Palaeophytochemical Constituents from the Miocene-fossil Wood of *Picea likiangensis* in Xun-dian of Yunnan, China

You-Xing Zhao,[†] Jian-Rong Luo,[†,‡] Cheng-Sen Li,[§] Tie-Mei Yi,[§] and Jun Zhou[†,*]

*[†]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China. *E-mail: jzhou@mail.kib.ac.cn*
[‡]Department of Pharmacology, Dali University, Dali 671000, China
[§]Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
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The analysis of chemical constituents in specific plant fossils provides crucial information for their probable diagenetic pathways. Natural products in plant fossils could retain their characteristic basic structural skeletons and be used as chemosystematic marker or biomarkers for their biological origin, though they may undergo rapid diagenetic processes during their fossilization.[1,2] Some natural flavonoids and other constituents were detected from Jurassic, Cretaceous, Pliocene, Miocene and Eocene plants, previously.[3-8] *Picea likiangensis* is an economically important conifer indigenous to the southwest and center regions of China.[9] A piece of Miocene-fossil wood of *P. likiangensis*, which was preserved morphologically, was discovered in an open Jinsuo coalmine in Xundian of Yunnan Province, China, raising questions about its phytochemical constituents. In this paper, we report the palaeophytochemical investigation on the Miocene-fossil wood of *P. likiangensis* by phytochemical methods as column chromatography to probe their organic constituents.

An ethanol extract prepared from the Miocene-fossil wood of *P. likiangensis* was purified repeatedly by column chromatography on silica gel and Sephadex LH-20 and then subjected to preparative thin-layer chromatography, yielding a new fluorene derivative (**1**) and other eleven known compounds (**2-12**) as shown in Figure 1.

Compound **1** was obtained as amorphous powder, with a melting point (mp) of 121-123 °C, and its molecular formula $C_{23}H_{20}O_2$ with fourteen unsaturation degrees was determined from a quasi-molecular ion peak at *m/z* 328 in its EI mass spectrum and the ^{13}C-NMR (DEPT) spectrum, which was supported by its HR-ESI MS observed at *m/z* 351.1357 (calculated 351.1360, $[M+Na]^+$). The IR spectrum of compound **1** showed the presence of aromatic rings (1611, 1494 and 1452 cm^{-1}) and hydroxyl groups (3385 and 3477 cm^{-1}). The ^{1}H-NMR spectrum of compound **1** showed eleven aromatic protons among which six protons at δ 6.00 (1H, d, J = 1.6 Hz), 6.47 (1H, dd, J = 1.6, 7.6 Hz), 6.96 (1H, d, J = 7.6 Hz), 6.15 (1H, d, J = 2.4 Hz), 6.52 (1H, dd, J = 2.4,

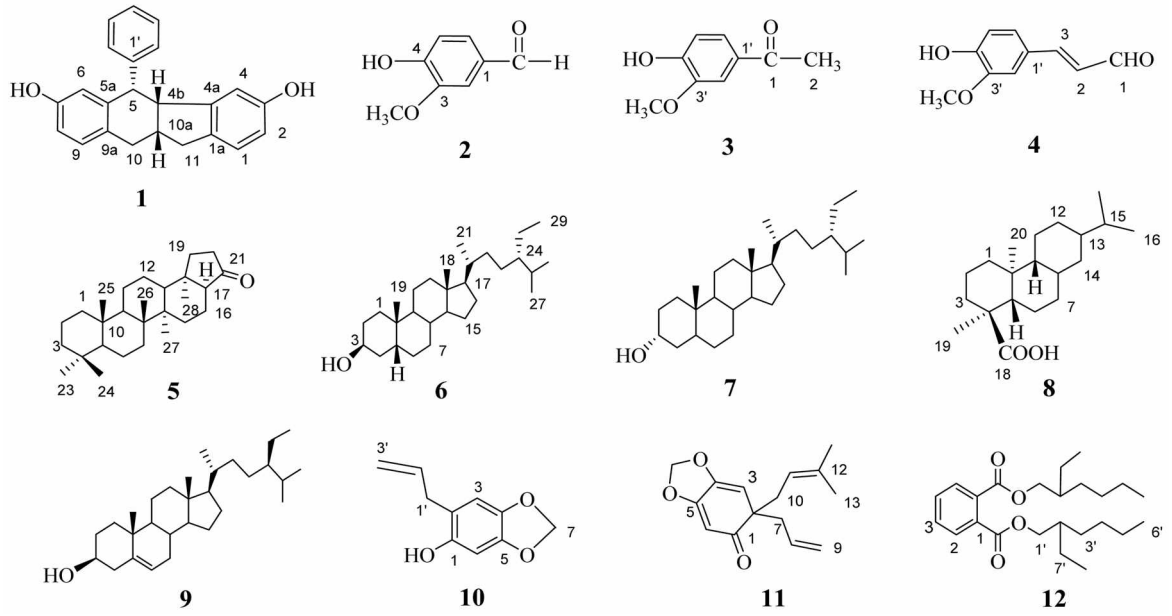

Figure 1. Structures of compounds **1-12**.

8.0 Hz) and 6.94 (1H, d, J = 8.0 Hz) indicated two typical tri-substituted aromatic groups. The ^{13}C-NMR and DEPT spectra of compound **1** revealed the presence of twenty-three carbon atoms including three aromatic rings and five saturated carbons. Two aromatic rings along with two methylene groups at δ 36.1 (t, C-10) and 37.6 (t, C-11) and three methine carbons at δ 54.4 (d, C-4b), 53.4 (d, C-5) and 47.7 (d, C-10a) suggested the skeleton of tetrahydrobenzo[b] fluorine in **1**, which was supported by the correlations of H-5 (δ 3.98) with C-6 (δ 117.5), and C-10a (δ 47.7); H-10 (δ 2.98) with C-9 (δ 131.1), C-5a (δ 143.4), C-11 (δ 37.6) and C-4b (δ 54.4); H-11 (δ 2.50 and 2.91) with C-1 (δ 125.5), C-4b (δ 54.4), C-4a (δ 148.2) and C-10 (δ 36.1); and H-4 (δ 6.00) with C-4b (δ 54.4) in the HMBC spectrum as shown in Figure 2 and the cross-peaks between H-10/H-10a, H-11/H-10a, H-10a/H-4b, H-4b/H-5, H-1/H-2 and H-8/H-9 in the ^{1}H-^{1}H COSY spectrum. Another aromatic ring was attached to C-5 based on the key HMBC correlations of H-5 (δ 3.98) with C-2 and 6 (δ 130.8). The two hydroxyl groups were adjacent to C-3 and C-7 as established by analysis of coupling constants of H-4 (δ 6.00, d, J = 1.6 Hz) and H-6 (δ 6.15, d, J = 2.4 Hz), respectively, and the corresponding carbon chemical shifts. The relative stereochemistry of compound **1** was deduced by the ROESY spectrum exhibiting cross-peaks of H-5/H-10a, H-5/H-4b, H-4b/H-10a, H-5/H-6, H-5/H-4 and H-5/H-2' as shown in Figure 2 and Table 1, indicating the *cis*-orientations of H-5/H-4b and H-4b/H-10a in **1**. Based on the above evidence, the structure of compound **1** was identified as 5-phenyl-5,10,10a,11-tetrahydro-4bH-benzo[b]fluorene-3,7-diol.

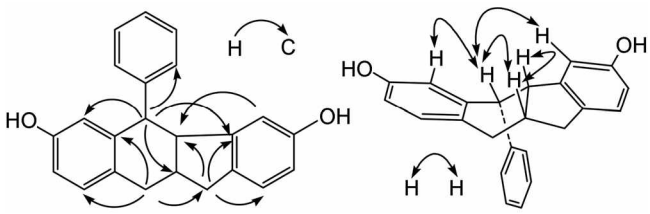

Figure 2. Selected HMBC and ROESY correlations of **1**.

Eleven known compounds (**2-12**) were elucidated to be 4-hydroxy-3-methoxybenzaldehyde (**2**),[10] acetovanillone (**3**),[11] (Z)-3-(4-hydroxy-3-methoxyphenyl) acrylaldehyde (**4**),[12] 22,29,30-trinor-21-hopanone (**5**),[13] 5β-24S-ethylchlestan-3β-ol (**6**),[14] 24α-ethylchlestan-3α-ol (**7**),[15] abietan-18β-oic acid (**8**),[16] 3β-sitosterol (**9**),[17] 2-allyl-4,5-(methylenedioxy) phenol (**10**),[18] 6-allyl-6-(3-methylbut-2-enyl)benzo[d] [1,3] dioxol-5(6H)-one (**11**),[19] bis(2-ethylhexyl)phthalate (**12**)[20] according to the analysis of their spectral data and literature, respectively. Bis(2-ethylhexyl)phthalate, a well-known plasticizer (DOP), belongs to phthalate diesters which are widely distributed in environment including animal tissues, excreta, food, and plastic containers.[21-23] Phthalate diesters can easily migrate among media.[24] Compound **12** isolated from this Miocene-fossil wood of *P. liktangensis* here might be its inner natural product or contaminated ones derived from environment such as coals and sediments during the storage and fossilization process in brown coal.

Table 1. ^{1}H ^{13}C NMR data and HMBC, ^{1}H-^{1}H COSY, ROESY correlations of **1** (400 MHz, in CD_3OD, δ ppm)

No.	δ_H	δ_C	HMBC	H-H COSY	ROESY
1	6.96 (d, 7.6 Hz)	125.5 (d)	C-2, 3, 4a, 11	H-2	H-2, 11
2	6.47 (dd, 1.6, 7.6 Hz)	114.0 (d)	C-3, 4, 1a	H-1	H-1
3	–	156.4 (s)	–	–	–
4	6.00 (d, 1.6 Hz)	112.4 (d)	C-2, 3, 1a, 4b	–	H-5, 4b, 2', 6'
5	3.98 (d, 11.5 Hz)	53.4 (d)	C-6, 5a, 4b, 10a, 1', 2', 6', 4a, 9a	H-4b	H-6, 4b, 10a, 2', 6'
6	6.15 (d, 2.4 Hz)	117.5 (d)	C-5, 7, 8, 9a	–	H-5, 2', 6'
7	–	156.2 (s)	–	–	–
8	6.52 (dd, 2.4, 8.0 Hz)	114.4 (d)	C-6, 7, 9a	H-9	H-9, 10
9	6.94 (d, 8.0 Hz)	131.1 (d)	C-7, 10, 5a	H-8	H-8, 10
10	2.98 (m)	36.1 (t)	C-9, 11, 10a, 4b, 9a,5a	H-10a	H-8, 9, 4b, 10a, 11
11	2.50 (dd, 11.4, 13.9 Hz) 2.91 (dd, 6.9, 13.9 Hz)	37.6 (t)	C-1, 10, 1a, 4a, 4b, 10a	H-10a	H-1, 10a, 10
1'	–	147.0 (s)	–	–	–
2'	7.31 (d, 7.5 Hz)	130.8 (d)	C-5, 1', 3', 4', 6'	H-3'	H-4, 5, 6, 4b, 3', 5'
3'	7.37 (dd, 7.4, 7.5 Hz)	129.8 (d)	C-1', 2', 4', 5'	H-2, 4	H-2', 4', 6'
4'	7.29 (t, 7.4 Hz)	127.6 (d)	C-2', 3', 5', 6'	H-3', 5'	H-3', 5'
5'	7.37 (dd, 7.4, 7.5 Hz)	129.8 (d)	C-1', 2', 4', 5'	H-4', 6'	H-2', 4', 6'
6'	7.31 (d, 7.5 Hz)	130.8 (d)	C-5, 1', 3', 4', 6'	H-5'	H-4, 5, 6, 4b, 3', 5'
1a	–	136.0 (s)	–	–	–
4a	–	148.2 (s)	–	–	–
4b	3.14 (t, 11.5)	54.4 (d)	C-4, 5, 10, 1a, 4a, 5a, 10a, 1'	H-5, 10a	H-4, 5, 10, 10a, 2', 6'
5a	–	143.4 (s)	–	–	–
9a	–	129.3 (s)	–	–	–
10a	2.22 (m)	47.7 (d)	C-5, 11, 9a,	H-10, 11, 4b	H-5, 10, 11, 4b

Experimental Section

General experimental procedures. Melting points were measured on an XRC-1 micro-melting apparatus and are uncorrected. IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV spectra were recorded on a UV 210A spectrometer. MS spectra were carried out on a VG Auto Spec-3000 spectrometer. The 1D and 2D NMR spectra were run on Bruker AM-400 MHz and DRX-500 MHz spectrometer using TMS as an internal. Silica gel (200-300 mesh, Marine Chemical Factory, Qingdao, China) were used for column chromatography.

Plant materials. The Miocene-fossil wood of *Picea liktangensis* was collected from an open Jinsuo coalmine in Xundian of Yunnan Province, People's Republic of China. The identity of this fossil wood material was verified by professor Cheng-Sen Li and a voucher specimen (KMJS-01) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation. The Miocene-fossil wood powder of *P. liktangensis* (23 Kg) was extracted with hot ethanol (80 L × 3) and filtered. The ethanol extraction was evaporated in vacuum to give the residue (380 g). The residue (200 g) was subjected to column chromatography on silica gel (200-300 mesh), eluted with gradient petroleum ether:acetone (3:2) to yield 5 fractions. Fraction 3 (8 g) was purified by repeated column chromatography on silica gel with petroleum ether:acetone (4:1) and petroleum ether: EtOAc (5:1) and on Sephadex LH-20 with MeOH to afford compound **1** (51 mg), **2** (25 mg), **3** (20 mg) and **4** (22 mg). Fraction 1 (31 g) was separated by repeated column chromatography on silica gel with petroleum ether:acetone (10:1) and petroleum ether:$CHCl_3$ (1:1) and on Sephadex LH-20 with MeOH:$CHCl_3$ (1:1) to give compound **5** (6 mg), **6** (20 mg), **7** (25 mg), **8** (12 mg) and **9** (1.2 g). Fraction 2 (4 g) was subjected to column chromatography on silica gel with petroleum ether:acetone (8:1) and petroleum ether:$CHCl_3$ (1:2) and on Sephadex LH-20 with MeOH:$CHCl_3$ (1:1) to yield compound **10** (15 mg), **11** (8 mg) and **12** (19 mg).

5-Phenyl-5,10,10a,11-tetrahydro-4bH-benzo[b]fluorene-3,7-diol (1). Amorphous powder, mp: 121-123 °C; $[\alpha]_D^{24}$ +6.98 (*c* 0.21, MeOH); IR (KBr) ν3477, 3385, 1611, 1494, 1452, 1340, 954, 872, 817, 736, 702 cm^{-1}; UV (MeOH) λ_{max} (logε): 204 (3.19), 283 (2.24), 322 (0.99), 353 (1.01), 368 (0.79) nm; ^{1}H-NMR (400 MHz, CD_3OD) and ^{13}C-NMR (100 MHz, CD_3OD) data see Table 1; HRESIMS *m/z*: 351.1357 (calcd. for $C_{23}H_{20}O_2Na$, 351.1360); EIMS *m/z* (%): 328 $[M]^+$ (62), 251 (4), 250 (12), 221 (32), 207 (19), 197 (68), 196 (90), 195 (100), 179 (36), 165 (31), 107 (27), 91 (19).

4-Hydroxy-3-methoxybenzaldehyde (2). Yellow needles (MeOH), ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.95 (3H, s, $OC\underline{H}_3$), 7.03 (1H, dd, *J* = 1.2, 8.4 Hz, H-6), 7.79 (1H, d, *J* = 8.4 Hz, H-5), 7.43 (1H, d, *J* = 1.2 Hz, H-2), 9.82 (1H, s, CHO); EIMS *m/z* (%): 152 ($[M]^-$, 93), 149 (40), 123 (20), 109 (17), 93 (14), 81 (28), 69 (41).

Acetovanillone (3). Yellow needles (MeOH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ2.93 (3H, s, H-2), 7.32 (1H, d, *J* = 8.6 Hz, H-5'), 7.90 (1H, d, *J* = 2.1 Hz, H-2'), 7.91(1H, dd, *J* = 2.1, 8.6 Hz, H-6'), 4.31 (3H, s, OCH_3); EIMS *m/z* (%): 166 ($[M]^+$, 49), 151 (100), 123 (26), 85 (18).

(Z)-3-(4-Hydroxy-3-methoxyphenyl)acrylaldehyde (4). Yellow needles (MeOH). ^{1}H-NMR (500 MHz, CD_3OD) δ 3.91 (3H, s, OCH_3), 7.24 (1H, d, *J* = 1.8 Hz, H-2'), 7.16 (1H, dd, *J* = 8.2, 1.8 Hz, H-6'), 6.84 (1H, d, *J* = 8.2 Hz, H-5'), 9.56 (1H, d, *J* = 7.9 Hz, H-1), 7.57 (1H, d, *J* = 15.7 Hz, H-3), 6.64 (1H, dd, *J* = 15.7, 7.9 Hz, H-2); EIMS *m/z* (%): 178 ($[M]^-$, 100), 177 (29), 163 (11), 161 (20), 135 (25), 107 (18).

22,29,30-Trinor-21-hopanone (5). White needles (MeOH). ^{1}H-NMR (500 MHz, $CDCl_3$) δ2.22 (2H, t, H-20), 1.80 (1H, t, H-17), 1.31 (3H, s, H-28), 1.18 (3H, s, H-27), 0.99 (3H, s, H-26), 0.96 (3H, s, H-25), 0.99 (3H, s, H-23); EIMS *m/z* (%): 384 $[M]^-$ (17), 369 (7), 206 (5), 191 (100), 177 (9), 149 (18), 123 (22), 95 (25), 81 (24).

5β-24S-Ethylchlestan-3β-ol (6). Colorless needles ($CHCl_3$), ^{1}H-NMR (400 MHz, $CDCl_3$) δ4.10 (1H, C-3), 0.64 (3H, s, H-18), 0.96 (3H, s, H-19), 0.90 (3H, d, *J* = 6.5 Hz, H-21), 0.82 (3H, d, *J* = 7.7 Hz, H-26), 0.80 (3H, d, *J* = 7.7 Hz, H-27), 0.85 (3H, t, *J* = 7.5 Hz, H-29); EIMS *m/z* (%): 416 $[M]^-$ (32), 402 (13), 401 (36), 383 (22), 316 (7), 290 (7), 248 (8), 233 (71), 215 (100), 165 (19), 107 (67), 81 (64), 69 (44).

24α-Ethylchlestan-3α-ol (7). Colorless needles ($CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$) δ0.89 (3H, d, *J* = 6.6 Hz, H-21), 0.84 (3H, t, *J* = 7.6 Hz, H-29), 0.82 (3H, d, *J* = 7.6 Hz, H-26), 0.80 (3H, d, *J* = 6.8 Hz, H-27), 0.75 (3H, s, H-19), 0.63 (3H, s, H-18); EIMS *m/z* (%): 416 $[M]^+$ (100), 401 (50), 383 (44), 316 (8), 290 (18), 248 (25), 233 (64), 215 (57), 165 (45), 107 (47), 83 (26), 69 (33).

Abietan-18β-oic acid (8). Colorless needles ($CHCl_3$), ^{1}H-NMR (400 MHz, $CDCl_3$) δ0.83 (6H, d, *J* = 7.0 Hz, H-16, 17), 0.84 (3H, s, H-20), 1.17 (3H, s, H-19); EIMS *m/z* (%): 306 $[M]^-$ (5), 191 (16), 163 (100), 164 (16), 149 (6), 121 (11), 109 (13), 95 (16), 81 (18).

2-Allyl-4,5-(methylenedioxy)phenol (10). Yellow oil, ^{1}H-NMR (400 MHz, $CDCl_3$) δ6.51 (1H, s, H-3), 6.35 (1H, s, H-6), 5.77 (2H, s, H-7), 3.22 (2H, d, *J* = 4.84 Hz, H-1'), 5.88 (1H, m, H-2'), 5.00 (1H, br s, H-3'), 4.97 (1H, m, H-3'); EIMS *m/z* (%): 178 $[M]^+$ (14), 177 (22), 164 (26), 163 (28), 161 (7), 151 (14), 138 (7).

6-Allyl-6-(3-methylbut-2-enyl)benzo[d][1,3]dioxol-5(6H)-one (11). Colorless oil, $[\alpha]_D^{24}$ +5.64 (*c* 0.37, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ5.45 (1H, s, H-3), 5.64 (1H, s, H-6), 2.63 (1H, dd, *J* = 7.2,13.2 Hz, H-7), 2.28 (1H, dd, *J* = 7.2,13.2 Hz, H-7), 5.57 (1H, m, H-8), 5.05 (1H, br s, H-9), 4.99 (1H, m, H-9), 2.54 (1H, dd, *J* = 7.4,13.7 Hz, H-10), 2.23 (1H, dd, *J* = 7.4,13.7 Hz, H-10), 4.94 (1H, t, *J* = 7.4 Hz, H-11), 1.60 (3H, s, H-13), 1.66 (3H, s, H-14), 5.84 (2H, d, *J* = 4.5 Hz, H-15); EIMS *m/z* (%): 246 $[M]^-$ (5), 205 (6), 189 (6), 179 (17), 178 (100), 177 (30), 151 (12), 147 (21), 69 (23).

Bis(2-ethylhexyl) phthalate (12). Colorless oil, ^{1}H NMR (100 MHz, $CDCl_3$) δ7.69 (2H, dd, *J* = 3.4, 5.7 Hz, H-2), 7.52 (2H, dd, *J* = 3.4, 5.7 Hz, H-3), 4.20 (4H, d, *J* = 6.5 Hz, H-1'), 1.66 (2H, m, H-2'), 1.28-1.38 (16H, m, H-3', 4', 5', 7'),

0.91 (6H, t, J = 7.4 Hz, H-8'), 0.88 (6H, t, J = 7.8 Hz, H-6'); EIMS m/z (%): 390 $[M]^+$ (2), 279 (10), 167 (35), 149 (100), 113 (10), 83 (6), 57 (16).

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