Systematic Phytochemical Investigation of *Abies spectabilis*

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Systematical phytochemical investigations on *Abies spectabilis* afforded 72 chemical constituents. On the basis of physical and spectroscopic data, including 1D and 2D homo- and heteronuclear NMR experiments (heteronuclear single quantum coherence (HSQC), ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple bond connectivity (HMBC), and nuclear Overhauser effect spectroscopy (NOESY)), and by comparison with the literature references, they were identified as 3 triterpenoids, 23 diterpenoids, 1 sesquiterpenoid, 13 flavonoids, 12 lignans, and 20 other components. Among these compounds, three were identified as new including abieta-7,13-diene-12α-methoxy-18-oic acid (1), 7α-methoxy-dehydroabietic acid (2), and 5-hydroxy-6-methyl-7,4′-dimethoxyflavone-8-*O*-β-D-glucopyranoside (3). These three new compounds (1—3) and all the known terpenoids (4—28) were tested for cytotoxic activities against four tumor cell lines: A549, COLO-25, QGY-25, and THP-1. However, none of them showed a positive effect (IC_{50}>100 μM).

Key words *Abies spectabilis*; Pinaceae; diterpenoid; flavonoid; cytotoxic activity

Abies is an important genus of the Pinaceae family comprising about 50 species around the world. Plants of this genus show high diversity in their secondary metabolites as well as pharmacological effects.[1)] Previously, we reported the occurrence of a unique sesquiterpenoid, a novel biflavanol, some diterpenoids, norditerpnoids, triterpnoids, and other compounds from three different *Abies* plants of *A. georgei*, *A. delabayi*, and *A. chensiensis*.[2—9)] *Abies spectabilis* (D. Don) spach is a tall evergreen tree distributed mainly in East Asia—Himalayas from Afghanistan to Nepal.[10)] To date, no phytochemical study has been reported on this species. As a continuation of the research analyzing the chemical constituents form *Abies* species in China, *A. spectabilis* was selected and subjected to a systematic phytochemical investigation, which led to the isolation of 3 new (Fig. 1) and 69 known chemical components. In this paper, we report the isolation and structure elucidation of the new compounds. Meanwhile, the cytotoxic activities of all three new compounds against four tumor cell lines are also described.

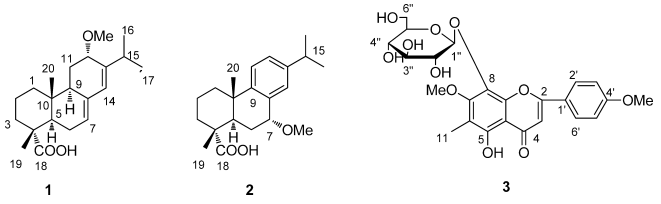

Fig. 1. Structrues of the New Compounds **1**—**3**

Results and Discussion

The $CHCl_3$ extract of whole plants of *Abies spectabilis* was subjected to silica gel, RP-18, and Sephadex LH-20 column chromatographic purification, as well as repeated preparative (prep.) TLC to yield two new diterpenes and 35 known compounds. By similar procedures, one new flavone and 34 known compounds were separated from the EtOAc extract.

Compound **1** had a molecular formula $C_{21}H_{32}O_3$, as evidenced by the positive high resolution-electrospray ionization-mass spectrum (HR-ESI-MS) at *m*/*z* 355.2244 [M+Na]$^+$, indicating six degrees of unsaturation. The IR spectrum indicated the presence of carboxyl (a broad band from 2600 to 3451, 1723 cm^{-1}) and conjugated olefinic bonds (1630, 1549 cm^{-1}). The ^{1}H, ^{13}C, and distortionless enhancement by polarization transfer (DEPT) NMR spectra of **1** (Table 1) indicated 21 carbon signals including two singlet methyls [δ_H 1.23 (3H, s, Me-19), 0.83 (3H, s, Me-20); δ_C 14.7 (q, C-19), 17.5 (q, C-20)], two doublet methyls [δ_H 1.03 (3H, d, J=6.9 Hz, Me-16), 1.06 (3H, d, J=6.9 Hz, Me-17); δ_C 22.0 (q, C-16), 22.6 (q, C-17)], one methoxy group [δ_H 3.36 (3H, s, 12-OMe); δ_C 56.6 (q, 12-OMe)]; five methylenes; six methines including two vinyls [δ_H 5.50 (H, br s, H-7), 5.85 (1H, s, H-14); δ_C 125.1 (d, C-7), 127.6 (d, C-14)] and one oxymethine [δ_H 3.83 (1H, t, J=2.9 Hz, H-12); δ_C 77.0 (d, C-12)]; and five quaternary carbons including one carbonyl (δ_C 182.9, s, C-19) and two olefinic groups [δ_C 136.1 (s, C-8), 143.2 (s, C-13)]. In the double quantunum fluorescein (DQF) correlation spectroscopy (COSY) experiment, the correlations of H-1 through H-2 to H-3; H-5 to H-6, H-9 through H-11 to H-12, and H-15 to H-16,17 established four fragments. The planar structure can be deduced as shown in Fig. 1 according to the heteronuclear multiple bond connectivity (HMBC) correlations traced from four methyls (Me-16,17,19,20), methoxyl, and olefinic protons. Based on the small coupling constant of H-12 ($^3J_{H11,H12}$= 2.9 Hz), the configuration of C12-OMe was deduced as *axial*-orientation.[11)] This could also be confirmed by the nuclear Overhauser effect spectroscopy (NOESY) correlations of H_3-20 to H_3-19/H-11 and H-9 to H-5/12-OMe. Therefore, compound **1** was concluded to be abieta-7,13-diene-12α-methoxy-18-oic acid.

Compound **2** was found to possess the molecular formula

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Table 1. The ^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR Spectroscopic Data for Compounds **1**—**3**

No.	**1**[a]		**2**[a]		No.	**3**[b]	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H
1	39.5 t	1.19 m; 1.90 m	39.1 t	2.35 (d, 13.0); 1.44 (dt, 13.2, 3.6)	2	163.8 s	
2	19.2 t	1.57 m	19.8 t	1.85 m; 1.71 (dt, 13.4, 3.0)	3	103.3 d	6.91 s
3	38.6 t	1.64 m; 1.80 m	37.7 t	1.92 m; 1.64 (d, 11.8)	4	182.4 s	
4	47.5 s		48.5 s		5	153.8 s	
5	46.4 d	2.10 m	41.4 d	2.46 (dd, 12.5, 2.0)	6	112.8 s	
6	25.9 t	1.09 m; 2.15 m	26.1 t	1.92 m	7	157.2 s	
7	125.1 d	5.50 br s	78.6 d	4.30 (dd, 3.8, 1.5)	8	128.7 s	
8	136.1 s		135.2 s		9	147.9 s	
9	45.3 d	2.22 (d, 13.7)	148.7 s		10	106.1 s	
10	35.1 s		38.6 s		11	8.1 q	2.05 s
11	26.9 t	1.88 m; 2.11 m	125.1 d	7.20 (d, 8.2)	1′	122.9 s	
12	77.0 d	3.83 (t, 2.9)	127.6 d	7.15 (dd, 8.2, 1.9)	2′, 6′	128.9 d	8.20 (d, 9.0)
13	143.2 s		147.2 s		3′, 5′	114.4 d	7.08 (d, 9.0)
14	127.6 d	5.85 s	129.8 d	7.08 (d, 1.9)	4′	162.5 s	
15	34.0 d	2.31 m	34.9 d	2.84 m	1″	103.6 d	4.87 (d, 7.8)
16	22.0 q	1.03 (d, 6.9)	24.3 q	1.21 (d, 7.0)	2″	76.4 d	3.29 m
17	22.6 q	1.06 (d, 6.9)	24.4 q	1.21 (d, 7.0)	3″	70.2 d	3.19 m
18	182.9 s		183.2 s		4″	74.1 d	3.40 m
19	17.5 q	1.23 s	17.4 q	1.26 s	5″	77.3 d	3.10 m
20	14.7 q	0.83 s	24.8 q	1.15 s	6″	61.2 t	3.59 m; 3.35 m
OMe	56.6 q	3.36 s	56.3 q	3.40 s	7-OMe	55.5 q	3.85 s
					4′-OMe	61.2 q	3.98 s

a) Recorded in CD_3OD. *b*) Recorded in DMSO-d_6.

$C_{21}H_{29}O_3$, as shown from the positive HR-ESI-MS at *m/z* 353.2087 $[M+Na]^+$. Its IR spectrum showed the presence of carboxyl (a broad band from 2700 to 3438, 1737 cm^{-1}) and benzene moiety (1630, 1513, 1442, 1383 cm^{-1}). The 1D NMR spectra of **2** (Table 1) indicated 21 carbon signals including four methyls [δ_H 1.21 (6H, d, J=7.0 Hz, Me-16,17), 1.26 (3H, s, Me-19), 1.15 (3H, s, Me-20); δ_C 24.3 (q, C-16), 24.4 (q, C-17), 17.4 (q, C-19), 24.8 (q, C-20)], one methoxy moiety [δ_H 3.40 (3H, s, 7-OMe); δ_C 56.3 (q, 7-OMe)]; four methylenes; six methines including one oxymethine [δ_H 4.30 (1H, dd, J=3.8, 1.5 Hz, H-7); δ_C 78.6 (d, C-7)] and three from an ABX benzenoid moiety [δ_H 7.20 (1H, d, J=8.2 Hz, H-11), 7.15 (1H, dd, J=8.2, 1.9 Hz, H-12), 7.08 (1H, d, J=1.9 Hz, H-14); δ_C 125.1 (d, C-11), 127.6 (d, C-12), 129.8 (d, C-14)]; and six quaternary carbons including one carbonyl (δ_C 183.2, s, C-18) and three from the rest of the ABX benzenoic moieties [δ_C 135.2 (s, C-8), 148.7 (s, C-9), 147.2 (s, C-13)]. These signals were very similar to those of 7α-hydroxydehydroabietic acid[12] except for the presence of an additional methyl at 7-OH. This was confirmed by the long-range correlation of 7-OMe to C-7 in the HMBC spectrum (Fig. 2). Accordingly, compound **2** was identified as 7α-methoxy-dehydroabietic acid.

Compound **3** was isolated as a yellow amorphous powder. The positive HR-ESI-MS at *m/z* 353.2087 $[M+Na]^+$ gave its molecular formula as $C_{24}H_{32}O_6$. The IR spectrum showed the presence of hydroxyls (3424 cm^{-1}), carbonyl (1736 cm^{-1}) and aromatic rings (1629, 1509 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectroscopic data of **1** (Table 1) were very similar to those of 5-hydroxy-7,4′-dimethoxy-6-methylflavone[13] except for an additional glucopyranose moiety. On acid hydrolysis, **3** afforded D-glucose which was detected by TLC with an authentic sample, and the configuration was determined by measurement of the optical rotation value. The β-anomeric configuration was judged by the large coupling constant (7.8 Hz) of the anomeric proton. In the HMBC spectrum, a long-range correlation was found for the anomeric proton (δ 4.87, d, J=7.8 Hz) of the glucose group to C-8 (δ 128.7). Therefore, the β-D-glucopyranose is apparently attached at the C-8 position of the flavone unit. Thus the structure of compound **3** was determined as 5-hydroxy-6-methyl-7,4′-dimethoxyflavone-8-*O*-β-D-glucopyranoside.

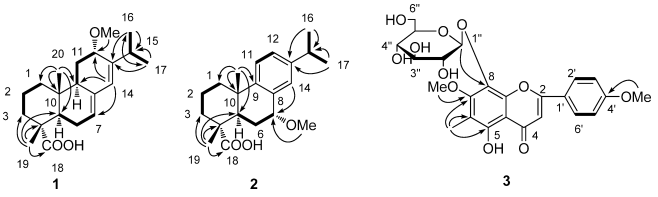

Fig. 2. Selected ^{1}H–^{1}H COSY (Bold) and HMBC (Arrow) Correlations of **1**—**3**

These three new compounds (**1**—**3**) and all the terpenoids (**4**—**28**) were tested for antitumor activities against the four tumor cell lines: A549, COLO-25, QGY-25, and THP-1. However, none of them showed positive effect (IC_{50}> 100 μM).

Experimental

General Experimental Procedures Optical rotations were recorded using a Perkin-Elmer 341 polarimeter, whereas UV spectra were obtained by a Shimadzu UV-2550 spectrometer. IR spectra were recorded on a Bruker Vector 22 spectrometer with KBr pellets. NMR spectra were recorded on Bruker Avance 300, 400, 500 or 600 NMR spectrometers in $CDCl_3$ or dimethyl sulfoxide (DMSO) with tetramethyl silane (TMS) as internal standard. ESI-MS were acquired on an Agilent LC/MSD Trap XCT mass spectrometer, whereas HR-ESI-MS were measured using a Waters Q-time-of-flight (TOF) micro mass spectrometer. RP-MPLC was carried out on a BüChi 615 pump and a 49×460 mm Büchi MPLC column filled with YMC-Gel ODS-A (50 μm; YMC, Milford, MA, USA). Materials for column chromatography were silica gel (100—200 mesh; Huiyou Silical Gel Development Co., Ltd. Yantai, People's Republic of China), Sephadex LH-20 (40—70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and Prep.

TLC (0.4—0.5 mm) was conducted with glass plates precoated with silica gel GF_{254} (Yantai). Compounds were visualized by exposure to UV light at 254 nm.

Plant Material The aerial parts of *A. spectabilis* (D. Don) spach were collected from Sejila Hills in Tibet in May 2007 and authenticated by Prof. Han-Ming Zhang in the Department of Pharmacognosy, Second Military Medical University. A voucher specimen (20070513002) was deposited at the Herbarium of the School of Pharmacy, Second Military Medical University, Shanghai, China.

Extraction and Isolation The air-dried and powdered aerial parts (17 kg) of *A. spectabilis* (D. Don) spach were extracted with 80% ethanol three times each for 3 h. After filtering, the extract was partitioned sequentially with $CHCl_3$ (20 l), EtOAc (40 l) and *n*-BuOH (30 l), respectively. The $CHCl_3$ extract was subjected to column chromatography over silica gel eluting with a gradient petroleum ether–$CHCl_3$ (98→0%) to give three fractions (Fr. 1—Fr. 3). Fr. 3 (10.9 g) was subjected to RP-MPLC eluting with (H_2O–MeOH, 50→100%), then purified by Sephadex LH-20 eluting with MeOH, and subjected to repeated prep. TLC (petrolum ether–EtOAc, 4 : 1) to afford 12α-methoxy-7,13-diene-18-oic acid (**1**, 8.8 mg), 7α-methoxy-dehydroabietic acid (**2**, 15.2 mg), dehydroabietic acid (**5**, 214.3 mg),[12] isopimaric acid (**13**, 17.7 mg),[14] 18-norabieta-8,11,13-trien-4-ol (**24**, 18.5 mg),[15] serratenedione (**27**, 4.0 mg),[16] 5-hydroxy-6-methyl-7,4′-dimethoxyflavone (**38**, 152.8 mg)[13] 4′-hydroxy-3′-methoxyacetophenone (**60**, 57.7 mg),[17] 5,9-nonadecadienoic acid (**70**, 10.9 mg).[18] Sixty-five known compounds were obtained from Fr. 2, Fr. 3 and the EtOAc extract. They were 18 diterpenoids: abieta-7,13-diene-18-oic acid (**4**, 680 mg),[19] 18-succinyloxyabieta-8,11,13-triene methyl ester (**6**, 110 mg),[20] 15,18-dihydroxyabieta-8,11,13-trien-7-one (**7**, 53 mg),[21] 15-hydroxy-7-oxo-8,11,13-abietatrien-18-oic acid (**8**, 26 mg),[22] abiesadine R (**9**, 102.4 mg),[9] 15-hydroxydehydroabietic acid (**10**, 68.9 mg),[12] abiesadine I (**11**, 157 mg),[9] 9,13β-epidioxy-8(14)-abieten-18-oic acid (**12**, 62.5 mg),[11] abiesadine X (**14**, 286 mg),[9] manool (**15**, 34.6 mg),[23] torreferol (**16**, 18.6 mg),[24] abiesadine Y (**17**, 40.7 mg), labd-13(*Z*)-ene-8α,15-diol (**18**, 88.6 mg),[25] manoyl oxide (**19**, 3.8 mg),[26] 8-*epi*-manoyl oxide (**20**, 3.8 mg),[27] phytol (**21**, 209.5 mg),[28] abiesanordine F (**23**, 124 mg),[4] 7α,15-dihydroxypodocarp-8(14)-en-13-one (**22**, 25.7 mg)[29]; 2 triterpenoids: 21-eptserratenediol dimethyl ether (**25**, 2 mg),[30] isoserratenedione (**26**, 9 mg)[31]; 1 sesquiterpenoid: icarisides B_5 (**28**, 28 mg)[32]; 12 flavonoids: 5-hydroxy-6-methyl-7,4′-dimethoxyflavone-8-O-β-D-glucopyranoside (**3**, 62.8 mg), isorhamnetin-3-β-D-(6-*O*-acetyl) glucoside (**29**, 242.7 mg),[33] juglalin (**30**, 44 mg),[34] kaempferol 3-*O*-(3″-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (**31**, 25.4 mg),[35] kaempferol 3-*O*-(2-*O*-*p*-coumaroyl)-α-L-arabinopyranoside (**32**, 12.5 mg),[36] kaempferol 3-*O*-(3″,6″-di-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (**33**, 60 mg),[35] tsugafolin (**34**, 10.3 mg),[37] 2-hydroxynaringenin (**35**, 990 mg),[38] pallasiin (**36**, 908 mg),[39] apigenin (**37**, 8.9 mg),[40] myricetin (**39**, 12.2 mg),[41] 4′,5-dihydroxy-3,7-dimethoxy-6-methylflavone (**40**, 70 mg)[42]; 12 lignans: (7′*S*,8′*R*)-dihydrodehydrodiconiferyl alcohol (**41**, 22.8 mg),[43] cedrusin (**42**, 358 mg),[44] (+)-pinoresinol (**43**, 32.6 mg),[45] (−)-massoniresinol (**44**, 58 mg),[46] daphneligin (**45**, 70.9 mg),[47] 1-(4-hydroxy-3-methoxyphenyl)-2-[2-methoxy-4-(3-hydroxypropyl) phenoxy]-3-propanol (**46**, 54.3 mg),[48] 3-methoxy-8,4′-oxyneoligna-3′,4,9,9′-tetraol (**47**, 211 mg),[49] (*erythro*) 4,7,9,9′-tetrahydroxy-3,3′-dimethoxy-8-*O*-4′-neolignan (**48**, 235.5 mg),[50] (*threo*) 4,4′,7,9,9′-pentahydroxy-3-methoxy-8-*O*-4′-neolignan (**49**, 201.2 mg),[50] (−)-isolariciresinol (**50**, 64.8 mg),[51] schizandriside (**51**, 40.2 mg),[52] icariside E (**52**, 96 mg)[53]; 10 phenols: benzoic acid (**53**, 10.749 g),[54] *p*-coumaric acid (**54**, 55.8 mg),[55] 4′-hydroxyacetophenone (**55**, 260 mg),[56] frambinone (**56**, 47.9 mg),[9] dihydroconiferyl alcohol (**57**, 74 mg),[57] caffeic acid (**58**, 63 mg),[58] 3′,4′-dihydroxyacetophenone (**59**, 143.7 mg),[59] ferulic acid (**61**, 143.2 mg),[55] 4,4′-dihydroxychalcone (**64**, 227 mg),[60] 1-(3,4-dihydroxyphenyl)-2-(3,5-dihydroxyphenyl)ethylene (**65**, 415 mg)[61]; 2 steroids: β-sitostenone (**69**, 21.6 mg),[62] β-sitosterol (**70**, 155.7 mg)[62]; and 7 other compounds: β-D-glucopyranosyl benzoate (**62**, 110 mg),[63] 1,1-dimethylallyl glucoside (**63**, 25 mg),[64] 2,4-octadecadienoic acid (**66**, 103.0 mg), methyl elaidate (**67**, 67.4 mg), 5,9-nonadecadienoic acid (**68**, 10.9 mg), *trans*-hexadecyl ferutic acid (**71**, 23 mg),[65] *cis*-hexadecyl ferutic acid (**72**, 14.6 mg).[66]

Abieta-7,13-diene-12α-methoxy-18-oic Acid (**1**): Amorphous powder; $[\alpha]_D^{24}$ −7.7 (c=0.27, MeOH); UV (MeOH) λ_{max} (log ε): 242 (3.73) nm; IR (KBr) ν_{max} 3451, 2925, 2868, 1723, 1630, 1549, 1383, 1229, 1083, 714 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; ESI-MS (positive) *m*/*z*: 355 $[M+Na]^+$; ESI-MS *m*/*z*: 331 $[M-H]^-$, 663 $[2M-H]^-$; HR-ESI-MS *m*/*z*: 355.2258 $[M+Na]^+$ (Calcd for $C_{21}H_{32}O_3Na$, 355.2244).

7α-Methoxy-dehydroabietic Acid (**2**): Amorphous powder; $[\alpha]_D^{24}$ −2.0 (c=0.08, MeOH); UV (MeOH) λ_{max}: 213 (3.49) nm; IR (KBr) ν_{max} 3438, 2969, 2926, 2867, 1737, 1630, 1513, 1495, 1442, 1383, 1366, 1228, 1216, 825 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; ESI-MS *m*/*z*: 353 $[M+Na]^+$, 683 $[2M+Na]^+$; ESI-MS (negative) *m*/*z*: 329 $[M-H]^-$, 659 $[2M-H]^-$; HR-ESI-MS (positive) *m*/*z*: 353.2108 $[M+Na]^+$ (Calcd for $C_{21}H_{30}O_3Na$, 353.2087).

5-Hydroxy-6-methyl-7,4′-dimethoxyflavone-8-*O*-β-D-glucopyranoside (**3**): Pale yellow powder; $[\alpha]_D^{20}$ +3.0 (c=0.20, MeOH); UV (MeOH) λ_{max} (ε): 218 (4.32), 270 (4.08), 306 (3.97) nm; IR (KBr) ν_{max} 3424, 1736, 1629, 1509, 1383, 1216, 1122, 1069, 827, 797, 607, 539 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; ESI-MS (positive) *m*/*z*: 491 $[M+H]^+$, 513 $[M+Na]^+$; ESI-MS (negative) *m*/*z*: 489 $[M-H]^-$, 525 $[M+Cl]^-$, 979 $[2M-H]^-$; HR-ESI-MS (positive) *m*/*z*: 491.1521 $[M+H]^+$ (Calcd for $C_{24}H_{27}O_{11}$, 491.1548).

Acid Hydrolysis of Compound 3 The experiment was carried out according to a previous method.[67] Briefly, compound **3** (10 mg) was dissolved in 5 ml HCl (10%), and kept refluxing at 75 °C. After 2 h, the reaction was stopped and the mixture was extracted by EtOAc. The remaining aqueous phase was concentrated to afford 4.4 mg of D-glucose, which was detected by TLC with an authentic sample. The configuration was determined by measurement of the optical rotation value, $[\alpha]_D^{20}$ +38.0 (c=0.22, H_2O).

Antitumor Assays A549, COLO-25, QGY-25, and THP-1 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The antitumor experiments were conducted according to previously reported procedures.[68]

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