Brine Shrimp Lethality Test Active Constituents and New Highly Oxygenated *Seco*-prezizaane-Type Sesquiterpenes from *Illicium merrillianum*

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In the study of bioactive substances in *Illicium* plants, the methanol extract of *I. merrillianum* showed brine shrimp lethality test (BST) activity at 200 μg/ml. Bioassay-guided fractionation of the BST active fractions resulted in the isolation of 4-*O*-methyleudesm-11-en-4α-ol, eudesmol-11-en-4α-ol and (−)-hinokinin as potent BST active compounds. On the other hand, four new highly oxygenated *seco*-prezizaane-type sesquiterpenes, merrilliortholactone (1), 2α-hydroxycycloparvifloralone (2), 2α-hydroxycycloparviflorolide (3), and 2α-hydroxyanisatin (4) were isolated from the BST-inactive polar fractions. The structures of new compounds were elucidated by extensive analyses of spectral data. Furthermore, the absolute configuration of 3 was established by the modified Mosher's method. Compounds 1—4 showed neither BST activity at 100 μg/ml nor neurite outgrowth-promoting activity.

Key words *Illicium merrillianum*; *seco*-prezizaane-type sesquiterpene; brine shrimp lethality

In recent years, we have engaged in a program to explore bioactive substances in *Illicium* species using the brine shrimp lethality test (BST)[1] and neurite outgrowth-promoting assay.[2] Three neurite outgrowth-promoting sesquiterpenes, isodunianin,[3] and merrilactone A[4] and 11-*O*-debenzoyl tashironin,[5] have so far been isolated from *I. tashiroi* and *I. merrillianum*, respectively. We have continued to investigate the chemical constituents of the pericarps of *I. merrillianum* collected in Yunnan Province, China, and locally used as an antirheumatic agent. To date, we have reported the isolation of 15 new *seco*-prezizaane-type and four novel anislactone-type sesquiterpenes from this plant.[4—8] Our screening program found that the methanol extract of *I. merrillianum* showed brine shrimp lethal activity. The percentage of deaths of the methanol extract was 37% at 200 μg/ml. This paper deals with the isolation of BST active constituents and structural elucidation of four new highly oxygenated *seco*-prezizaane-type sesquiterpenes designated as merrilliortholactone (**1**), 2α-hydroxycycloparvifloralone (**2**), 2α-hydroxycycloparvifllorolide (**3**), and 2α-hydroxyanisatin (**4**).

The methanol extract of *I. merrillianum* was divided into fractions A—M by column chromatography on silica gel. The BST of these fractions revealed the LC_{50} of fractions A, C, and D to be lower than 50 μg/ml, but the LC_{50} of fractions B and E—M was higher than 100 μg/ml. The BST results indicated that the less polar fractions contained more potent bioactive compounds than the polar fractions. Thus bioassay-guided fractionation of the active fractions A, C and D using a combination of various chromatographic methods led to the isolation of 11 previously known compounds: 3,7-dimethyl-1,5-octadiene-3,7-diol; methyl palmitate, geranyl acetate; α-cadinol methyl ether; caryophyllene oxide; eudesm-11-en-4α-ol; 4-*O*-methyleudesm-11-en-4α-ol; (−)-hinokinin, 4-allyl-2,6-dimethoxyphenol; *trans*-cinnamic acid; and tashironin.[9] Their brine shrimp lethal activities are shown in Table 1. Two eudesmane-type sesquiterpenes, eudesm-11-en-

Table 1. Brine Shrimp Lethal Activity of Compounds Obtained from Fractions A, C, and D

Compounds	LC_{50} (μg/ml)
3,7-Dimethyl-1,5-octadiene-3,7-diol	>100
Methyl palmitate	>100
Geranyl acetate	82.6
α-Cadinol methyl ether	52.8
Caryophyllene oxide	51.7
Eudesm-11-en-4α-ol	32.8
4-*O*-Methyleudesm-11-en-4α-ol	16.2
(−)-Hinokinin	24.3
4-Allyl-2,6-dimethoxyphenol	>100
trans-Cinnamic acid	>100
Tashironin	65.2

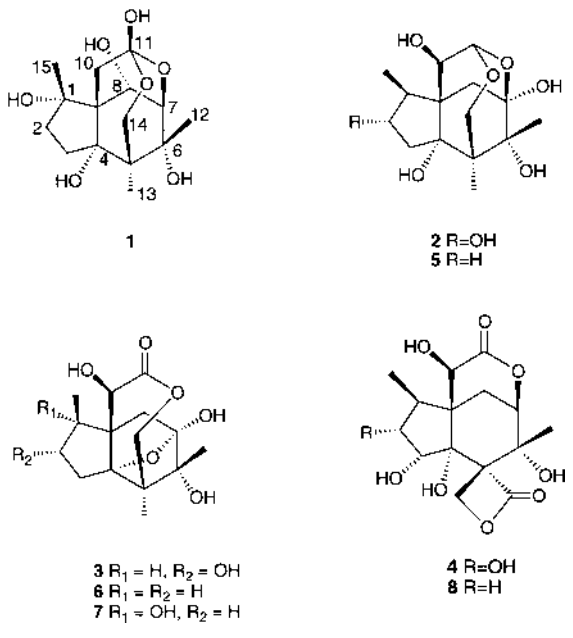

Chart 1. Structures

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Table 2. ^{1}H-NMR Spectral Data for **1**—**4** (600 MHz, CD_3OD)

H	1	2	3	4
1		2.11 qd (7.1, 7.0)	2.45 qd (9.6, 7.1)	2.23 qd (7.4, 6.9)
2	1.94—2.20 m	4.01 ddd (9.1, 7.0, 1.5)	4.04 ddd (9.6, 9.6, 3.3)	3.79 dd (7.9, 7.4)
3α	1.78 ddd (14.3, 9.9, 4.7)	1.37 dd (13.7, 1.5)	1.63 dd (14.8, 3.3)	4.64 d (7.9)
3β	2.33 ddd (14.3, 11.5, 6.0)	2.66 dd (13.7, 9.1)	2.42 dd (14.8, 9.6)	
7	3.70 d (3.3)			4.23 dd (3.6, 1.7)
8α		2.37 d (13.2)	1.57 d (14.0)	2.50 dd (14.8, 1.7)
8β	4.43 d (3.3)	1.43 d (13.2)	1.71 d (14.0)	2.08 dd (14.8, 3.6)
10α	1.90 d (14.8)	3.75 d (5.5)	4.40 s	4.14 s
10β	1.67 d (14.8)			
11		5.02 d (5.5)		
12	1.42 s	1.31 s	1.26 s	1.49 s
13	1.09 s	1.05 s	0.90 s	
14α	3.50 d (13.2)	3.41 d (12.4)	3.89 d (13.5)	4.04 d (6.6)
14β	3.94 d (13.2)	3.89 d (12.4)	4.97 d (13.5)	4.42 d (6.6)
15	1.33 s	1.08 d (7.1)	1.03 d (7.1)	1.10 d (6.9)

Table 3. ^{13}C-NMR Spectral Data for **1**—**4**[a)]

C	1	2	3	4
1	86.2	50.1	54.6	48.3
2	38.2	79.8	78.8	77.2
3	32.0	42.9	38.2	70.7
4	91.2	88.5	94.3	85.0
5	49.4	47.3	52.5	48.3
6	78.4	81.6	80.1	75.4
7	80.4	99.3	109.9	82.5
8	75.3	37.9	38.9	27.6
9	51.4	53.9	56.8	48.3
10	41.5	70.2	75.0	69.9
11	111.8	97.6	174.9	175.7
12	22.1	16.5	18.8	21.9
13	15.1	15.9	17.8	169.3
14	68.7	68.9	69.7	65.6
15	23.8	11.4	11.0	11.7

a) 150 MHz, CD_3OD.

4α-ol and 4-*O*-methyleudesm-11-en-4α-ol, and one lignan, (−)-hinokinin,[10] were the most active among these compounds.

Preliminary TLC analyses of the polar fractions E—M that did not show brine shrimp lethal activity indicated that these fractions are rich in *seco*-prezizaane-type and anislactone-type sesquiterpenes, as characteristic of *Illicium* species.[11,12] Thus extensive purification of fraction K resulted in the isolation of four novel highly oxygenated *seco*-prezizaane-type sesquiterpenes **1**—**4**.

The molecular formula $C_{15}H_{24}O_7$ of merrilliortholactone (**1**) was assigned on the basis of high-resolution (HR)-FAB-MS at *m/z* 339.1390 $[M+Na]^+$. The NMR spectral data (Tables 2, 3) of **1** revealed the presence of three methyl groups, an isolated methylene group (C-10: δ_C 41.5), an oxygenated methylene group (C-14: δ_C 68.7), and two vicinal oxygen-bearing methines [C-7, C-8: δ_H 3.70 (d, J=3.3 Hz), 4.43 (d, J=3.3 Hz)], whereas no carbonyl group was detected in its IR and ^{13}C-NMR (Table 3) spectra. These spectral data indicate that **1** belongs to the cycloparvifloralone subtype in *seco*-prezizaane-type sesquiterpenes.[13]

The five-membered ring in the molecule of **1** was easily assigned from ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear multiple-bond connectivity (HMBC) experiments. The presence of a C-11 ortholactone group was clarified by ^{13}C-NMR data at δ_C 111.8 and HMBC correlations of the signal of C-11 with the proton signals of H-7, H-10, and H-14. The remaining HMBC correlations shown in Fig. 1 also substantiated the positions of the other five quaternary carbons (C-1, C-4, C-5, C-6, C-9). The relative configurations on the C-5, C-7, C-9, and C-11 chiral centers were automatically established by forming the (11)7,14-ortholactone ring. The nuclear Overhauser effect spectroscopy (NOESY) correlations between H-15 and H-10β, H-8 and H-10β, and H-12 and H-14α allowed us to assign all the relative configurations for CH_3-15, CH_3-12, and H-8 as β. Thus the structure of merrilliortholactone (**1**) was elucidated to be 1α,8α-dihydroxy-3,10-dedihydroxy-(11)7,14-ortholactonefloridanolide, which was the first example of *seco*-prezizaane-type sesquiterpene with a hydroxyl group at the C-8 position.

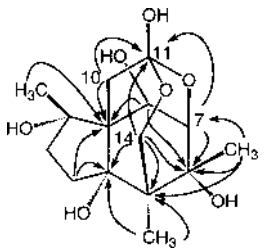

Fig. 1. Representative HMBC Correlations of **1**

HR-FAB-MS of compound **2** showed the quasimolecular ion $[M+Na]^+$ at *m/z* 339.1390, corresponding to the molecular formula $C_{15}H_{24}O_7$. The ^{13}C-NMR data (Table 3) contained two acetal carbons at δ_C 99.3 and 97.6, indicating the presence of double acetal moieties similar to those of cycloparvifloralone (**5**).[13] Comparing the ^{13}C-NMR data of **2** with those of **5**, **2** consisted of the same structural parts as **5** except for the oxygen-bearing C-2 (δ_C 79.8). Additionally, the HMBC experiment confirmed this tentative structure to be 2-hydroxycycloparvifloralone. The NOESY correlations between H-15 and H-10 as well as H-12 and H-14 established β configurations of CH_3-15, OH-10, and CH_3-12. Furthermore, the OH-2 was elucidated to take an α-configuration based on the large coupling constant (9.1 Hz) between H-2 and H-3β, and distinct NOESY cross peaks between H-15 and H-2 as well as H-2 and H-3β. The above spectral data

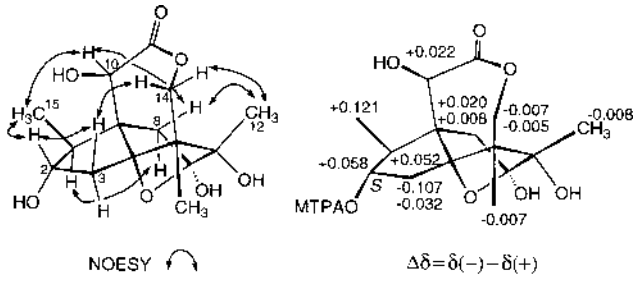

Fig. 2. Key NOESY Correlations of **3** and $\Delta\delta=\delta(-)-\delta(+)$ Values (ppm) Obtained from the MTPA Esters of **3**

determined the structure of **2** to be 2α-hydroxycycloparviflo-ralone.

Compound **3** had the molecular formula $C_{15}H_{22}O_7$, as established by HR-FAB-MS. IR and ^{13}C-NMR spectra indicated the presence of a carbonyl group (1725 cm^{-1} and δ_C 174.9). The ^{1}H-NMR spectrum (Table 2) showed the presence of three methyl groups and two isolated methylene groups (H_2-8, H_2-14), which are typical of *seco*-prezizaane-type sesquiterpenes. Furthermore, the ^{13}C-NMR data of **3** were found to be similar to those of cycloparviflorolide (**6**)[13] and cyclomerrillianolide (**7**)[6] except for C-1, C-2, and C-3 being shifted downfield to δ_C 54.6, 78.8, and 38.2, respectively. These differences implied that **3** has a hydroxyl group substituted at the C-2 position of **6**. Routine analyses of two-dimensional (2D) NMR (^{1}H–^{1}H COSY, heteronulcear single quantum bond correlation (HSQC), HMBC) supported that the plane structure of **3** corresponded to a 2-hydroxyl derivative of **6**. Both **3** and **6** are unique sesquiterpenes with a seven-membered lactone ring and an acetal group in the molecule. The relative configuration of **3** was determined by NOESY experiment as follows: The cross peaks observed between H-15 and H-10 as well as H-12 and H-14 indicated that CH_3-15, CH_3-12, and OH-10 all took β-orientations (Fig. 2). Finally, the absolute configuration of **3** was confirmed by the modified Mosher's method.[14] Treatment of **3** with α-methoxy(trifluoromethyl)phenylacetic acid (MTPA), dimethylaminopyridine (DMAP), and dicyclohexylcarbodiimide (DCC) afforded (+)-MTPA monoester and (−)-MTPA monoester, respectively. Comparing the ^{1}H-NMR data of the (+)- and (−)-MTPA ester derivatives (Fig. 2), C-2 chirality could be assigned as the *S* configuration and thereby the absolute structure of 2α-hydroxycycloparviflorolide can be represented as **3**.

Compound **4** had the molecular formula $C_{15}H_{20}O_9$, as established by HR-FAB-MS. IR and ^{13}C-NMR (Table 3) spectral data showed the presence of a β-lactone (1821 cm^{-1}; δ_C 169.3) and a δ-lactone (1730 cm^{-1}; δ_C 175.7), typical of anisatin-subtype sesquiterpenes. Comparing the NMR signals of **4** with those of anisatin (**8**),[15] δ_C 41.9 for C-2 in **8** was not only replaced by δ_C 77.2 in **4** but also H-2 was appeared in the lowfield at δ_H 3.79 (dd, *J*=7.9, 7.4 Hz), thereby indicating the presence of an additional hydroxyl group on the C-2 position. Thus **4** was deduced to be 2-hydroxyanisatin. This structure was confirmed by ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. The CH_3-15, CH_3-12, H-2, and H-3 were verified to take the same β-orientations as anisatin (**8**) by the NOESY correlations between CH_3-15 and H-10, CH_3-12 and H-14α, H-2 and H-3, and H-3 and H-14β.

It is noted that compounds **1**—**4** have no effect on neurite outgrowth and cell viability in the primary cultures of the rat cerebral neurons at 10—0.1 μM.

Experimental

General Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. IR spectra were measured on a Jasco FT-IR 5300 IR spectrometer. NMR spectra were recorded on a Varian Unity 600 NMR spectrometer instrument. Chemical shifts were given as δ (ppm) with tetramethylsilane (TMS) as an internal standard. The MS were recorded on a JEOL AX-500 MS spectrometer. Column chromatography was carried out on Kiselgel 60 (70—230, 230—400 mesh), Wakogel C-300, and Sephadex LH-20.

BST The bioassays were carried out by the previously reported method.[16]

Neurite Outgrowth-Promoting Bioassay The bioassays were performed on a serum-free medium (neurobasal medium supplemented with B-27[2]) as previously reported.[5]

Plant Material The ripe fruits of *I. merrillianum* A. C. Smith were collected in Yunnan, China, in September 1998, and a voucher specimen (94041) is available in the Beijing University of Chinese Medicine.

Extraction and Isolation The dried powdered pericarps of *I. merrillianum* (3.7 kg) were extracted with methanol at room temperature to give 1 kg of pale yellow extract. The extract (525 g) was chromatographed on 400 g silica gel (70—230 mesh) eluted successively with CH_2Cl_2, CH_2Cl_2/EtOAc (3 : 1, 1 : 3), EtOAc, EtOAc/MeOH (4 : 1), and MeOH to yield 13 fractions (A—M). BST {% deaths=[(test−control)/control]×100%} showed the LC_{50} of fractions A, C, and D to be lower than 50 μg/ml.

Bioassay-guided fractionation of the BST-active fractions by various chromatographic methods led to the isolation of methyl palmitate (4.5 mg) and caryophyllene oxide (1.0 mg) from fraction A, α-cadinol methyl ether (19 mg) and geranyl acetate (2.2 mg) from fraction C, (−)-hinokinin (6.6 mg), tashironin (2.2 mg), 4-allyl-2,6-dimethoxyphenol (2.0 mg), *trans*-cinnamic acid (2.0 mg), eudesm-11-en-4α-ol (6.1 mg), 4-*O*-methyleudesm-11-en-4α-ol (11 mg), and 3,7-dimethyl-1,5-octadiene-3,7-diol (6.1 mg) from fraction D (300 mg). Fraction K (5.2 g) was subjected to column chromatography on silica gel and eluted with $CHCl_3$/MeOH (3 : 1) to afford fractions 1—6. Then combined fractions 3 and 4 (720 mg) were subjected to column chromatography on Sephadex LH-20 and eluted with methanol to afford fractions 7—13. Fraction 9 (16 mg) was separated by repeated chromatography on silica gel using $CHCl_3$/MeOH (6 : 1) and ether/MeOH (10 : 1) as eluent to give **1** (4 mg) as colorless powder. Fraction 5 (79 mg) was chromatographed on Sephadex LH-20 eluted with MeOH to give fractions 14—18. Fraction 16 (25 mg) was purifed by preparative TLC on RP-8 (100% EtOH) to yield **2** (7.8 mg), **3** (3.0 mg), and **4** (3.5mg) as colorless amorphous solids.

Merrilliortholactone (**1**): $[\alpha]_D^{21}$ −9° (*c*=0.84, MeOH); IR ν_{max} (film): 3407, 1050 cm^{-1}; HR-FAB-MS *m/z*: 339.1390 $[M+Na]^+$ (Calcd for $C_{15}H_{24}O_7Na$: 339.1420); ^{1}H- and ^{13}C-NMR: see Tables 2 and 3.

2α-Hydroxycycloparvifloralone (**2**): $[\alpha]_D^{22}$ +3° (*c*=0.75, MeOH); IR ν_{max} (film): 3372 cm^{-1}; HR-FAB-MS *m/z*: 339.1390 $[M+Na]^+$ (Calcd for $C_{15}H_{24}O_7Na$: 339.1419); ^{1}H- and ^{13}C-NMR: see Tables 2 and 3.

2α-Hydroxycycloparviflorolide (**3**): $[\alpha]_D^{22}$ +45° (*c*=1.52, MeOH); IR ν_{max} (film): 3439, 1725 cm^{-1}; HR-FAB-MS *m/z*: 337.1260 $[M+Na]^+$ (Calcd for $C_{15}H_{22}O_7Na$: 337.1263); ^{1}H- and ^{13}C-NMR: see Tables 2 and 3.

MTPA Esterification of **3**: DMAP (1.1 mg), DCC (1.6 mg) and (−)-MTPA (3.2 mg) were added to a solution of **3** (1.5 mg) in anhydrous CH_2Cl_2. After being stirred at room temperature for 18 h, the reaction mixture were purified by preparative TLC on silica gel (EtOAc) to afford 0.8 mg of (−)-MTPA ester derivative. The (+)-MTPA ester derivative (1.1 mg) was prepared under the same conditions except that the reaction time was 20 h; (+)-MTPA ester: ^{1}H-NMR ($CDCl_3$) δ: 0.940 (3H, s, CH_3-13), 0.946 (3H, d, *J*=7.1 Hz, CH_3-15), 1.328 (3H, s, CH_3-12), 1.666 (1H, d, *J*=14.3 Hz, H-8β), 1.806 (1H, dd, *J*=15.4, 3.3 Hz, H-3α), 1.902 (1H, d, *J*=14.3 Hz, H-8α), 2.641 (1H, dd, *J*=15.4, 9.6 Hz, H-3β), 2.866 (1H, dq, *J*=9.1, 7.1 Hz, H-1), 3.948 (1H, d, *J*=13.7 Hz, H-14α), 4.616 (1H, d, *J*=4.4 Hz, H-10), 4.940 (1H, d, *J*=13.7 Hz, H-14β), 5.207 (1H, ddd, *J*=9.6, 9.6, 3.3 Hz, H-2). (−)-MTPA ester: ^{1}H-NMR ($CDCl_3$) δ: 0.933 (3H, s, CH_3-13), 1.067 (3H, d , *J*=7.1 Hz, CH_3-15), 1.320 (3H, s, CH_3-12), 1.674 (1H, d, *J*=14.3 Hz, H-8β), 1.699 (1H, dd, *J*=15.4, 3.6 Hz, H-3α), 1.922 (1H, d, *J*=14.3 Hz, H-8α), 2.609 (1H, dd, *J*=15.4, 9.6 Hz, H-3β), 2.918 (1H, dq, *J*=9.6, 7.1 Hz, H-1), 3.943 (1H, d, *J*=14.3 Hz, H-14α), 4.638 (1H, d, *J*=4.7 Hz, H-10), 4.933 (1H, d, *J*=13.7 Hz, H-14β), 5.265 (1H, ddd, *J*=9.6, 9.6, 3.6 Hz, H-2).

2α-Hydroxyanisatin (**4**): $[\alpha]_D^{20}$ −12° (*c*=0.19, MeOH); IR ν_{max} (film): 3374, 1821, 1730 cm^{-1}; HR-FAB-MS *m/z*: 367.1029 $[M+Na]^+$ (Calcd for

$C_{15}H_{20}O_9Na$: 367.1005); ^1H- and ^{13}C-NMR: see Tables 2 and 3.

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