most importantly, cytotoxicity assay showed that isoamoxyl group at C-20 could increase the lipophilicity of *ent*-kauranoids leading to apparent improvement of cytotoxicity, and this discovery could therefore serve as a scaffold for the synthesis of more potent modified diterpenoids.

5. Experimental

5.1. General

Melting points were obtained on an XRC-1 apparatus and were uncorrected. Optical rotations were carried out on a Perkin–Elmer model 241 polarimeter. IR spectra were measured in a Bio-Rad FTS-135 spectrometer with KBr pellets. MS were recorded on a VG Auto spec-3000 spectrometer or on a Finnigan MAT 90 instrument. 1D and 2D NMR spectra were taken on a Bruker AM-400 and a Bruker DRX-500 instrument with TMS as internal standard, respectively. Semipreparative HPLC was performed on an Agilent 1100 liquid chromatograph with a Zorbax SB-C_{18}, 9.4 mm× 25 cm column. Column chromatography were performed either on silica gel (200–300 mesh. Qingdao Marine Chemical Inc., China), silica gel H (10–40 μm, Qingdao Marine Chemical Inc., China), or Lichroprep RP-18 gel (40–63 μm, Merck, Dramstadt, Germany).

5.2. Extraction and isolation

The leaves of *I. rubescens* were purchased in Hehuachi herbal market, Chengdu, Sichuan Province, People's Republic of China, in January 2004, and were identified by Prof. Xi-Wen Li. A voucher specimen was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences. The powdered air-dried leaves of *I. rubescens* (4.5 kg) were extracted with 70% aq acetone (3×20 L) at rt overnight. The extract was partitioned between H_2O and EtOAc. The EtOAc layer (165 g) was chromatographed on MCI-gel CHP 20P (90% CH_3OH–H_2O, 100% CH_3OH). The 90% CH_3OH fraction (145 g) was chromatographed over silica gel (200–300 mesh, 1.5 kg), eluted in a step gradient manner with $CHCl_3$–acetone (1:0 to 0:1) to afford fractions I–VIII. Fraction II was submitted to repeated chromatography over silica gel (petroleum–acetone, from 30:1 to 10:1; cyclohexane–2-propanol, 60:1) and RP-18, followed by semipreparative and preparative HPLC to yield compounds **2** (1.1 g), **3** (7 mg), **4** (11 mg), **5** (8 mg), and **12** (5 mg). Fraction III was first submitted to chromatography over RP-18 (CH_3OH–H_2O, from 0:1 to 1:0) and silica gel ($CHCl_3$–acetone, from 40:1 to 20:1), followed by semipreparative HPLC to yield compounds **6** (13 mg), **15** (25 mg), **29** (4 mg), **30** (24 mg), and **31** (46 mg). In the same way, fraction IV yielded compounds **10** (16 mg), **13** (1.21 g), **14** (0.42 g), **25** (7 mg), **26** (6 mg), **27** (305 mg), and **28** (3 mg). Compound **16** (12.3 g) was obtained from fraction V by recrystallization from CH_3OH. The remnant of fraction V was separated by silica gel chromatography and semipreparative HPLC to afford compounds **17** (13 mg), **18** (13 mg), **19** (6 mg), **20** (7 mg), **22** (562 mg), and **24** (11 mg). Compounds **7** and **8** (45 mg), **9** (26 mg), **11** (7 mg), **21** (3 mg), **23** (23 mg) were obtained from fraction VI. Compound **1** (9 mg) was obtained from fraction VII by repeated silica gel chromatography and semipreparative HPLC.

5.2.1. Hebeirubescensin A (1). Amorphous powder; $[\alpha]_D^{19}$ −23.2 (*c* 0.12, CH_3OH); IR (KBr) ν_{max} 3387, 2948, 2875, 1711, 1643, 1552, 1453, 1369, 1094 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) see Table 1; HRESIMS (positive ion) *m/z* 514.2430 (calcd for $C_{26}H_{37}NO_8Na$ $[M+Na]^+$, 514.2416).

5.2.2. Hebeirubescensin B (2). Colorless prisms; mp 194–195 °C; $[\alpha]_D^{19}$ −2.67 (*c* 0.26, CH_3OH); IR (KBr) ν_{max} 3395, 2956, 2871, 1710, 1643, 1458, 1092, 1063, 985 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 473.2511 (calcd for $C_{25}H_{38}O_7Na$ $[M+Na]^+$, 473.2515).

5.2.3. Hebeirubescensin C (3). Amorphous powder; $[\alpha]_D^{19}$ −27.5 (*c* 0.32, CH_3OH); IR (KBr) ν_{max} 3417, 2954, 2870, 1711, 1644, 1463, 1142, 1095, 1060, 992 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 473.2513 (calcd for $C_{25}H_{38}O_7Na$ $[M+Na]^+$, 473.2515).

5.2.4. Hebeirubescensin D (4). Amorphous powder; $[\alpha]_D^{19}$ −26.2 (*c* 0.32, CH_3OH); IR (KBr) ν_{max} 3375, 2957, 2933, 1711, 1645, 1459, 1370, 1218, 1091, 996 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 459.2353 (calcd for $C_{24}H_{36}O_7Na$ $[M+Na]^+$, 459.2358).

5.2.5. Hebeirubescensin E (5). Amorphous powder; $[\alpha]_D^{19}$ −43.1 (*c* 0.38, CH_3OH); IR (KBr) ν_{max} 3374, 2956, 2934, 1710, 1642, 1458, 1370, 1083, 1052, 969 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 473.2514 (calcd for $C_{25}H_{38}O_7Na$ $[M+Na]^+$, 473.2515).

5.2.6. Hebeirubescensin F (6). Amorphous powder; $[\alpha]_D^{19}$ −31.6 (*c* 0.15, CH_3OH); IR (KBr) ν_{max} 3315, 2955, 1628, 1463, 1366, 1104, 982 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 475.2671 (calcd for $C_{25}H_{40}O_7Na$ $[M+Na]^+$, 475.2671).

5.2.7. Hebeirubescensin G (7). Amorphous powder; IR (KBr) ν_{max} 3332, 2945, 2871, 1709, 1645, 1460, 1364, 1094, 1055, 909 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see

Table 2; HRESIMS (positive ion) *m/z* 403.1738 (calcd for $C_{20}H_{28}O_7Na$ $[M+Na]^+$, 403.1733).

5.2.8. Hebeirubescensin H (8). Amorphous powder; IR (KBr) ν_{max} 3332, 2945, 2871, 1709, 1645, 1460, 1364, 1094, 1055, 909 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 403.1738 (calcd for $C_{20}H_{28}O_7Na$ $[M+Na]^+$, 403.1733).

5.2.9. Hebeirubescensin I (9). Amorphous powder; $[\alpha]_D^{19}$ −12.5 (*c* 0.10, CH_3OH); IR (KBr) ν_{max} 3363, 2931, 2869, 1622, 1454, 1359, 1073, 1025, 983 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 419.2040 (calcd for $C_{21}H_{32}O_7Na$ $[M+Na]^+$, 419.2046).

5.2.10. Hebeirubescensin J (10). Amorphous powder; $[\alpha]_D^{19}$ −19.1 (*c* 0.20, CH_3OH); IR (KBr) ν_{max} 3423, 2926, 1629, 1449, 1204, 1104, 984 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 403.2090 (calcd for $C_{21}H_{32}O_6Na$ $[M+Na]^+$, 403.2096).

5.2.11. Hebeirubescensin K (11). Amorphous powder; $[\alpha]_D^{19}$ −10.8 (*c* 0.15, CH_3OH); IR (KBr) ν_{max} 3332, 2945, 2871, 1645, 1460, 1364, 1094, 1055, 909 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 389.1943 (calcd for $C_{20}H_{30}O_6Na$ $[M+Na]^+$, 389.1940).

5.2.12. Hebeirubescensin L (12). Amorphous powder; $[\alpha]_D^{19}$ −57.2 (*c* 0.42, CH_3OH); IR (KBr) ν_{max} 3539, 2949, 1740, 1372, 1249, 1057, 899 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) see Supplementary data; ^{13}C NMR (C_5D_5N, 100 MHz) see Table 2; HRESIMS (positive ion) *m/z* 499.2315 (calcd for $C_{26}H_{36}O_8Na$ $[M+Na]^+$, 499.2308).

5.3. Conversion of 7 and 8 into ponicidin

Hebeirubescensins G and H (**7** and **8**, 10 mg) were added to acetone (5 ml) and TsOH (2.0 mg), then stirred and kept at rt for 24 h, and then evaporated the solvent and subjected to RP-18 column eluted with CH_3OH–H_2O (40:60) to give ponicidin (**27**) (2.5 mg).

5.4. Cytotoxicity assay

Cytotoxicity of compounds against suspended tumor cells was determined by trypan blue exclusion method and against adherent cells was determined by sulforhodamine B (SRB) assay. Cells were plated in 96-well plate 24 h before treatment and continuously exposed to different concentrations of compounds for 72 h. After compound treatment, cells were counted (suspended cells) or fixed and stained with SRB (adherent cells) as described by Monks et al.[28]

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