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Bisrubescensins A–C: Three New Dimeric *ent*-Kauranoids Isolated from *Isodon rubescens*

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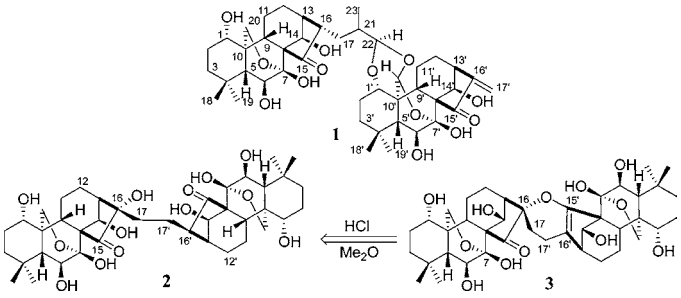
ABSTRACT



A phytochemical study of the secondary metabolites produced by the species of *Isodon rubescens* has led to the isolation of three new dimeric *ent*-kauranoids and two known ones. The most important of these compounds are bisrubescensin A (1), which contains an unprecedented C_{23} *ent*-kaurane unit, and bisrubescensin C (3), which is the precursor of bisrubescensin B (2) from the viewpoint of biosynthesis. Their structures were determined on the basis of extensive spectroscopic analysis and chemical evidence.

The genus *Isodon*, which includes about 150 species, is one of the most widespread members of the Labiatae (Lamiaceae) family and has attracted considerable attention as a prolific source of new natural products with diverse structures and biological properties. In particular, diterpenoids are thought by many to be the largest pool of chemically diverse and physiologically interesting metabolites.[1,2] For the past 30 years, as part of research for the discovery of novel natural products as useful leads for the development of therupeutic agents to treat cancer, more than 50 *Isodon* species of China have been phytochemically investigated by our group, and about 500 new diterpenoids (mainly *ent*-kauranoids) have been isolated and characterized.[3]

Isodon rubescens, a perennial herb of this genus that is notable for being abundant in *ent*-kaurane diterpenoids, is well-known in China as an anticancer and antiinflammation folk medicine.[4] Several varieties of this species collected from different regions of China have been systematically investigated by our group.[5] About 60 new diterpenoids including several compounds with novel skeletons, such as dimeric *ent*-kuaronoids (xindongnins M−O),[5a] 8,15-*seco*-*ent*-

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kauranoids (rubescensins U and V),[5b] 15,16-*seco*-*ent*-kauranoid (rubescensin S),[5c] and 20-*nor*-*ent*-kauranoid (rubescensin N),[5d] have been isolated and identified. With an aim to isolate structurally interesting and bioactive diterpenoids, as well as key intermediates to clarify the biogenetic pathways, purification of an extract of *I. rubescens* led to the isolation of three new dimeric *ent*-kauranoids, bisrubescensins A−C (**1**−**3**) and two known *ent*-kauranoids, oridonin (**4**)[6] and rabdoternin F (**5**).[7] Among them, **1** has a unique C_{23} *ent*-kaurane diterpenoid unit. Moreover, the isolation of two biogenetically related dimers, **2** and **3**, which have the unique linkage of single carbon−carbon bond between two subunits, provide important support to the biosynthetic pathways of xindongnins M−O proposed previously.[5a] This paper describes the isolation and structure elucidation of bisrubescensins A−C (**1**−**3**) on the basis of spectroscopic analysis and chemical transformation.

Bisrubescensin A (**1**) was isolated as amorphous powder. The IR spectrum of **1** showed absorptions for hydroxy (3418 cm^{-1}), ketone (1723 cm^{-1}), conjugated ketone (1706 cm^{-1}), and olefinic (1643 cm^{-1}) groups. The molecular formula $C_{43}H_{60}O_{13}$ was determined by HRESIMS for the $[M + Na]^+$ ion at *m*/*z* 807.3929, which requires 14 degrees of unsaturation in the molecule. The ^1H and ^{13}C NMR spectra of **1** (Table 1) showed 43 carbon resonances due to two carbonyl groups, an oleflnic group, 11 oxygenated carbons including two quaternary and one methylene carbons, and four tertiary and one secondary methyl groups. Careful analysis of NMR and MS spectra showed that **1** seemed to be a dimeric *ent*-kaurane diterpenoid.

The gross structure of **1** was elucidated by the analysis of 2D NMR data and by comparison with the NMR data of **4** and **5**. Each pair of these ^1H and ^{13}C NMR signals of **1** (Table 1) seemed to be due to each half of molecule (parts **a** and **b**, Figure 1). In part **a**, the close similarities of the NMR data of the A and B rings with those of known compound **4** suggested a similar structure for rings A and B, but with differences in the chemical shifts at C-12 and C-13 of C ring. These changes, along with the completely different carbon and proton chemical shifts of C-15, C-16, and C-17, indicated that the structures of compounds **1** and **4** differ in the D ring of the molecule. The substructure of the D ring was revealed by 2D NMR experiments (Figure 1). The $^1H-^1H$ COSY correlations of H-17 with H-21 and HMBC correlations of H-21 with C-16 and C-17 showed that C-17 was connected to C-21 through a single carbon−carbon bond. Furthermore, the HMBC cross-peaks of H_3-23 (δ_H 1.14, d, J = 6.5 Hz) with C-21 (δ_C 36.0, d), C-17 (δ_C 28.2, t), and C-22 (δ_C 95.9, d) allowed us to determine the gross structure of part **a**. Subtraction of the twenty three carbons associated with part **a** left 20 carbons, which were suggestive of a normal diterpene skeleton. Comparison of the remaining carbon and proton NMR chemical shifts of **1** to those of **5** indicated the gross structure of part **b**, which was also confirmed by the careful analysis of 2D-NMR data. The connection between parts **a** and **b** was achieved by the HMBC correlations of H-22 (δ_H 5.05) with C-1′ (δ_C 76.8, d) and C-20′ (δ_C 99.0, d) and of H-20′ (δ_H 5.96, s) with

Table 1. ^1H and ^{13}C NMR Data for Compound **1** in C_5D_5N

	1 (*J* in Hz)			**1** (*J* in Hz)	
no.	δ_H mult	δ_C	no.	δ_H mult	δ_C
1	3.65, t like (8.1)	72.8	1′	3.83, dd (12.5, 5.0)	76.8
2	1.80−1.90,[a] m, 2H	30.5	2′	2.02, m	22.7
				1.54, m	
3α	1.45,[b] m	39.4	3′α	1.38,[d] m	38.6
3*β*	1.33,[d] m		3′*β*	1.25,[c] m	
4		34.1	4′		33.1
5	1.44[b]	61.6	5′	1.45[b]	57.5
6	4.18, dd (10.8, 5.7)	74.7	6′	4.25, dd (11.1, 9.3)	75.5
7		98.1	7′		100.6
8		62.6	8′		61.8
9	1.89[a]	53.9	9′	1.87[a]	53.9
10		41.5	10′		38.6
11α	1.88[a]	19.5	11′α	2.94, m	23.8
11*β*	1.51		11′*β*	1.86,[a] m	
12α	1.98, m	20.6	12′α	2.46, m	31.6
12*β*	1.66, m		12′*β*	1.46,[b] m	
13	2.79, m	38.9	13′	3.18, m	43.8
14	5.43, br s	74.2	14′	5.56, br s	72.9
15		225.1	15′		208.8
16	3.59, m	49.6	16′		153.3
17a	2.49, m	28.2	17′ a	5.50, br s	119.8
17b	1.79,[a] m		17′ b	6.26, br s	
18	1.24,[c] s	33.1	18′	1.26,[c] s	33.8
19	1.12, s	21.9	19′	0.99, s	22.2
20a	4.40, d (10.5)	64.2	20′	5.96, s	99.0
20b	4.75, d (10.5)				
21	2.19, m	36.0			
22	5.05[e]	95.9			
23	1.14, d (6.5)	15.3			

[a−d] Signals overlapped. [e] Overlapped with peak of H_2O.

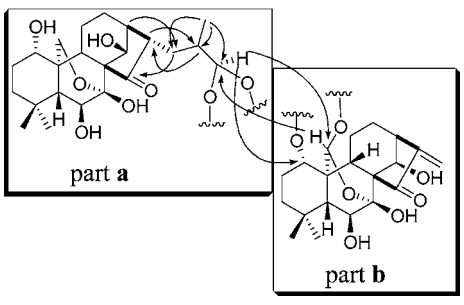

Figure 1. Selected HMBC correlations of **1**.

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C-22, giving rise to the connectivities of C-22 to C-1′ and C-20′ through two ether bonds. ROESY correlations of H-22 with H-2′α and H-20′ also supported the connectivities of the two parts. Thus, the gross structure of bisrubescensin A (**1**) was assigned as a new dimeric diterpenoid consisting of a C_{23} diterpene unit.

The ROESY spectrum was used to establish the relative stereochemistry. Although we have not yet determined the absolute stereochemistry, we have shown here the molecules with *ent*-stereochemistry as was found in the *ent*-kauranes from the genus *Isodon*. 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 molecular models (Figure 2),

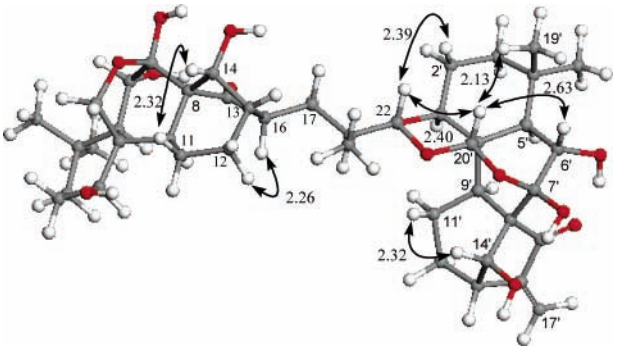


Figure 2. Key ROESY correlations of **1** and corresponding interatomic distance (Å).

energy minimized using the PCFF force field in Material Studio 3.0.1 overlaid with key correlations observed in the ROESY spectrum. The configuration of C-22 was inferred to be *R*, judging from ROESY correlations of H-22 with H-20 and H-2′α, which were all supported by calculated interatomic distances of approximately 2.40 Å. The ROESY cross-peaks of H-16 with H-12β confirmed the β-orientation of H-16 in **1**. All of the other chiral centers of **1** were in complete agreement with those of **4** and **5**. However, the relative configuration at C-21 in **1** could not be determined from spectroscopic data alone, and the small amount of material has thus far precluded chemical degradation efforts to elucidate this final structure feature.

Bisrubescensin B (**2**) was obtained as an amorphous solid, and the molecular formula was established to be $C_{40}H_{58}O_{13}$ by its HRESIMS data (*m*/*z* 769.3751 $[M + Na]^+$). Its ^{13}C NMR spectrum showed 40 carbon signals, which exhibited two diterpene units. The characteristic signals of four methyl groups (C-18,18′ and C-19,19′), six unoxygeated methine carbons (C-5,5′, C-9,9′, and C-13,13′), and six quaternary carbons (C-4,4′, C-8,8′, and C-10,10′) in the ^{13}C NMR spectrum showed that each diterpene unit has an *ent*-kaurane skeleton. Comparison of the ^1H NMR data of **2** with those of **4** suggested that each unit resembled to **4**, except for the absence of olefinic signals attributable to H-16 and H-17. The signals corresponding to this double bond were also absent from the ^{13}C NMR spectrum of **2**, where four signals at δ_C 37.2 (t), 19.9 (t), 52.0 (d), and 80.1 (s) suggested that both diterpene units have a 16,17-dihydrooridonin moiety. In addition, the protons of two methylene groups (H_2-17 and H_2-17′) showed $^1H-^1H$ COSY correlations with each other indicating a single bond between these two carbons, which was further confirmed by a series of HMBC correlations of H_2-17 with C-15, C-16, and C-17′, of H_2-17′ with C-15′, C-16, and C-16′, and of H-16′ with C-17.

The relative stereochemistry of **2** was deduced by ROESY experiment. Key ROESY correlations between H-17 and H-12β indicated that the OH-16 was in an α-orientation. Similarly, the β-orientation of 16′-methylene group was also deduced by the ROESY correlation between H-17′ and H-12′β. Thus, the stereochemistry of C-16 and C-16′ could be superimposed on that of xindongnin M, the absolute stereochemistry of which was determined by single X-ray diffraction.[5a] This result was also supported by the fact that the chemical shifts of C-16 (δ_C 80.1) in **2** closely resembled those of xindongnin M (δ_C 80.5).[5a] Therefore, the structure of bisrubescensin B was concluded as **2**.

From ^{13}C NMR data (Table 2), it was clear that bisrubescensin C (**3**) was closely related to **2**, since the chemical shifts of rings A, B, and C were nearly identical (Table 2). On the basis of IR, MS, and NMR data, compound **3** differed from **2** by the presence of an additional tetrasubstituted double bond (δ_C 153.6, s and δ_C 115.5, s), a loss of methine and ketone carbons, and an additional ring. In the HMBC spectrum, a series of key correlations were clearly observed: H-17 with C-15, 16, 16′, and 17′, H-17′ with C-15′, 16′, 16, and 17′, and H-13′ with C-15′. As a consequence, C16−O−C15′−C16′−C17′−C17 formed a six-membered dihydropyran ring. To determine the stereochemistry of C-16, the ROESY spectrum was employed. Unfortunately, no

Table 2. ^{13}C NMR Data for Compounds **2** and **3** in C_5D_5N

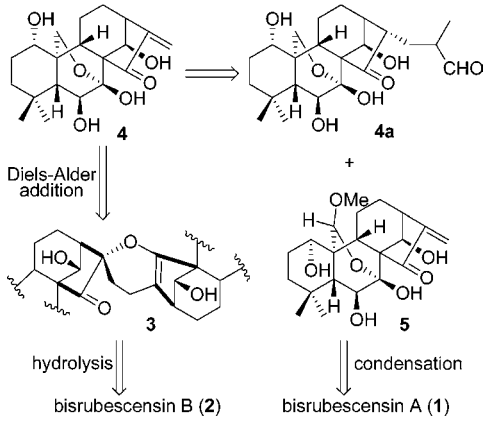
no.	**2**	**3**	no.	**2**	**3**
1	73.0	72.8	1′	72.7	72.6
2	30.5	30.7[d]	2′	30.5	30.6[d]
3	39.3	39.3	3′	39.3	39.5
4	34.0[a]	33.8	4′	34.1[a]	34.1
5	60.7	60.1	5′	61.5	61.6
6	74.8	75.1	6′	74.5	74.4
7	98.0[b]	98.2	7′	98.1[b]	99.3
8	62.7	63.2	8′	62.6	56.4
9	54.6	54.1	9′	53.8	49.3
10	41.6	42.0[e]	10′	41.4	41.9[e]
11	19.2	19.3	11′	19.3	21.0
12	20.3	21.3	12′	19.9	22.3
13	42.8	44.0	13′	37.6	44.0
14	73.4	73.0	14′	74.1	76.9
15	225.7	217.8	15′	225.0	153.6
16	80.1	86.3	16′	52.0	115.5
17	37.2	30.2	17′	19.9	17.5
18	33.3[c]	33.9	18′	33.1[c]	33.1
19	21.8	22.4	19′	22.2	21.9
20	64.1	64.1	20′	64.1	64.0

[a−e] Signals may be interchangeable.

ROESY correlation was observed to confirm the stereochemistry of C-16. The close biogenetic relationship of **3** to **2** might indicate that the absolute stereochemistry at C-16 for **3** is the same as that of **2**, so we hydrolyzed **3** with HCl to a single product, which was identical to **2** (by NMR and HPTLC). This fact indicated that the configuration of C-16 in **3** was the same as that of **2**. Thus, bisrubescensin C (**3**) was determined to be a dimer of **4** linked by a six-membered dihydropyran ring.

The biosynthetic pathway of bisrubescensin A (**1**) is outlined in Scheme 1. The key step is the formation of **4a**,

Scheme 1. Proposed Biogenetic Pathway for **1**−**3**



followed by condensation reaction between aldehyde group of **4a** and hydroxy and methoxy groups of **5** producing dimer **1**. The co-occurrence of compounds **2**−**4** in the same plant suggests a similar biosynthetic pathway. The six-membered dihydropyran ring of **3** could be formed via an intermolecular Diels−Alder cycloaddition between the olefin group and the α,β-unsaturated ketone group in **4**. The hydrolysis of this heterocycle yields **2** (Scheme 1). Therefore, it was interesting to investigate the biogenetic Diels−Alder reaction leading to synthesis of compounds **2** and **3** in vitro. The Diels−Alder dimerization reactions of **4** were carried out in several solutions (CH_3OH; Me_2CO; $CHCl_3$; CH_3OH and silica gel; Me_2CO and silica gel; $CHCl_3$ and silica gel) under a nitrogen atomosphere or air at room temperature for 4 weeks, and the reactions were monitored by HPTLC with **2** and **3** as control, but no change was observed in any of conditions. Thus, the synthesis of this type of dimer may be catalyzed by a Diels−Alderase in *Isodon* species. Recently, considerable efforts have been made to prove and to identify the enzymatic Diels−Alder reaction in the biosynthesis of natural products,[8] and several natural Diels−Alderase such as solanapyrone synthase,[9] lovastatin nonaketide synthase,[10] and macrophomate synthase[11] have been purified and characterized. Therefore, the function and catalytic mechanism of Diels−Alderase in *Isodon* species are another interesting topic to be investigated.

Compounds **1**−**3** were tested for cytotoxicity against A549, HT-29, and K562 cells using the sulforhodamine B (SRB) method as reported previously.[12] Compound **1** exhibited significant inhibitory activity against those tumor cell lines with IC_{50} values of 0.54, 1.48, and 1.85 μM, respectively. Compounds **2** and **3** were completely inactive with IC_{50} values of >100 μM.

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Supporting Information Available: Detailed description of the experimental procedures, copies of the NMR (1D and 2D) and MS spectra of compounds **1**−**3**, and ^{1}H NMR data for compounds **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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