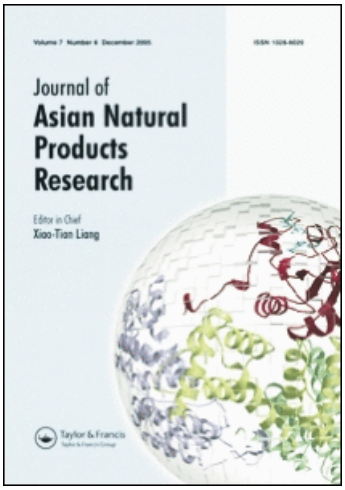

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Hong-Xiang Lou[a]; Guang-Yao Li[a]; Feng-Qiang Wang[a]
[a] Department of Natural Product Chemistry, School of Pharmacy, Shandong University, Shandong Jinan, China

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A CYTOTOXIC DITERPENOID AND ANTIFUNGAL PHENOLIC COMPOUNDS FROM *FRULLANIA MUSCICOLA* STEPH

HONG-XIANG LOU*, GUANG-YAO LI and FENG-QIANG WANG

Department of Natural Product Chemistry, School of Pharmacy, Shandong University, Shandong Jinan 250012, China

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A novel *ent*-labdane type diterpenoid, muscicolone (**1**), as well as two bibenzyls (**2**, **3**) and four flavonoids (**4**–**7**) were isolated from the liverwort *Frullania muscicola* Steph. Their structures were established by analysis of the spectral data of IR, UV, 1D-, 2D-NMR, and ORD. The stereostructure of **1** was confirmed by X-ray crystallographic analysis. Bioassay experiments showed that compound **3** and **4** have potent inhibitory effects against some fungi while **1** showed cytotoxic effects to some human tumor cells.

Keywords: Hepaticae; Liverworts; *Frullania muscicola*; Diterpenoid, Bibenzyls; Flavonoids; Cytotoxic, Anti-fungi

INTRODUCTION

Liverworts are rich sources of novel terpenoids and aromatic compounds, which often exhibit interesting pharmacological effects [1]. In the course of our investigation on the biologically active substances from the Chinese liverworts (Scheme 1) [2], one novel *ent*-labdane type diterpenoid, muscicolone (**1**), was isolated from *Frullania muscicola*, together with two previously known bibenzyls (**2**, **3**) and four flavonoids (**4**–**7**). Bioassay experiments showed that compound **3** and **4** have potent inhibitory effects against some fungi while **1** showed cytotoxic effects to some human tumor cells.

RESULTS AND DISCUSSION

From the ether extract of *F. muscicola*, a new *ent*-labdane diterpenoid named muscicolone (**1**) was isolated along with six previously known aromatic compounds. Compounds **2** and **3** were identified as 3,4-dimethoxy-4-hydroxybibenzyl and 3-hydroxy-4′-methoxybibenzyl, respectively, on the basis of spectral data analysis and by comparing their spectral data with

*Corresponding author. Tel.: +86-531-2942012. Fax: +86-531-2942019. E-mail: hxlou@jn-public.sd.cninfo.net.

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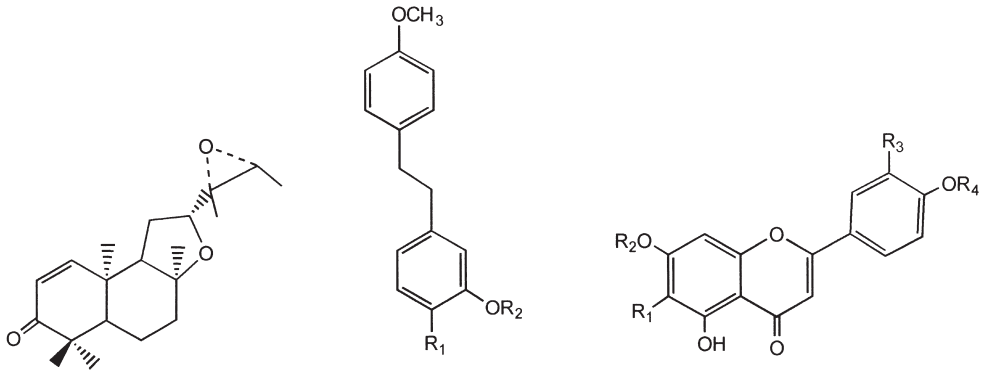

1

2 R_1=OH, R_2=CH_3

3 R_1=H, R_2=H

	R_1	R_2	R_3	R_4
4	H	CH_3	H	CH_3
5	OMe	H	H	CH_3
6	OMe	H	OMe	CH_3
7	OMe	H	H	H

SCHEME 1 Compounds isolated from the Chinese liverworts *F. muscicola.*

TABLE I NMR data for compound **1***

No	*Carbon* δ_C	*Correlated* H† δ_H	H *coupled with* C‡	H *coupled with* H¶
1	158.5 d	H-1 6.91 d (10.1)	H_3-20	H-2
2	126.1 d	H-2 5.78 d (10.1)		H-1
3	205.1 s		H-1, H_3-18, H_3-19	
4	45.1 s		H-2, H-5, H_3-18, H_3-19	
5	54.2 d	H-5 1.76 m		
6	21.6 t	H_α-6 1.46 m	H_3-18	H-5 H_β-6
		H_β-6 1.75 m		H-5, H_α-6, H_α-7
7	39.6 t	H_α-7 1.96 m	H-5, $H_{\alpha,\beta}$-6, H_3-17	H_β-7, H_β-6
		H_β-7 1.44 m		H_α-7
8	80.7 s		H_3-17	
9	55.5 d	H-9 1.74 m	H-1, H-7, $H_{\alpha,\beta}$-11, H_3-17, H_3-20	$H_{\alpha,\beta}$-11
10	39.1 s		H-1, H-2, H-5, H-9, H_3-20	
11	23.0 t	H_α-11 1.73 m	H-9, H-12	H-9, H-12
		H_β-11 1.78 m		
12	80.7 d	H-12 4.06 d t (7.4, 3.3)	$H_{\alpha,\beta}$-11, H_3-16,	$H_{\alpha,\beta}$-11
13	27.9 q	H_3-13 1.10 s	H_3-14	
14	21.2 q	H_3-14 1.02 s	H-5, H_3-13	
15	18.9 q	H_3-15 1.03 s	H-5, H-9, H_α-11	
16	24.4 q	H_3-16 1.15 s	H_α-6	
17	61.2 s		H_3-18	
18	14.7 q	H_3-18 1.30 s	H-12, H-19	
19	56.0 d	H-19 2.86 q (5.4)	H_3-20, H_3-18	H_3-15
20	13.8 q	H_3-20 1.25 d (5.4)	H-19	H-14

* ^{1}H-NMR at 500 Hz ($CDCl_3$, TMS) and ^{13}C-NMR at 125 Hz ($CDCl_3$, TMS), respectively.
† HMQC, Figures in parentheses are coupling constants (Hz).
‡ HMBC.
¶ ^{1}H–^{1}H COSY.

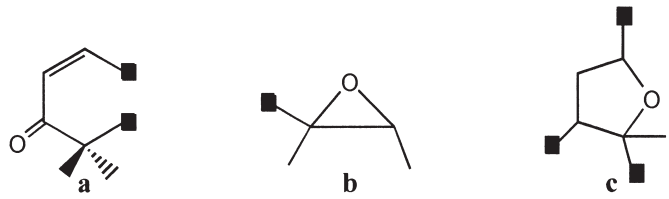

FIGURE 1 Partial structure of compound **1**.

that reported in the literature [3,4]. Four flavonoids were identified as 7,4′-dimethoxyl-apigenin (**4**), 6,4′-dimethoxyl-scutellarin (**5**), 5,7,4′-trihydroxy-6,3′-dimethoxyl-flavonoids (**6**), and 6-methoyl-scutellarin (**7**), respectively, according to the ^{1}HNMR and ^{13}CNMR data.

The molecular formula of **1** was determined to be $C_{20}H_{30}O_3$ by HRMS $[M^+] = m/z$: 318.2186 (calcd: 318.2195). Its ^{1}H-NMR and DEPT spectra data (Table I) showed the presence of six methyls, three methenes, six methines and five quaternary carbons. The IR spectrum indicated the presence of an α,β-unsaturated ketone (1674 cm^{-1}), to which the two protons of olefinic methines (δ_H 6.91, d, $J = 10.1$ Hz, δ_C (158.5, C-1), (δ_H 5.78, d $J = 10.1$ Hz, δ_C 126.1 C-2) and one carbonyl (δ_C 205.1) were attributable. HMQC and HMBC experiments connected the six methyls to four quaternary carbons and one methine, respectively. The structure of an α,β-unsaturated ketone was determined by the signals of ν_{max} (cm^{-1}) 1704, 1674 in IR spectrum, which was confirmed by the signals of δ_H 6.91(1H, d, $J = 10.1$ Hz, H-1), 5.78 (1H, d, $J = 10.1$ Hz, H-2) in ^{1}H-NMR and δ_C 158.5 (d, C-1), 126.1 (d, C-2), 205.1 (s) in ^{13}C-NMR. The correlation between two methyl groups at δ_H 1.10(3H, s) and 1.02 (3H, s) with the carbonyl signal at δ_C 205.1 (s) confirmed the structure of **a** in Fig. 1. According to its unsaturation degrees and absence of hydroxyl signals in the IR and ^{1}H-NMR spectra, the two remaining oxygen atoms in the structure must be in an ether linkage forming a tetracyclic diterpene. The correlations of δ_H 1.25 (3H, d, $J = 5.4$ Hz,

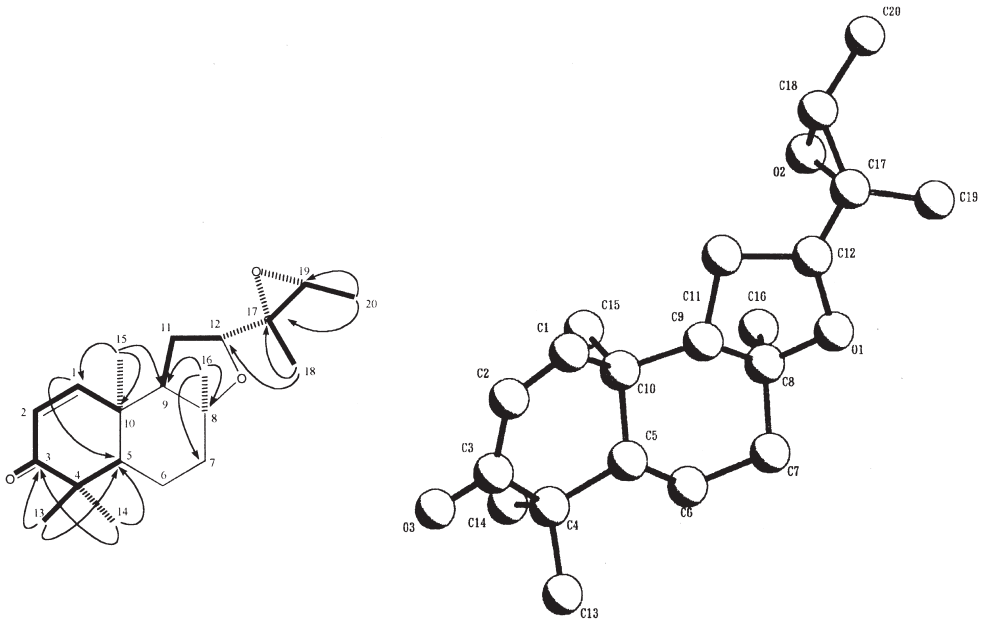

FIGURE 2 ^{1}H-^{13}C long-range correlations observed by HMBC spectrum of compound **1** and its X-ray crystallographic structure.

TABLE II Antibacterial activities of compounds **2** and **3** in dilution assays

	MIC (μg/ml)						
Sample	*A**	*B*	*C*	*D*	*E*	*F*	*G*
2	n	n	n	n	n	400	n
3	n	n	100	50	n	200	n
Pakyonol	n	n	n	n	n	n	n
Ref. [1]†	100	25	25	12.5	n	n	n

* A: *Escherichia coli*, B: *Staphylococeus aureus*, C: *Bacillus subtilis*, D: *B. spore cereus*, E: *Shigella flexneri*, F: *Sarcina lutea*, G: α-*Streptococcus hemolyticus*.
† Reference compounds: Marchantin A.
n = No inhibition detected adove 1000 μg/m.

H3-20) with δ_C 56.0 (C-19) and 61.2 (C-17); δ_H 1.30 (3H, s, H3-18) with C-17 confirmed the structure of **b** in Fig. 1. Two signals at δ_C 61.2(C) and 56.01 (CH) in ^{13}C-NMR, as well as one proton at δ_H 2.86 (q, $J = 5.37$ Hz) in the ^{1}H-NMR were attributable to the tricyclic epoxide ring. ^{1}H–^{1}H COSY and correlations of 1.15 (3H, s, H3-16) with δ_C 55.5 (d, C-9), 56.0 (s, C-8) and 80.7 (d, C-12) in HMBC spectrum established the partial structure of **c**. From these observations, a labdane skeleton as depicted in Fig. 2 was finally decided. The structure of **1** was finally determined by X-ray crystallographic analysis, which established the relative arrangement of the various partial structures and the stereochemistry. Its stereoscopic drawing of **1** is shown as in Fig. 2. The negative Cotton effect at 370 nm ($n \rightarrow \pi^*$, $\phi = 4100$) and a positive one at 308 nm ($\pi \rightarrow \pi^*$, $\phi = 7900$) confirmed its absolute configuration according to the octant rule for ketones [5,6].

Frullania species are reported containing plenty of sesquiterpene lactones and/or bibenzyls [7–10]. Flavonoids from some plant species belong to this genus are also reported [11,12]. The labdane-type diterpenoids, highly oxidized manoyloxides, isolated from the liverwort, *F. hamachiloba* have the same absolute configuration with those found in higher plant [13]. The present species is, however, very specific, since the major component is an *ent*-labdane type diterpene together with the previously known bibenzyls and flavonoids, no sesquiterpene lactones were found. The present diterpene is the first labdane-type diterpene possessing an enantiomeric configuration from *Frullania* species. This affords another example that *Frullania* species produce both normal and enantiomeric series of diterpenoids.

Bioassay experiments on the inhibitory effects on seven strains of bacteria and six kinds of fungi of two bibenzyls **3** and **4** as well as a bisbibenzyl pakyonol from the liverwort *F. muscicola* [2] showed that compound **4** possess potent inhibitory activities against selected fungi and bacteria, while compound **3** and pakyonol showed moderate inhibitory effects (Tables II and III). Similar results about the bibenzyl from the New Zealand liverwort *Plagiochila stephensoniana* had been reported [14]

TABLE III Antifungi activities of compounds **2** and **3** in dilution assays

	MIC (μg/ml)					
Sample	*H**	*I*	*J*	*K*	*L*	*M*
2	n	200	50	400	200	200
3	200	6.25	6.25	25	25	12.5
Pakyonol	n	n	200	100	n	400
Ref. [2]†	31.25	n	n	n	62.5	n

* H: *Candida allbicans*, I: *Trichophyton rubyum*, J: *Trichophyton gypseum*, K: *Microsporum gypseum*, L: *Microsporum lanosum*, M: *Epidermophyton floccoum*.
† Reference compounds: 3-methyloxy-4′-hydroxybibenzyl.
n No inhibition detected above 1000 μg/ml.

In vitro experiment by MTT method, compound **1** was found to have weakly cytotoxic effects to the human tumor cells of KB, PG, HT-29 and BEL-7402 with the IC50 at 20–60 μg/ml, respectively.

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points were determined with an X4-type micro-melting point apparatus (uncorrected). Optical rotations were measured with a Perkin–Elmer 241 MC ultravio-spectrometer. UV and IR spectra were determined with a Hitachi U-2000 spectrophotometer and a Perkin–Elmer IR-783 spectrometer, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded with an INOVA 300 NMR spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) and INOVA 500 NMR spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts were given in δ (ppm), based on the TMS standard. MS was performed with a ZAB-2F mass spectrometer. TLC was carried out on pre-coated silica gel thin layer (Qingdao Ocean Chemical Co.) with *n*-hexane-EtOAc (4:1–1:1) and detected by spraying 10% H_2SO_4 alcohol reagents. Column chromatography was performed over silica gel (200–300 mesh, Qingdao Ocean Chemical Co.) and Sephadex LH-20 (Pharmacia Biotech), respectively.

X-ray crystallographic analysis was with a MAC DIP SHELX-86 diffractometer using radiation MoKα. The crystal data, experimental conditions and refinement details were shown in Table IV.

Plant Material

Plant of *F. muscicola* Steph. was collected in November 1998 at Mount Tai (Altitude above 1000 m), Shandong province, China. The voucher specimens are deposited at the School of Pharmacy, Shandong University.

TABLE IV Crystal data, experimental conditions and refinement details

	Compound **1**
Chemical formula	$C_{20}H_{30}O_3$
Formula weight	318.46
Cell parameters	
A (Å)	7.248(1)
B (Å)	15.705(1)
C (Å)	16.132(1)
β (°)	
V (Å^3)	1836.3(3)
Space group	$P2_1$
Z	4
D_x (g/cm^3)	1.152
Size (mm)	0.5 × 0.5 × 0.7
Diffractometer	SHELXS-86
Radiation	Graphite-monochromated MoKα
θ-range	$2\theta_{max} = 50.0°$
Number of unique reflections	1796
Number of observed reflections	1749
Criterion for observed	
Reflections	$\|F\|^2 \geq 8\sigma\|F\|^2$
R_r	0.061
$R_f(w = 1/\sigma^2\|F\|)$	0.066

Extraction and Isolation

The air-dried liverwort *F. muscicola* Steph. (1.7 Kg) was ground mechanically and extracted three times with boiling 95% aqueous ethanol (7 l, 4 l × 2), each time for 3 h. These combined extracts (180 g) were diffused in warm water, participated with petroleum, ether, EtOAc, and butanol successively. The ether fraction (65 g) was subjected to chromatography over silica gel (160 g) and eluted with a mixture of hexane and ethyl acetate to yield 11 fractions (Fr. I–XI). Further isolation and purification were performed by repeated silica gel column chromatography with a mixture of benzene and acetone, and Sephadex LH-20 (80 g) column chromatography with a mixture of chloroform and methanol (1:1), respectively, compounds **2** (240 mg) and **3** (200 mg) were obtained from Fr. II (3.2 g from the elution with a mixture of hexane and ethyl acetate (98:2)). Compounds **1** (600 mg) as well as **4** (300 mg) from Fr. IV (6 g, hexane: ethyl acetate = 95 : 5), **5** (60 mg) from Fr. V (2 g, hexane: ethyl acetate = 9 : 1), **6** (120 mg) and **7** (210 mg) from Fr. VI (6 g, hexane: ethyl acetate = 8 : 2) were, respectively, obtained.

Muscicolone (1)

Colorless needles (MeOH); mp 142–143°C; Anal. C 75.49, H 9.46; Calcd. for $C_{20}H_{30}O_3$: C 75.43, H 9.49. IR ν_{max} (KBr) cm^{-1}: 3022, 2990, 2975, 2947, 2929, 2887, 2873, 2863, 1708, 1675, 1634, 1485, 1454, 1391, 1386, 1379, 1250, 1156, 1076, 999, 990, 864, 841, 830, 689; EI-MS *m/z*: 318 (M^+), 303 (80, M^+-15), 274 (2), 247 (20), 203 (65), 189 (15), 175 (11), 161 (13), 149 (50), 137 (30), 123 (25), 43 (100); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: Table I; ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: Table I.

3,4′-Dimethoxyl-4-hydroxylbibenzyl (2)

Colorless needles (*n*-hexane); mp 75–76°C; $C_{16}H_{18}O_3$, IR ν_{max} (KBr) cm^{-1}: 3420 (OH), 3072, 3032, 1614, 1591, 1516, 1490, 1480, 1282, 1270, 1254, 1242, 822, 801, 784, 759, 733; EI-MS *m/z*: 258 (15, M^+), 207, 181, 165, 151, 137 (8), 121 (100); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 2.85–2.90 (4H, m, CH_2CH_2), 3.78 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 5.72 (1H, br.s, OH), 6.70 (1H, dd, J = 2.69 Hz, 8.06 Hz, H-6), 6.74 (1H, d, J = 2.69 Hz, H-2), 6.78 (1H, d, J = 8.06 Hz, H-5), 6.82 (2H, d, J = 8.73, H-3′, 5′), 7.14 (2H, d, J = 8.73, H-2′, 6′); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 32.5 (CH_2, C-1″), 35.4 (CH_2 C-2″), 55.5 (OCH_3), 56.3 (OCH_3), 108.7 (C-2), 113.93 (C-3′, C-5′), 119.5 (C-5), 122.7 (C-6), 128.0 (C-1), 129.7 (C-2′, C-6′), 134.8 (C-1′), 143.8 (C-3), 146.6 (C-4), 158.0 (C-4′).

3-Hydroxy-4′-methoxylbibenzyl (3)

Colorless plates (*n*-hexane); $C_{15}H_{16}O_2$, mp, 65.5–66°C; EI-MS *m/z*: 228 (97, M^+), 204 (10), 179 (4), 161 (60), 145, 133, 121 (100), 107 (5), 105 (5), 91 (10), 77 (15); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 2.84 (4H, m, CH_2CH_2), 3.80 (3H, s, OCH_3), 5.26 (1H, br.s, OH), 6.65 (1H, d, J = 2.0 Hz, H-2), 6.67 (1H, dd, J = 8.1, 2.0 Hz, H-4), 6.76 (1H, dd, J = 7.4, 2.0 Hz, H-6), 6.84 (2H, d, J = 8.7 Hz, H-3′, 5′), 7.09 (2H, d, J = 8.73 Hz, H-2′, 6′), 7.15 (1H, t, J = 7.39, 8.06 Hz, H-5); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 36.7 (CH_2), 37.9 (CH_2), 55.3 (OCH_3), 112.8 (C-2), 113.8 (C-3′, C-5′), 115.4 (C-4), 120.9 (C-6), 129.3 (C-2′, C-6′), 129.5 (C-5), 133.9 (C-1′), 143.7 (C-1), 155.4 (C-4′), 157.7 (C-3).

7,4′-Dimethoxyl-apigenin (4)

Yellowish needles ($CDCl_3$–MeOH); $C_{17}H_{14}O_5$, mp, 171–172°C; IR ν_{max} (KBr) cm^{-1}: 3698, 3088, 3050, 3005, 2989, 2947, 2845, 1668, 1607, 1571, 1510, 1443, 1384, 1339, 1315, 1272, 1196, 1189, 1163, 835, 575; EI-MS *m/z*: 298 (100, M^+), 283 (2), 279 (10), 255 (12), 227 (2), 166 (10, a^+), 149 (5), 137 (7), 135 (20), 133 (20, b^+), 117 (8, b-15); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.80(2H, d, $J = 9.4$ Hz, H-2′, 6′), 6.74(2H, d, $J = 9.4$ Hz, H-3′, 5′), 6.54(1H, s, H-3), 6.44(1H, d, $J = 2.3$ Hz, H-8), 6.33(1H, d, $J = 2.3$ Hz, H-6), 3.86(3H, s, OMe) and 3.85(3H, s, OMe); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 164.3(C-2), 104.5(C-3), 182.7(C-4), 162.4(C-5), 98.3(C-6), 165.7(C-7), 92.9(C-8), 157.9(C-9), 105.8(C-10), 123.8(C-1′), 128.3(C-2′, 6′), 114.8(C-3′, 5′), 162.9(C-4′), 56.1(OMe), 55.8(OMe).

6,4′-Dimethoxyl-scutellarin (5)

Yellowish needles ($CDCl_3$–MeOH); $C_{17}H_{14}O_6$, mp, 223–224°C; IR ν_{max} (KBr) cm^{-1}: 3331, 3058, 3017, 2987, 2845, 1662, 1606, 1515, 1473, 1432, 1358, 1275, 1250, 1187, 1080, 1022, 832; EI-MS *m/z*: 314 (100, M^+), 299 (63, M^+-15), 296 (40, M^+-18), 285 (8), 271 (40, M^+-43), 167 (15, a^+-15), 133 (25, $b^+ + 1$), 118 (7, b^+-15); ^{1}H-NMR (300 MHz, $CDCl_3$) 7.99 (2H, d, $J = 8.8$ Hz, H-2′, 6′), 7.10(2H, d, $J = 8.8$ Hz, H-3′, 5′), 6.66(1H, s, H-3), 6.62(1H, s, H-8), 3.89(3H, s, OMe) and 3.84(3H, s, OMe); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 164.0(C-2), 103.1(C-3), 182.7(C-4), 153.0(C-5), 131.8(C-6), 153.2(C-7), 94.4(C-8), 157.8(C-9), 104.5(C-10), 123.2(C-1′), 128.4(C-2′, 6′), 114.7(C-3′, 5′), 162.8(C-4′), 60.0 (OMe), 55.4(OMe).

5,7,4′-Trihydroxy-6,3′-dimethoxylflavonoids (6)

Yellowish needles (MeOH); $C_{17}H_{14}O_7$, mp, 232–234°C IR ν_{max} (KBr) cm^{-1}: 3420, 3087, 2995, 2968, 2935, 1662, 1619, 1577, 1512, 1494, 1459, 1431, 1371, 1304, 1275, 1211, 1162, 846, 703; EI-MS *m/z*: 330 (100, M^+), 315 (66, M^+-15), 312 (40, M^+-18), 301 (5), 287 (37, M^+-43), 167 (18, a^+-15), 149 (20, $b^+ + 1$), 139 (15)); ^{1}H-NMR (300 MHz, $CDCl_3$) 7.61(1H, d, $J = 2.0$ Hz, H-2′), 7.59(1H, dd, $J = 2.0$, 8.0 Hz, H-6′), 6.99(1H, d, $J = 8.0$ Hz, H-5′), 6.68(1H, s, H-3), 6.61(1H, s, H-8), 3.98(3H, s, OMe) and 3.86(3H, s, OMe); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 165.2(C-2), 103.9(C-3), 183.6(C-4), 153.8(C-5), 129.3(C-6), 154.0(C-7), 94.9(C-8), 157.8(C-9), 105.5(C-10), 123.5(C-1′), 110.6(C-2′), 149.0(C-3′), 162.9(C-4′), 151.5(C-4′), 116.4(C-5′), 121.4(C-6′), 60.7(OMe), 56.6(OMe).

6-Methoxyl-scutellarin (7)

Yellowish powder, $C_{16}H_{12}O_6$, mp, 280–284°C(MeOH); IR ν_{max} (KBr) cm^{-1}: 3337, 3088, 3020, 2942, 2837, 1648, 1610, 1582, 1560, 1492, 1371, 1296, 1286, 1252, 1178, 1158, 1111, 1096, 829, 597, 569; EI-MS *m/z*: 300 (92, M^+), 285 (52, M^+-15), 282 (35, M^+-18), 270 (10), 257 (33, M^+-43), 167 (15, a^+-15), 139 (15), 119 (24), 118 (20, b^+-15), 89 (7), 69 (100); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.92(2H, d, $J = 8.9$ Hz, H-2′, 6′), 7.00 (2H, d, $J = 8.9$ Hz, H-3′, 5′), 6.62(1H, s, H-3), 6.61(1H, s H-8), 3.84(3H, s, OMe); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 164.8(C-2), 103.1(C-3), 183.1(C-4), 153.5(C-5), 131.8(C-6), 154.2(C-7), 94.4(C-8), 157.8(C-9), 105.3(C-10), 122.7(C-1′), 128.9(C-2′, 6′), 116.4(C-3′, 5′), 161.7(C-4′), 60.3(OMe).

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