Cytotoxic Constituents of Chinese Propolis

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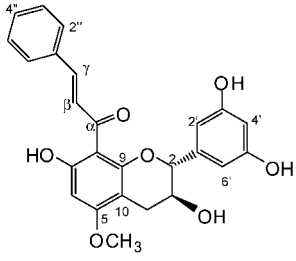
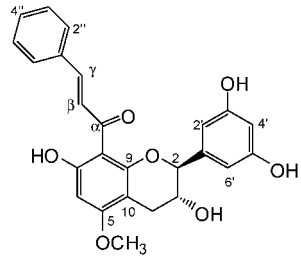
A pair of new flavanol racemates (**1a** and **1b**) and a new flavanol racemic mixture (**2**) were isolated from crude propolis from Henan Province, People's Republic of China. Also obtained were nine known compounds, including two flavones, four flavonols, two flavanols, and isoferulic acid. Spectroscopic analysis was employed to assign the structures of these new compounds and the absolute configurations of **1a** and **1b**. Cytotoxicity of the isolated compounds against the HeLa human cervical carcinoma cancer cell line was evaluated, with only compounds **1a**, **1b**, **2**, and rhamnetin (**3**) being active.

Propolis is a complex mixture of beeswax with small amounts of sugars and plant exudates collected by honeybees (*Apis mellifera*). The product has a pleasant aromatic odor and a yellow-green to dark brown color depending on the source and age.[1] The major constituents of propolis are flavonoids,[2,3] organic acids, phenols, various kinds of enzymes, vitamins, and minerals.[4] Propolis is a traditional remedy in folk medicine, and there is evidence indicating that it has antibacterial,[5] antiviral,[6] antifungal,[7] anti-inflammatory,[8] local anesthetic,[9] antioxidant,[10] immunostimulant,[11] cariostatic,[12] cytotoxic,[13] and anti-*Helicobacter pylori*[14] activities.

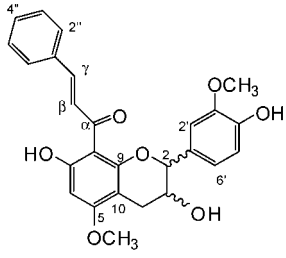
A detailed chemical investigation of the EtOH extract of the crude propolis from Henan Province in mainland China resulted in the isolation of a pair of new flavanol racemates (**1a** and **1b**) and a new flavanol racemic mixture (**2**), together with nine known compounds. The cytotoxicity against the HeLa human cervical carcinoma cell line was evaluated for all the compounds obtained.



1a



1b



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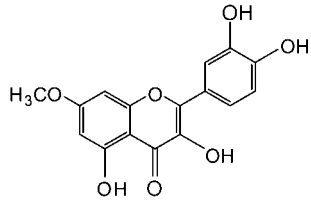

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Compound **1** was obtained as a yellow powder. Its molecular formula was determined as $C_{25}H_{22}O_7$ by HREIMS (*m*/*z* 434.1366 M^+), indicating 15 degrees of unsaturation. The UV spectrum revealed an absorption maximum at 341 nm. Inspection of the ^1H and ^{13}C NMR spectra together with the DEPT and HSQC spectroscopic data revealed the presence of 25 carbon signals, due to one methoxyl group, one methylene unit, two oxygenated tertiary carbons, two *trans*-olefinic carbons, nine aromatic tertiary carbons, and nine aromatic quaternary carbons (including five oxygenated), together with one ketone carbon. The occurrence of a flavan-3-ol skeleton in the molecule could be determined from the characteristic signals at δ_H 2.90 (1H, dd, J = 5.6, 16.0 Hz, H-4a), 2.41 (1H, dd, J = 9.6, 16.0 Hz, H-4b), 4.62 (1H, d, J = 9.2 Hz, H-2), and 4.00–4.02 (1H, m, H-3).[15] In the ^1H NMR spectrum, the proton signals at δ 6.80 (2H, brs) and 6.93 (1H, brs) implied the presence of a 1′,3′,5′-trisubstituted ring B.[16] The proton signal at δ 6.17 (1H, s) suggested the presence of pentasubstituted ring A. In the HMBC spectrum, the correlation between the proton at δ_H 3.84 (OCH_3-5) and the carbon at δ_C 163.9 (C-5) indicated that the methoxy group is attached to C-5. The correlations between three hydroxyl protons at δ_H 14.10 (OH-7), 9.02 (OH-5′), and 9.05 (OH-3′) and carbons at δ_C 166.1 (C-7), 145.8 (C-5′), and 145.4 (C-3′) suggested that the three hydroxyl groups are substituted at C-7, C-3′, and C-5′, respectively. A correlation to a ketone carbonyl carbon at δ_C 192.0 (C-α) from two *trans*-olefinic protons at δ_H 7.58 (1H, d, J = 15.6 Hz, H-γ) and 7.89 (1H, d, J = 15.6 Hz, H-β) indicated the presence of α,β-unsaturated ketone group. Aromatic proton signals appeared in the region δ_H 7.33−7.09 ppm (5H, m), together with their carbon signals at δ_C 128.4 ppm (CH × 2), 129.0 ppm (CH × 2), 130.4 ppm (CH × 1), and 134.8 ppm (C × 1), and suggested the presence of a benzene ring. Correlations from the proton signal at δ_H 7.58 (H-γ) to the carbon signals at δ_C 134.8 (C-1″) and 128.4 (C-2″, 6″) and from the proton signal at δ_H 7.89 (H-β) to the carbon signal at δ_C 134.8 (C-1″) indicated a (2*E*)-4-phenylprop-2-en-1-one moiety. Although a correlation between signals at δ_H 7.89 (H-β) and δ_C 104.8 (C-8) was not observed in the HMBC spectrum, a HMBC correlation from δ_H 6.17 (H-6) to δ_C 163.9 (C-5) and a ROESY interaction between proton signals at δ_H 6.17 (H-6) and δ_H 3.84 (OCH_3-5) were evident, from which it was concluded that the (2*E*)-4-phenylprop-2-en-1-one unit is affixed at C-8. This conclusion was also supported by a *W* coupling between signals for δ_H 6.17 (H-6) and δ_C 192.0 (C-α) in the HMBC spectrum. The 2,3-*trans* configuration of **1** was determined by a $J_{2,3}$ coupling constant of 9.2 Hz.[17] However, the optical rotation value of this compound was determined to be zero, and the CD spectrum showed no Cotton effect. Since **1** was obtained as a racemate, **1a** and **1b** were obtained using a chiral HPLC column. The optical rotation values and CD data of these two optical isomers [**1a**, $[\alpha]^{23}_D$ −58

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2** (in DMSO-d_6)

	1		**2**	
position	δ_H[a] (J in Hz)	δ_C[b], mult.	δ_H[a] (J in Hz)	δ_C[b], mult.
2	4.62, d (9.2)	82.8, CH	4.70, d (9.2)	82.8, CH
3	4.00−4.02, m	65.0, CH_3	4.16−4.10, m	64.6, CH_3
4a	2.90, dd (5.6, 16.0)	29.3, CH_2	2.94, dd (6.0, 16.0)	29.4, CH_2
4b	2.41, dd (9.6, 16.0)		2.43, dd (9.6, 16.4)	
5		163.9, qC		163.7, qC
6	6.17, s	92.8, CH	6.18, s	92.7, CH
7		166.1, qC		165.4, qC
8		104.8, qC		104.8, qC
9		156.4, qC		156.3, qC
10		101.6, qC		101.6, qC
1′		129.2, qC		129.1, qC
2′	6.80, s	115.5, CH	7.14, d (1.6)	112.3, CH
3′		145.4, qC		147.7, qC
4′	6.93, s	115.3, CH		146.9, qC
5′		145.8, qC	6.80, d (8.0)	115.3, CH
6′	6.80, s	119.8, CH	6.98, dd (2.0, 8.0)	121.4, CH
1″		134.8, qC		134.8, qC
2″	7.09−7.11, m	128.4, CH	7.05−7.07, m	128.3, CH
3″	7.24−7.28, m	129.0, CH	7.22−7.26, m	128.8, CH
4″	7.31−7.33, m	130.4, CH	7.34−7.36, m	130.4, CH
5″	7.24−7.28, m	129.0, CH	7.22−7.26, m	128.8, CH
6″	7.09−7.11, m	128.4, CH	7.05−7.07, m	128.3, CH
γ	7.58, d (15.6)	142.5, CH	7.57, d (15.6)	142.3, CH
β	7.89, d (15.6)	127.2, CH	7.88, d (15.6)	127.4, CH
C═O		192.0, qC		192.8, qC
OCH_3-5	3.84, s	56.3, CH_3	3.86, s	56.2, CH_3
OH-7	14.10, s		14.50, s	
OH-3′	9.05, s			
OCH_3-3′			3.77, s	55.7, CH_3
OH-4′			9.50, s	
OH-5′	9.02, s			

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz.

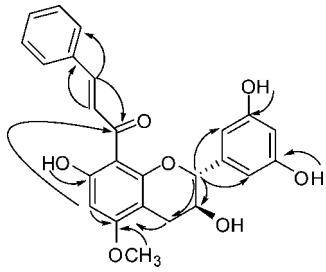

Figure 1. Selected HMBC (H→C) correlations for **1a**.

(c 0.11, CH_3CN); **1b**, $[\alpha]^{23}_D$ +62 (c 0.12, CH_3CN)] were opposite, as shown in Figure S1, Supporting Information. The absolute configurations of **1a** and **1b** were deduced from the comparison of their CD spectra with that of (2*R*,3*S*)-catechin-7-*O*-β-D-glucopyranoside.[18] Compound **1a** had positive Cotton effects at ca. 229 and 341 nm and a negative Cotton effect at ca. 275 nm. In turn, **1b** gave negative Cotton effects at ca. 229 and 341 nm and a positive Cotton effect at ca. 275 nm. Thus, the structure of **1a** was assigned as 8-[(*E*)-4-phenylprop-2-en-1-one]-(2*R*,3*S*)-2-(3,5-dihydroxyphenyl)-3,4-dihydro-2*H*-2-be-nzopyran-5-methoxyl-3,7-diol, and the structure of **1b** was assigned as 8-[(*E*)-4-phenylprop-2-en-1-one]-(2*S*,3*R*)-2-(3,5-dihydroxyphenyl)-3,4-dihydro-2*H*-2-benzopyran-5-methoxyl-3,7-diol.

Compound **2** was obtained as a yellow powder. The molecular formula of $C_{26}H_{24}O_7$ was determined by HREIMS (m/z 448.1521 M^+), indicating 15 degrees of unsaturation. The UV spectrum revealed an absorption maximum at 341 nm, and the NMR data displayed great similarities with those of **1**. In the ^{1}H and ^{13}C NMR spectra, a small difference from **1** was observed at C-3′ in the B ring, on which the hydroxyl group in **1** was replaced by a methoxy group in **2**. Moreover, the hydroxyl group at C-5′ in **1** was moved to C-4′ in **2**. This deduction was confirmed by the proton signals in the ^{1}H NMR spectrum at δ_H 7.14 (d, J = 1.6 Hz, H-2′), 6.80 (d, J = 8.0 Hz, H-5′), and 6.98 (dd, J = 2.0, 8.0 Hz, H-6′) and the HMBC correlations from the proton at δ_H 3.77 (OCH_3-3′) to the carbon at δ_C 147.7 (C-3′) and from the protons at δ_H 6.98 (H-6′) and 7.14 (H-2′) to the carbon at δ_C 146.9 (C-4′). The 2,3-*trans* configuration of **2** was determined by a $J_{2,3}$ coupling constant of 9.2 Hz.[17] The observation of a zero optical rotation value and the lack of a Cotton effect was similar to **1**, suggesting **2** to be a racemate. We were not able to isolate the individual racemates because of the trace amount available, but they were separated by a chiral HPLC column, as shown in Figure S2, Supporting Information. Thus, the structure of **2** was assigned as a mixture of 8-[(*E*)-4-phenylprop-2-en-1-one]-(2*R*,3*S*)-2-(3-methoxyl-4-hydroxyphenyl)-3,4-dihydro-2*H*-2-benzopyran-5-methoxyl-3,7-diol and 8-[(*E*)-4-*p*-henylprop-2-en-1-one]-(2*S*,3*R*)-2-(3-methoxyl-4-hydroxyphenyl)-3,4-dihydro-2*H*-2-benzopyran-5-methoxyl-3,7-diol.

The nine known compounds were identified as rhamnetin (**3**),[19] galangin,[20] isoferulic acid,[21] pinocembrin,[22] chrysin,[23,24] 5-methoxy-3,7-dihydroxyflavanone,[25] apigenin,[26] isorhamnetin,[27,28] and quercetin,[26] respectively, by comparison of their spectroscopic data with the literature values.

All the isolated compounds were evaluated for cytotoxicity against the human HeLa cervical cancer cell line using the MTT method. All compounds were inactive ($IC_{50} \geq 10$ μM), except for compounds **1a**, **1b**, **2**, and **3**, which were found to be cytotoxic, with IC_{50} values of 7.3 ± 0.05, 7.1 ± 0.04, 6.4 ± 0.04, and 7.8 ± 0.06 μM, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were obtained using a Shimadzu UV-240 spectrophotometer. CD spectra were collected on a JASCO J-20 spectropolarimeter. IR spectra were recorded on a Perkin-Elmer 577 spectrometer. NMR spectra were recorded on a Bruker AM-400 spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. HREIMS were recorded on a Finnigan/MAT-95 instrument. Chiral

HPLC was performed on a Chiralpak AS-H column (250 × 4.6 mm, 5 μm, Daicel Chiral Technologies Co., Ltd.) for **1** and a Chiralpak OJ-H column (250 × 4.6 mm, 5 μm, Daicel Chiral Technologies Co., Ltd.) for **2**. Silica gel (200−300 mesh, Qingdao Haiyang Chemical Co., Ltd.) and Sephadex LH-20 gel (Amersham Biosciences) were also used for column chromatography. TLC analysis was run on 60 F254 precoated silica gel plates (Merck), and spots were visualized by heating after spraying with 10% H_2SO_4−EtOH. All solvents used for isolation were of analytical grade.

Plant Material. Crude propolis was collected in Changge County, Henan Province, People's Republic of China, in March 2007. A voucher specimen (SC0052007) was deposited at Shanghai Research Center for Modernization of Traditional Chinese Medicine, Shanghai Institute of Materia Medica, SIBS, CAS, Shanghai.

Extraction and Isolation. The propolis (1 kg) was extracted three times with 5 L of 95% EtOH in an ultrasonic water bath for 1 h in two days. Then, the solvent was evaporated and the resulting residue (500 g) was suspended in H_2O and then successively extracted with petroleum ether and AcOEt. The AcOEt fraction (400 g) was subjected to column chromatography (silica gel; $CHCl_3$−MeOH, 100:1 → 2:1) to obtain fractions A−F. Fr. A (50 g) was submitted to repeated column chromatography (silica gel, petroleum ether−acetone 20:1 → 1:1; Sephadex LH-20 MeOH−$CHCl_3$, 1:1) to afford three major fractions, Fr. A1−A3. Fr. A1 was subjected to column chromatography over silica gel (petroleum ether−EtOAc, 15:1) and Sephadex LH-20 (MeOH−$CHCl_3$, 1:1), to yield chrysin (2 g) and galangin (200 mg). Fr. A2 was subjected to column chromatography over silica gel (petroleum ether−EtOAc, 10:1) and further purified by Sephadex LH-20 ($CHCl_3$−MeOH, 1:1) to afford pinocembrin (300 mg) and isorhamnetin (20 mg). Fr. A3 was subjected to column chromatography over silica gel (petroleum ether−EtOAc, 5:1) to obtain isoferulic acid (150 mg). Fr. B (10 g) was subjected to column chromatography over silica gel ($CHCl_3$−MeOH, 50:1 → 2:1) to obtain two major fractions, Fr. B1 and B2. Fr. B1 was further purified by passage over Sephadex LH-20 (MeOH−$CHCl_3$, 1:1) to afford 5-methoxy-3,7-dihydroxyflavanone (25 mg) and apigenin (60 mg). Fr. B2 was separated on a silica gel column eluting with $CHCl_3$−MeOH (15:1) to afford quercetin (50 mg). By analogous separation and purification procedures as for Fr. B, Fr. C (10 g) afforded **1** (50 mg), **2** (4 mg), and rhamnetin (**3**, 50 mg). Compound **1** was separated by a Chiralpak AS-H column [*n*-hexane−EtOH (0.1% DEA), 80:20] to obtain **1a** (8 mg) and **1b** (8 mg). **2** was analyzed by a Chiralpak OJ-H column [*n*-hexane−EtOH (0.1% DEA), 60:40].

8-[(*E*)-4-Phenylprop-2-en-1-one]-(2*R*,3*S*)-2-(3,5-dihydroxyphenyl)-3,4-dihydro-2*H*-2-benzopyran-5-methoxyl-3,7-diol (1a): yellow powder; $[\alpha]^{23}_D$ −58 (*c* 0.12, CH_3CN); UV (CH_3CN) λ_{max} (log ϵ) 341 (4.56) nm; IR (KBr) ν_{max} 3423, 2925, 1560, 1350, 1228, 1211 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 434 $[M]^+$ (20), 283 (100), 179 (100), 152 (20), 123 (20), 149 (16), 77 (16); HREIMS *m*/*z* 434.1366 (calcd for $C_{25}H_{22}O_7$, 434.1366).

8-[(*E*)-4-Phenylprop-2-en-1-one]-(2*S*,3*R*)-2-(3,5-dihydroxyphenyl)-3,4-dihydro-2*H*-2-benzopyran-5-methoxyl-3,7-diol (1b): yellow powder; $[\alpha]^{23}_D$ +62 (*c* 0.11, CH_3CN); UV, IR, ^1H and ^{13}C NMR, and EIMS data were the same as **1a**.

8-[(*E*)-4-Phenylprop-2-en-1-one]-2-(3-methoxyl-4-hydroxyphenyl)-3,4-dihydro-2*H*-2-benzopyran-5-methoxyl-3,7-diol (2): yellow powder; UV (CH_3CN) λ_{max} (log ϵ) 341 (4.58) nm; IR (KBr) ν_{max} 3423, 2937, 1630, 1587, 1550, 1350, 1228, 1147 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 448 $[M]^+$ (27), 284(24), 283 (100), 179 (96), 166 (60), 137(32), 66 (16); HREIMS *m*/*z* 448.1521 (calcd for $C_{26}H_{24}O_7$, 448.1522).

Cytotoxicity Assay. Compounds were evaluated for their cytotoxic activity according a protocol described in the previous literature,[29] with doxorubicin as positive control (IC_{50} 0.04 μM against HeLa cells).

Supporting Information Available: NMR and mass spectra of **1a**, **1b**, and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) *Committee of National Pharmacopoeia. China Pharmacopoeia*; Chemical Industry Press: Beijing, 2005; Part 1, pp 249−250.
(2) Usia, T.; Banskota, A. H.; Tezuka, Y.; Midorikawa, K. *J. Nat. Prod.* **2002**, *65*, 673–676.
(3) Bankova, V. S.; Popov, S. S.; Marekov, N. L. *J. Nat. Prod.* **1983**, *46*, 471–474.
(4) Bankova, V. S.; Marcucci, M. C. *Apidologie* **2000**, *31*, 3–15.
(5) Scazzocchio, V.; D'Auria, F. D.; Alessandrini, D.; Pantanella, E. *Microbiol. Res.* **2006**, *161*, 327–333.
(6) Amoros, M.; Simoes, C. M. O.; Girre, L.; Sauvager, F.; Cormier, M. *J. Nat. Prod.* **1992**, *55*, 1732–1740.
(7) Silici, S.; Koc, N. A.; Ayangil, D.; Cankaya, S. *J. Pharmacol. Sci.* **2005**, *99*, 39–44.
(8) Tan-No, K.; Nakajima, K. T.; Shoii, T.; Nakagawasai, O.; Niijima, F.; Ishikawa, M.; Endo, Y.; Sato, T.; Satoh, S.; Tadano, K. *Biol. Pharm. Bull.* **2006**, *29*, 96–99.
(9) Jeong, C. S.; Jung, J. H.; Jung, K. H. *J. Appl. Pharmacol.* **2000**, *8*, 223–227.
(10) Pietta, P. G. *J. Nat. Prod.* **2000**, *63*, 1035–1046.
(11) Sforcin, J. M.; Orsi, R. O.; Bankova, V. *J. Ethnopharmacol.* **2005**, *98*, 301–305.
(12) Feres, M.; Figuereido, L. C.; Barreto, I. M.; Coelho, M. H.; Araujo, M. W.; Cortelli, S. C. *J. Int. Acad. Periodontol.* **2005**, *7*, 90–96.
(13) Orsolic, N.; Sarnovic, A. B.; Basic, I. *Planta Med.* **2006**, *72*, 20–27.
(14) Abdel-Latif, M. M.; Windle, H. J.; Homasany, B. S.; Sabra, K. *Br. J. Pharmacol.* **2005**, *146*, 1139–1147.
(15) Chen, H. F.; Tanaka, T.; Nonaka, G. I.; Fujioka, T.; Mihashi, K. *Phytochemistry* **1993**, *33*, 183–187.
(16) Yi, J. H.; Zhang, G. L.; Li, B. G. *Acta Pharm. Sin.* **2002**, *37*, 352–354.
(17) Friedrich, W.; Galensa, R. *Eur. Food Res. Technol.* **2002**, *214*, 388–393.
(18) Slade, D.; Ferreira, D.; Marais, J. P. *Phytochemistry* **2005**, *66*, 2177–2215.
(19) Chi, J. P.; Xue, B. W.; Chen, H. S. *Chin. Pharm. J.* **1996**, *31*, 264–266.
(20) Ning, A.; Yang, S. L.; Zou, Z. M.; Xu, L. Z. *Chin. Trad. Herbal Drugs* **2006**, *37*, 663–664.
(21) He, Z. R.; Pan, W. D.; Lu, Y. Z.; Zhang, C. G. *Chin. Trad. Herbal Drugs* **2002**, *33*, 405–406.
(22) Zhou, L. D.; Guo, J.; Yu, J. G. *China J. Chin. Mat. Med.* **1999**, *24*, 162–164.
(23) Chen, L. J.; Games, D. E.; Jones, J. *J. Chromatogr. A* **2003**, *988*, 95–105.
(24) Aksnes, D. W.; Standnes, A.; Anderson, O. M. *Magn. Reson. Chem.* **1996**, *34*, 820–823.
(25) Nagy, M.; Suchy, V.; Uhrin, D.; Ubik, K.; Budesinsky, M.; Grancai, D. *Chem. Papers* **1988**, *42*, 691–696.
(26) Miyazawa, M.; Hisama, M. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 2091–2099.
(27) Lin, W. H.; Deng, Z. W.; Lei, H. M.; Fu, H. Z.; Li, J. *J. Asian Nat. Prod. Res.* **2002**, *4*, 287–295.
(28) Hsich, T. J.; WU, Y. C.; Lo, W. L.; Chen, S. C.; Kuo, S. H.; Chen, C. Y. *Chin. Pharm. J. (Taipei)* **2004**, *56*, 17–23.
(29) Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 581–601.

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