FLAVONOID COMPONENTS OF PROPOLIS

S. A. Popravko, A. I. Gurevich, and M. N. Kolosov

Khimiya Prirodnykh Soedinenii, Vol. 5, No. 6, pp. 476–482, 1969

UDC 547.972.2

Propolis or bee glue [1–5] has scarcely been studied hitherto; only recently has a report appeared on the isolation from it of galangin (3,5,7-trihydroxyflavone) [6].

In a chromatographic study of samples of propolis collected in various zones of the European part of the USSR, we have found that a number of compounds are present in it in practically all cases, regardless of the region inhabited by, and the nature of, the bees. We have studied these compounds as characteristic constant components of propolis, since it is just these compounds that must determine its general biological activity.

In this paper we describe the flavonoids most widely represented among these constant components of propolis. They are all present in approximately the same amount (1.5–2%) and were isolated by adsorption chromatography on silica gel. The spectral characteristics of these compounds and their derivatives are given in Tables 1 and 2.

A comparison of their empirical formulas, functional compositions, and spectral properties has shown that the compounds under consideration are flavonoids of three types: trisubstituted flavones, tetrasubstituted flavones, and flavanone derivatives.

The compounds of the first group are derivatives of apigenin (4',5,7-trihydroxyflavone), being its 4'-methyl and 4',7-dimethyl ethers (I) and (II). The presence of a chelate hydroxy group in position 5 in them was established from the nature of the change in the UV spectrum on the formation of complexes with $AlCl_3$ (cf. [11]) and the positions of the other two substituents in the aromatic rings was shown by the NMR spectra of the corresponding acetates. In these spectra there are the signals of the four protons of a p-disubstituted benzene ring (two two-proton doublets at about 7.0 and 7.8 ppm) and of two meta protons which, in the diacetate (I—Ac_2) are displaced in the weak-field direction. The structure of compounds I and II were shown finally by the direct identification of the first of them with a sample of acacetin and the second with the product of the methylation of acacetin with methyl iodide in the presence of potassium carbonate.

The compounds of the second group are various methyl ethers of kaempferol (3,5,7,4'-tetrahydroxyflavone), as was established by their exhaustive methylation to give one and the same tetramethyl ether (X) by means of CH_3I + + NaH in dimethyl sulfoxide. Under the action of diazomethane they all formed the 3,7,4'-trimethyl ether of kaempferol (IX) and, consequently, contain a free hydroxyl in position 5. In addition to this, with the exception of compound V they all have a hydroxyl group in position 3, as is shown by the magnitude of the bathochromic shift of the long-wave absorption (with no change in its extinction) on the formation of the chelate with $AlCl_3$ (see Table 1 and [11]). One of these 3,5-dihydroxyflavones does not contain additional hydroxy groups, thanks to which its conversion into the tetramethyl ether X unambiguously shows that it has the structure of 7,4'-di-O-methylkaempferol (VI). In its isomer having a methoxy group in position 3 (see above) the phenolic hydroxyl, 7-OH, must be free, since the 8-H proton in the acetate of this compound is descreened more strongly than in the acetate of 3,5-dihydroxy-4',7-dimethoxy-flavone (VI) (see Table 2). Consequently, the second methoxy is located at C-4', i.e., the substance possesses structure V; we confirmed the correctness of this conclusion by independent synthesis of V by Lynch's method [12]. The two other flavones of this group each contain three hydroxyls, and as follows from what has been said, consist of the isomeric 4'- and 7-monomethyl ethers of kaempferol. The choice between the two possible structures for them, III and IV, was made on the basis that one of these substances is unstable in 0.002 N ethanolic EtONa and, consequently, in addition to the 3-OH contains a free hydroxyl in position 4' [11]. Thus, it is rhamnocitrin (IV), and its isomer is kaempferide (III).

Table 1. UV and IR Spectra of the Flavonoids of Propolis and of Their Acetates

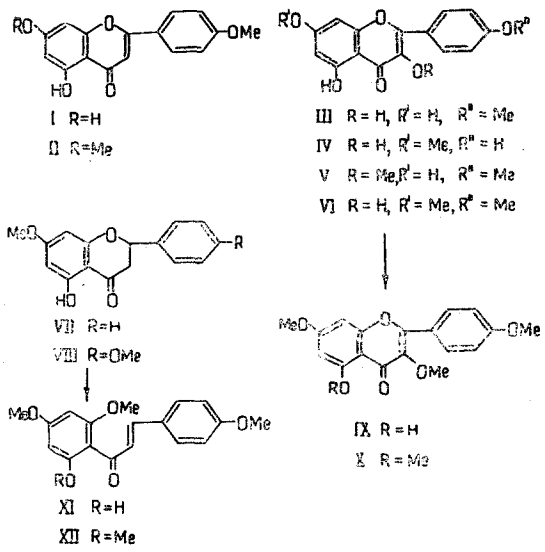
Compound* (empirical formula)	λ_{max}^{**}, mμ (log ε)		ν_{max}^{KBr}, cm^{-1}
	in 96% ethanol	in a 0.002 M solution of $AlCl_3$ in 96% ethanol	
I	269, 298, 330	280, 302, 335, 380	3180, 1670–1654,
($C_{16}H_{12}O_5$)	(4.30; 4.15; 4.27)	(4.24; 4.26; 4.27; 4.01)	1610, 1568, 1512
I–Ac_2	230, 259, 324		1768, 1645, 1612
($C_{20}H_{16}O_7$)	(4.18; 4.18; 4.03)		1518
II	270, 330	278, 300, 337, 380	3100–2600, 1670,
($C_{17}H_{14}O_5$)	(4.47; 4.53)	(4.41; 4.40; 4.47; 4.20)	1608, 1515
II–Ac	248, 257, 321		1760, 1632, 1608,
($C_{19}H_{16}O_6$)	(4.25; 4.26; 4.53)		1516
III	269, 367	278, 302, 350, 420	3200–3100, 1652,
($C_{16}H_{12}O_6$)	(4.38; 4.28)	(4.42; 4.17; 4.21; 4.29)	1587, 1570, 1507***
III–Ac_3	253, 305		1772–1764, 1635,
($C_{22}H_{18}O_9$)	(4.39; 4.28)		1505
IV	268, 368	270, 303i, 350, 422	3300–3100, 1650,
($C_{16}H_{12}O_6$)	(3.95; 4.01)	(4.06; 3.64; 3.76; 4.07)	1615, 1580, 1500***
IV–Ac_3	255, 303		1770, 1632, 1516***
($C_{22}H_{18}O_9$)	(4.38; 4.44)		
V	270, 351	279, 303i, 343, 401	3130, 1650, 1614,
($C_{17}H_{11}O_6$)	(4.35; 4.25)	(4.34; 4.18; 4.28; 4.11)	1577, 1500***
V–Ac_2	255, 339		1767, 1640–1620,
($C_{21}H_{18}O_8$)	(4.38; 4.43)		1612, 1518***
VI	269, 320i, 369	270, 303, 350, 425	3330, 1664, 1630,
($C_{17}H_{14}O_6$)	(4.46; 4.24; 4.48)	(4.48; 4.01; 4.19; 4.49)	1598, 1514
VI–Ac_2	230, 255, 322		1765, 1640, 1610,
($C_{21}H_{18}O_8$)	(4.38; 4.31; 4.44)		1584, 1515
VII	289, 320		3060, 1646, 1624,
($C_{16}H_{14}O_4$)	(4.36; 3.61)		1580, 1525
VII–Ac	273, 304i		1770, 1682, 1620,
($C_{18}H_{16}O_5$)	(4.13; 3.75)		1570
VIII	216, 220, 320 i		3105, 3095, 1630,
($C_{17}H_{16}O_5$)	(4.55; 4.33; 3.64)		1582, 1524
VIII–Ac	224, 275, 305i		1767, 1681, 1628–
($C_{19}H_{18}O_6$)	(4.57; 4.35; 3.93)		1620, 1588, 1565***

*Ac) acetate; Ac_2) diacetate; Ac_3) triacetate.
**i) inflection.
***In a mull with paraffin oil.

Table 2. NMR Spectra of Derivatives of the Flavonoids of Propolis*

Compound	2–H	3–H	6–H	8–H	2′–H and 6′–H	3′–H and 5′–H	4′–H	3–OMe or 3–OAc**	5–OMe or 5–OAc**	7–OMe or 7–OAc**	4′–OMe or 4′–OAc**
I–Ac_2		6.54 (s)	6.82 (d, 2)	7.29 (d, 2)	7.77 (d, 9)	6.98 (d, 9)			(2.44)	(2.34)	3.84
II–Ac		6.50 (s)	6.59 (d, 2)	6.83 (d, 2)	7.77 (d, 9)	7.00 (d, 9)			(2.43)	3.85	3.89
III–Ac_3			6.63 (d, 2)	6.84 (d, 2)	7.76 (d, 9)	7.27 (d, 9)		(2.38)	(2.48)	(2.37)	3.93
IV–Ac_3			6.58 (d, 2)	6.80 (d, 2)	7.81 (d, 9)	7.20 (d, 9)		(2.20)	(2.30)	3.84	(2.20)
V–Ac_2			6.81 (d, 2)	7.28 (d, 2)	8.06 (d, 9)	7.00 (d, 9)		3.79	(2.46)	(2.33)	3.86
VI–Ac_2			6.61 (d, 2)	6.82 (d, 2)	7.78 (d, 9)	6.98 (d, 9)		(2.32)	(2.44)	3.87	3.88
VII	5.43 (q, 12 and 4)	a: 3.10 (q, 12 and 17) e: 2.81 (q, 4 and 17)	6.08 (s)	6.08 (s)	7.42 (s)	7.42 (s)	7.42 (s)			3.82	
VII–Ac	5.47 (q, 12 and 5)	a: 3.02 (q, 12 and 17) e: 2.67 (q, 5 and 17)	6.36 (d, 2.5)	6.51 (d, 2.5)	7.53 (s)	7.53 (s)	7.53 (s)		(2.38)	3.82	
VIII–Ac	5.35 (q, 12 and 4)	a: 3.00 (q, 12 and 17) e: 2.63 (q, 4 and 17)	6.22 (d, 2.5)	6.35 (d, 2.5)	7.32 (d, 9)	6.88 (d, 9)			(2.36)	3.77	3.77
X			6.24 (d, 2)	6.40 (d, 2)	8.01 (d, 9)	6.92 (d, 9)		3.89	3.86)	3.84	3.84

*The spectra were measured in $CDCl_3$ with Me_4Si as internal standard. The chemical shifts are expressed in ppm on the δ scale; the nature of the signal and the magnitude of J in Hz are shown in brackets; s) singlet; d) doublet; q) quartet; a) axial; e) equatorial; Ac) acetate; Ac_2) diacetate; Ac_3) triacetate. The assignment of the signals of the aromatic protons and of the methoxy groups is based on literature data [7-10].
**Chemical shifts of the acetoxy groups given in brackets.



Finally, the compounds of the third group are mono- and dimethoxy derivatives of hydroxyflavanone. The presence in them of a chelate hydroxyl at 5-C follows from the UV and IR spectra, and the positions of the methoxy groups follow from the NMR spectra of the corresponding acetates, which show that positions 6,8,2',3',4',5', and 6' are unsubstituted in one of them (VII) and positions 6,8,2',3',5', and 6' (VIII) in the other. The structure of the latter compound was also shown by its conversion into 2,4,6,4'-tetramethoxychalcone (XII) by the action of CH_3I + NaH in dimethyl sulfoxide and the independent synthesis of this chalcone from 2,4-dimethylphloracetophenone and p-methoxybenzaldehyde via the trimethyl ether XI.

The flavonones VII and VIII are optically active, VII being (–)-pinostrobin [13], while VIII has previously been described only in the form of the racemate obtained by partial synthesis from naringenin [14]. The optical rotatory dispersion curves of the flavanones and their 5-acetates have a positive Cotton effect at about 330 mμ, which shows the S configuration of their asymmetric center 2 [15]. At the same time in these compounds the Cotton effects in the 280–290 mμ region have different signs: VII) positive; VIII) negative.

EXPERIMENTAL

Chromatography was carried out on a 0.5-mm nonfixed layer of silica gel of "vodnaya kremnevaya kislota" ["aqueous silicic acid"] type (100–150 mesh, activity grade II), in the solvent systems: 1) ethyl acetate-heptane (2 : 3), and 2) benzene–ethyl acetate (9 : 1). The acetates were obtained by the action of Ac_2O in pyridine (2 days at 20° C). The molecular weights were determined mass spectrometrically. The analytical results of all the compounds corresponded to the calculated figures.

Samples of kaempferol and acacetin were kirdly given to us by G. L. Kuznetsova and A. P. Prokopenko. The IR spectra were taken by L. B. Senyavina. The mass spectra were measured by V. N. Bochkarev.

1. Isolation of the components of propolis. Eighteen grams of finely ground shavings of propolis was extracted with 0.5 *l* of 96% ethanol at room temperature for 3 days. The solution was filtered and evaporated, the residue (8–9 g) was dissolved in 50 ml of acetone and 50 g of silica gel was added, and the mixture was dried in the air and transferred to a column (1000 × 40 mm) containing 300 g of the same adsorbent. Elution was then carried out with 1.5 *l* of benzene–petroleum ether (1 : 4), 3 *l* of benzene, 6 *l* of benzene-ethyl acetate (10 : 1), and, finally, 1.5 *l* of acetone, 250-ml fractions being collected. The first two fractions contained substances of nonflavonoid nature the total weight of which was 0.4–0.8 g, and fractions 3–7 contained substances II, VI, IX, and X. Compounds III-V were isolated from fractions 15–18, and acacetin (I) from fractions 19–21. Final purification was carried out by TLC.

5,7-Dihydroxy-4'-methoxyflavone (acacetin) (I)–mp 262–264° C (from benzene); R_f 0.48 (1), 0.28 (2); diacetate $C_{20}H_{16}O_7$, mp 205–206° C (from MeOH); R_f 0.27 (1), 0.26 (2), mol. wt. 368.

5-Hydroxy-7,4'-dimethoxyflavone (II), $C_{17}H_{14}O_5$, mp 172–174° C (from ethanol); R_f 0.62 (1), 0.73 (2), mol. wt. 298.

Acetate, $C_{19}H_{16}O_6$, mp 197–198° C (from ethanol), R_f 0.26 (1), 0.21 (2).

3,5,7-Trihydroxy-4'-methoxyflavone (kaempferide) (III), $C_{16}H_{12}O_6$, mp 225–227° C (from benzene); R_f 0.46 (1), 0.41 (2).

Triacetate, $C_{22}H_{18}O_9$, mp 206–208° C (from benzene); R_f 0.24 (1), 0.23 (2), mol. wt. 426.

3,5,4'-Trihydroxy-7-methoxyflavone (rhamnocitrin) (IV), $C_{16}H_{12}O_6$, mp 222–224° C (from benzene); R_f 0.47 (1), 0.40 (2), mol. wt. 300.

Triacetate, $C_{22}H_{18}O_9$, mp 205–206° C (from ethanol); R_f 0.24 (1), 0.21 (2).

5,7-Dihydroxy-3,4'-dimethoxyflavone (V), $C_{17}H_{14}O_6$, mp 225–226° C (from benzene), R_f 0.49 (1), 0.58 (2), mol. wt. 314.

Diacetate, $C_{21}H_{18}O_8$, mp 160–162° C (from benzene–heptane); R_f 0.39 (1), 0.36 (2).

3,5-Dihydroxy-7,4'-dimethoxyflavone (VI), $C_{17}H_{14}O_6$, mp 176–177° C (from benzene); R_f 0.71 (1), 0.80 (2), mol. wt. 314.

Diacetate, $C_{21}H_{18}O_8$, mp 195–196° C (from benzene); R_f 0.27 (1), 0.37 (2).

(–)-5-Hydroxy-7-methoxyflavanone[(–)-pinostrobin] (VII), $C_{16}H_{14}O_4$, mp 99–100° C (from ether–heptane); R_f 0.75 (1), 0.81 (2); $[\alpha]_D^{26}$ –31° (c 0.1; in $CHCl_3$); $[\alpha]_{589}^{26}$ –22.7°, $[\alpha]_{343}$ +268°, $[\alpha]_{320}$ –59°, $[\alpha]_{292}$ +945°, $[\alpha]_{280}$ –652°, $[\alpha]_{258}$ +504° $[\alpha]_{240}$ –296° (c 0.1; in ethanol), mol wt. 270.

Acetate, $C_{18}H_{16}O_5$, mp 141–143° C (from benzene); R_f 0.61 (1), 0.67 (2).

(–)-5-Hydroxy-7,4'-dimethoxyflavanone (VIII), $C_{17}H_{16}O_5$, mp 114–115° C (from benzene), R_f 0.73 (1); 0.83 (2); $[\alpha]_{589}^{26}$ –182°, $[\alpha]_{350}$ +1020°, $[\alpha]_{294}$ –12 400°, $[\alpha]_{275}$ +10 100°, $[\alpha]_{243}$ +5950° (c 0.1; in ethanol), mol. wt. 300.

Acetate, $C_{19}H_{18}O_6$, mp 120–121° C (from cyclohexane); R_f 0.52 (1), 0.59 (2); $[\alpha]_{589}^{26}$ +79°, $[\alpha]_{347}$ +1780°, $[\alpha]_{317}$ –10 600°, $[\alpha]_{282}$ +5550°, $[\alpha]_{247}$ +4290° (c 0.1; in ethanol).

2. Tetramethyl ether of kaempferol (X). To a solution of 30 mg of kaempferol or one of compounds III–VI in 1.5 ml of absolute dimethyl sulfoxide and 0.5 ml of methyl iodide was added 14 mg of sodium hydride. The mixture was stirred at 20° C for 30 min and was then acidified with 0.2 ml of AcOH and was diluted with 10 ml of benzene, 10 ml of ethyl acetate, and 20 ml of water. The organic layer was separated off, washed with water and with saturated solutions of $NaHCO_3$ and NaCl, dried with Na_2SO_4, and evaporated. The residue was triturated with ether and filtered off. The yield of the tetramethyl ether X was 25 mg (70%); mp 159–160° C (from benzene–heptane); λ_{max}^{EtOH} 265, 337 mμ (lg ε 4.38, 4.40); ν_{max}^{KBr} 1636, 1605, 1580, 1511, 1490 cm^{-1}.

Found, %: C 66.8; H 5.1. Calculated for $C_{19}H_{18}O_6$, %: C 66.6; H 5.3.

3. 3,7,4'-Trimethoxy-5-hydroxyflavone (IX). A) To a solution of 100 mg of compound III or one of compounds IV–VI in 5 ml of absolute tetrahydrofuran was added 2 ml of a 0.6 M ethereal solution of diazomethane. The mixture was left at 20° C for 3 hr and was then evaporated, and the residue was crystallized from ethanol. This gave 50 mg (48%) of the trimethyl ether IX, mp 141–142° C; λ_{max}^{EtOH} 269, 349 mμ (lg ε 4.30, 4.25); ν_{max}^{Nujol} 3100, 1665, 1607, 1592, 1504 cm^{-1}.

Found, %: C 65.8; H 5.0. Calculated for $C_{18}H_{16}O_6$, %: C 65.9; H 4.9.

B) To a solution of 300 mg of compound V in 5 ml of acetone and 2 ml of methyl iodide was added 800 mg of anhydrous K_2CO_3. The mixture was heated to the boil for 20 hr and was then filtered and evaporated, and the residue was chromatographed in the ethyl acetate–petroleum ether (1 : 1) system. The zone with R_f 0.75–0.85 furnished 35 mg of the trimethyl ether (IX) (yield 10%) and the zone with R_f 0.25–0.30 gave 60 mg of the tetramethyl ether X (yield 18%).

4. 5-Hydroxy-4',7-dimethoxyflavone (II). To a solution of 30 mg of acacetin (I) in 4 ml of acetone and 1 ml of

methyl iodide was added 150 mg of anhydrous K_2CO_3. The mixture was stirred at 20° C for 3 hr, filtered, and evaporated, and the residue was chromatographed in system 1. This gave 17 mg (54%) of the methyl ether II.

5. 2,4,6,4'-Tetramethoxychalcone (XIII). A) To a solution of 200 mg of the flavanone X in 1.5 ml of methyl iodide and 3 ml of dimethyl sulfoxide was added 140 mg of sodium hydride. The mixture was stirred at 20° C for 1.5 hr and was then acidified with 0.5 ml of AcOH and diluted with benzene, after which the solution was washed repeatedly with water, dried with Na_2SO_4, and evaporated, and the residue was triturated with a mixture of benzene and petroleum ether. This gave 140 mg (64%) of the chalcone XII with mp 117–118° C (from benzene–petroleum ether); R_f 0.38 (1); λ_{max}^{EtOH} 227, 330 mμ (lg ε 4.31, 4.45); λ_{max}^{Nujol} 1674, 1600, 1518 cm^{-1}.

Found, %: C 70.2; H 6.1; CH_3O 35.7; mol. wt. 328. Calculated for $C_{19}H_{20}O_5$, %: C 69.5; H 6.1; 4 CH_3O 37.8; mol. wt. 328.

B) A solution of 0.7 g of KOH in 2 ml of 50% ethanol was added to 250 mg of 2,4-dimethylphloracetophenone [16] and 350 mg of p-methoxybenzaldehyde in 4 ml of ethanol. The mixture was heated to the boil in an atmosphere of argon for 30 min, cooled, acidified with acetic acid, diluted with ethyl acetate, washed with water and saturated NaCl solution, dried with Na_2SO_4, and evaporated, and the last traces of water were eliminated by azeotropic distillation with benzene. The unpurified chalcone XI obtained was methylated under the conditions of the preceding experiment using 2 ml of methyl iodide and 60 mg of sodium hydride in 6 ml of dimethyl sulfoxide. The chalcone XII was isolated by chromatography in system 1; yield 130 mg (31%).

CONCLUSIONS

The following flavonoids have been isolated from propolis and identified: acacetin (I), 7,4'-dimethoxyflavone (II), kaempferide (III), rhamnocitrin (IV), 5,7-dihydroxy-3,4'-dihydroxyflavone (V), 3,5-dihydroxy-7,4'-dimethoxyflavone (VI), (–)-pinostrobin (VII), and (–)-5-hydroxy-7,4'-dimethoxyflavanone (VIII).

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17 September 1968

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