A STUDY ON FLAVONOIDS OF PROPOLIS

V.S. BANKOVA, S.S. POPOV, and N.L. MAREKOV

Institute of Organic Chemistry with Centre Of Phytochemistry, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

ABSTRACT.—Seven known propolis flavonoids have been isolated and identified. A selective elution, used in hplc analysis of propolis flavonoids have permitted the detection of three new flavonoids in this object. Structural assignments for two of them [3,7-dihydroxy-5-methoxyflavanone (**8**) and 2,5-dihydroxy-7-methoxyflavanone (**9**)] have been given.

Propolis, a resinous hive product collected by bees, is known to have been used in folk medicine as early as 300 BC. Recently, it was reported to possess such versatile biological activities as antibacterial, antiviral, fungicidal, local anaesthetic, antiulcer, immunostimulating (1), hypotensive (2) and cytostatic properties (3). Some reports were published about successful clinical use of propolis to aid the healing of wounds, ulcers, and tuberculosis, for the treatment of micotic infections and eczema, in stomatology, etc. (4).

These valuable properties of propolis created an interest in its chemical composition. Fatty and phenolic acids and their esters, aromatic aldehydes and alcohols, sesquiterpenes, napththalene and stilbene compounds, as well as a large number of flavonoids, were identified in propolis. Till now, flavonoids were the sole propolis constituents that had been chemically investigated in detail. The interest in propolis flavonoids is due to their significant physiological activities, which can be responsible for a large part of propolis activity. Pinocembrin and galangin determine the antibacterial activity of propolis (5); pinocembrin has also fungicidal (6) and local anesthetic activities (7). Quercetin, kaempferide, and pectolinarigenin have spasmolytic activity (1); acacetin has antiinflamatory activity (1); and luteolin and apigenin possess antiulcer activity (1).

In this paper, we describe an investigation of flavonoids of Bulgarian propolis.

RESULTS AND DISCUSSION

Methanol extract from propolis was extracted consecutively with petroleum ether and ethyl ether. The last extract, which contained the main part of the flavonoids, was chromatographed on polyamide column, and the obtained flavonoid fractions were purified on silica gel columns. The final purification was achieved by recrystallization. Seven main flavonoid constituents were isolated and identified (mp, hplc, tlc, pmr, uv, and ms) as pinocembrin (**1**), galangin (**2**), chrysin (**3**), tectochrysin (**4**), quercetin (**5**), and isorhamnetin (**6**) (8), as well as a small amount of kaempferol (**7**) (7). All these flavonoids have been found previously in propolis.

Recently, we published a method for hplc analysis of propolis flavonoids on reversed-phase columns (9) and examined a series of solvent systems on Partisil PXS/10/25 ODS column, including those described previously (10-13). The most suitable mobile phase for the separation of propolis flavonoids proved to be water-methanol-acetic acid (60:75:5). We also found that, when water-methanol-acetic acid (65:30:5) was used as a mobile phase, the flavones and flavonols, two major flavonoid groups of propolis, cannot be eluted from the column. Making use of this technique, we obtained a simplified chromatogram revealing peaks that were overlapped by the peaks of the major flavonoids, and this enabled us to investigate some unknown minor flavonoids of propolis.

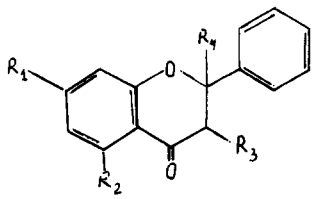
Preparative hplc under these conditions yielded pinocembrine and three other flavonoids (I, II, and III) in quantities large enough for ms. These spectra indicated that the last three flavonoids are new for propolis. Larger amounts of these flavonoids were isolated from propolis by detailed column chromatography, and their structures were investigated as follows.

FLAVONOID I.—Its mass spectrum was typical for dihydroflavonols—relatively low molecular ion peak (*m/z* 286) and elimination of HCO· from it (*m/z* 257). The last fragment eliminated ring B, together with a proton, to the ion with *m/z* 179, which was an indication for an unsubstituted ring B. The base peak (*m/z* 167) resulted from retro-Diels-Alder fragmentation and indicated the presence of a hydroxyl and a methoxy group in ring A. This was confirmed by further elimination of CH_3· and CHO· from this ion. The presence of an ion with *m/z* 120, containing ring B together with C-2 and C-3, located an additional OH group at the last two carbon atoms; the elimination of HCO· from this ion (*m/z* 91) localized this OH group at C-3. From all these data it can be concluded that flavonoid I is dihydroflavonol with a hydroxyl and a methoxyl group in ring A. This was in accordance with the 250 MHz pmr spectrum. Two multiplets at δ 7.31 (H-3′, H-4′, and H-5′) and at δ 7.41 (H-2′ and H-6′) confirmed the lack of substitution in ring B. H-2 (δ 5) and H-3 (δ 4.26) had $J_{2,3}=11$ Hz, indicating that they are trans-diaxially related to each other, as in other naturally occurring dihydroflavonols. The methoxyl group appeared as 3-H singlet at δ 3.68, and the broad peak at δ 5.27 probably was due to a 3-OH group. Two weakly separated one-proton doublets: at δ 6.02 and δ 5.88 are characteristic of H-8 and H-6, respectively, in 5,7-dioxygenated dihydroflavonols.

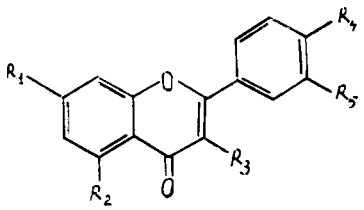
The uv maxima at 287 and 312 (sh) nm are typical for dihydroflavonols. The bathochromic shift (253 sh, 326 nm) in the NaOAc spectrum was an indication for the presence of a free 7-OH group. The bathochromic shift with $AlCl_3$ (278 sh, 317 nm) and decomposition of the complex with HCl, as well as a lack of complexing with H_3BO_3 was an indication for the presence of a free 3-OH group and a substituted 5-OH group. On the basis of all this data, a structure of 5-methoxy-3,7-dihydroxyflavanon (**8**) was proposed for flavonoid I. Accordingly, the mp of the dihydroflavanol alpinon, with the isomeric structure (3,5-dihydroxy-7-methoxyflavanon), was reported to be 186-7° (10), while flavonoid I melted at over 220°.

FLAVONOID II.—This is a flavanone according to its uv spectrum [289 and 323 (sh) nm]. From the molecular ion peak (*m/z* 286), we can conclude that it is an isomer of flavonoid I, but its ms fragmentations were very different. The molecular ion (a weak peak) eliminates an OH group and water, the first process being more intensive. This was an indication for the presence of a tertiary-OH group. The ions with *m/z* 167 (A_1+H), 166 (A_1), and 138 (A_1-CO) showed a presence of a hydroxyl and a methoxyl group in ring A. The base peak was at *m/z* 105 and the corresponding ion eliminated CO to the ion with *m/z* 77. This unusual fragmentation is characteristic (15) of one relatively new group of flavonoids, those containing a 2-OH group, discovered in 1972 (16). A comparison of their mass spectra to that of flavonoid II confirms that the latter belongs to the same group and contains a hydroxyl and a methoxyl group in ring A. The absence of a band-I bathochromic shift in the uv spectrum after the addition of NaOAc showed a lack of free 7-OH group, and the bathochromic shift in the $AlCl_3$ and $AlCl_3$-HCl spectra (312 and 384 nm) was an indication for the presence of a free 5-OH group.

On the basis of these data we can assume that flavonoid II is 2,5-dihydroxy-7-methoxyflavanone (**9**), which is in agreement with its mp [167-170°, Lit. 170-2° (17)]. This flavonoid has not been found in propolis till now. It is known to be a constituent of *Populus nigra* buds (17).



1 $R_1=R_2=OH; R_3=R_4=H;$
8 $R_1=R_3=OH; R_2=OCH_3; R_4=H;$
9 $R_1=OCH_3; R_2=R_4=OH; R_3=H;$



2 $R_1=R_2=R_3=OH; R_4=R_5=H;$
3 $R_1=R_2=OH; R_3=R_4=R_5=H;$
4 $R_1=OCH_3; R_2=OH; R_3=R_4=R_5=H;$
5 $R_1=R_2=R_3=R_4=R_5=OH;$
6 $R_1=R_2=R_3=R_4=OH; R_5=OCH_3;$
7 $R_1=R_2=R_3=R_4=OH; R_5=H;$

FLAVONOID III.—This was found in hplc fraction containing pinocembrin as a main constituent. Its mass spectrum gave evidence for a flavon, flavonol, or isoflavon structure, containing a hydroxyl and a methoxy group in ring A. As flavones and flavonols cannot be eluted from the column under these conditions, flavonoid III may be an isoflavone, which is somewhat strange, as isoflavones have not been found in plants that were expected to be sources of propolis. Further work for isolation of this compound in an amount sufficient for full structural assignment is in progress.

The flavonoid composition of Bulgarian propolis differs from that of propolis collected and investigated in the USSR (18), which can be connected with its origin. It is now accepted that because bees collect propolis from resinous tree buds in different geographical regions, the origin of propolis can be different. For example, in the USSR it probably originates from Betula buds, and in the Ukrainian SSR from Betula and Populus buds (18). In France, propolis probably was collected from Populus and Salix buds (19). In a separate communication (8) we reported the results of qualitative and quantitative analysis of propolis, collected in different regions in Bulgaria as well as such analysis of flavonoids of some probable sources of Bulgarian propolis—buds of *Populus nigra, Populus nigra* var. *italica, Betula verrucosa, Aesculus hippocastanum* and *Salix* spp. We found that the flavonoid content of all these tree buds, with exception of *Populus nigra* buds, differ considerably from that of propolis, indicating that bees do not collect propolis from these trees. *P. nigra* flavonoids have almost the same composition as that of propolis, when the flavonoid composition of wide-spread *P. nigra* var. *italica* was different qualitatively and, especially, quantitatively from it. The data of *Populus* flavonoids are similar to those found by Wollenweber (20).

EXPERIMENTAL

The ir spectra were taken in KBr and uv spectra in MeOH; pmr spectra of DMSO-d_6 solutions were determined at 60 MHz and those of flavonoids **8** and **9** at 250 MHz. Mass spectra were measured at 70 eV by direct inlet. The analytical instrument was an Pye Unicam LC 3 liquid chromatograph, housing a reversed-phase column Partisil PXS/10/25 ODS, particle size 10 μm. The composition of the eluent was H_2O-MeOH-AcOH (60:75:5 or 65:30:5), monitored at 275 nm.

ISOLATION OF FLAVONOID MIXTURE.—Propolis (10 g) was cut into small pieces and extracted with boiling methanol (150 ml) for 1 h. The extract was filtered warm, concentrated at 50 ml, diluted with 10 ml water, and then extracted with petroleum ether (3 x 100 ml) and ethyl ether (3 x 100 ml). The last extract (4.85 g) contained almost all of the propolis flavonoids.

ISOLATION OF FLAVONOIDS.—A portion of the flavonoid fraction (2 mg) was purified by preparative hplc (water-methanol-acetic acid—65:30:5). Three peaks were obtained, and the corresponding fractions were evaporated. The residue was investigated by ms.

The main part of the flavonoid fraction of propolis (4.5 g) was chromatographed on a polyamide column (1:30—column I), eluted with $C_2H_4Cl_2$-MeOH-MeCOEt (1200 ml, 20:2:1; 1000 ml, 20:4:2; 600 ml, 20:6:3; 600 ml, 20:8:4; 500 ml, 20:10:5; 400 ml, 20:12:6). Fractions of 80 ml each were collected.

The second fractions was evaporated, and the residue after recrystallization from EtOH yielded 73 mg tectochrysin (**4**), mp 161-4°.

The fifth fraction was rechromatographed on a polyamide column (1:50), eluted with $CHCl_3$ containing 0.5 % MeOH. The obtained flavonoid fractions were rechromatographed on a silicagel column (1:100) with chloroform-ethyl acetate (10:1), and fractions of 40 ml each were collected. Fractions 3 and 4 were combined and the residue, after evaporation, was recrystallized from $CHCl_3$, yielding 12 mg of 2,5-dihydroxy-7-methoxyflavanone (**9**) with mp 167-170°.

The sixth fraction from column I was rechromatographed on a polyamide column (1:50) and eluted with $CHCl_3$ containing 2% MeOH. The fraction, enriched in flavonoids, was rechromatographed on a silica gel column (1:100), eluted with $CHCl_3$-MeOH (10:1), and fractions of 40 ml each were collected. The second fraction contained 600 mg of almost pure pinocembrin (**1**, mp 194-6° (acetone). 3,7-Dihydroxy-5-methoxyflavanone (**8**) (14 mg) was isolated from fractions 9 and 10 after evaporation and recrystallization of the residue from $CHCl_3$-EtOAc.

^{1}H-nmr (DMSO-d_6): 3.68 (3H, s); 4.26 (1H, d); 5 (1H, d); 5.27 (1H, s), 5.88 (1H, d); 6.02 (1H, d); 7.31 (3H, m); 7.41 (2H, m); ms (70 eV) *m/z* (%): 286 (M^+, 14), 257 (21), 179 (10), 167 (100), 166 (12), 152 (4), 149 (2), 138 (9), 120 (14), 91 (22), 77 (4).

The seventh fraction of column I was rechromatographed on a polyamide column (1:50), eluted with $CHCl_3$ containing 3% MeOH and fractions, containing flavonoids, combined. They were rechromatographed on a silica gel column (1:100), eluted with $CHCl_3$-EtOAc 8:1, and fractions of 40 ml each were collected. Fractions 6-9 were combined and chromatographed on a silica gel column (1:100), eluted with $CHCl_3$-EtOAc (5:1). The obtained flavonoid fractions were evaporated and the residue recrystallized from $CHCl_3$-EtOAc, yielding 160 mg of chrysin (**3**), mp 284-7°.

Fractions 8 and 9 from column I were chromatographed together on polyamide column (1:50), eluted with $CHCl_3$ containing 10% MeOH, and fractions of 60 ml each were collected. The tenth fraction was rechromatographed on a silica gel column (1:100), eluted with $CHCl_3$-EtOAc (10:1), and fractions of 50 ml each were collected. From the first two fractions, 105 mg galanging (**2**) were isolated, mp 220-222°.

Fractions 18-21 from column I were chromatographed on a polyamide column (1:50), eluted with $CHCl_3$ containing 3% MeOH. The obtained flavonoid fraction after recrystallization from EtOH yielded 18 mg of isorhamnetin (**6**) with mp 302-6°.

Fractions 27-31 from column I were chromatographed on a polyamide column (1:50) and eluted with $CHCl_3$-MeOH (3:1). Fractions, containing flavonoids were collected and, after recrystallization from EtOH, yielded 24 mg of kaempferol with mp 275-8°.

Fractions 35-43 from column I were rechromatographed on a polyamide column (1:50), eluted with $CHCl_3$-MeOH (5:2), and fractions of 60 ml each were collected. Fraction 7 was evaporated, and the residue, after recrystallization from EtOH, yielded 32 mg of quercetin (**5**) with mp 298-302°.

ACKNOWLEDGMENTS

The authors are grateful to UNDP (Project BUL 77/009) for the partial support of this work.

LITERATURE CITED

1. E.L. Chisalberti, *Bee World*, **60**, 59 (1979).
2. S. Nikolov, E. Georgieva, M. Koleva, V. Vasilev, S. Todorov, and S. Drjanska, "Mezd. Kongress Pchelov.," Grenoble 1975, Apimondia, Bucharest, 1976, p 235.
3. B. Hlada, M. Bylka, W. Ellnain-Wojtaszek, L. Skuzypezak, P. Szafarek, and L. Kovalski, *Arzneim.-Forsh.*, **30**, 1847 (1980).
4. "A remarkable hive product: Propolis," Apimondia, Bucharest, 1978.
5. V. Vilanueva, M. Barbier, M. Gonnet, and P. Lavie, *Ann. Inst. Pasteur*, **118**, 84 (1970).
6. J. Metzner, E. Schneidewind, and E. Friedfich, *Pharmazie*, **32**, 730 (1977).
7. M. Paintz and J. Metzner, *Pharmazie*, **34**, 839 (1979).
8. V. Bankova, S. Popov, and N. Marekov, First Int. Conf. Chem. Biotech. Biologically Active Nat. Prod., Varna 1981, Bulg. Acad. Sci., 1981, p 104.
9. V. Bankova, S. Popov, and N. Marekov, *J. Chromatogr.*, **242**, 135 (1982).
10. M. Vanhaelen and R. Vanhaelen-Fastre, *J. Chromatogr.*, **187**, 255 (1980).
11. D. Strack and J. Krause, *J. Chromatogr.* **156**, 359 (1978).
12. J. Fisher and T. Wheatan, *J. Agr. Food Chem.*, **24**, 898 (1976).
13. L. Wulf and C. Nagel, *J. Chromatogr.*, **116**, 271 (1976).
14. H. Kotake, T. Sakan and T. Kubota, *Chem. Ind.* (London), 1562 (1954).
15. M. Hauteville, M. Chadenson, and J. Chopin, *Bull. Soc. Chim. Fr.*, **II** 1803 (1975).
16. M. Chadenson, M. Hautville, and J. Chopin, *C.R. Acad. Sci. Paris, Ser. C*, **275**, 1291 (1972).
17. E. Wollenweber and K. Egger, *Phytochemistry*,, **10**, 225 (1971).
18. S. Popravko, "A remarkable hive product: Propolis," **15** (1978).
19. P. Lavie, *ibid.*, 41.
20. E. Wollenweber, *Biochem. Syst. Ecol.*, **3**, 35 (1975).

Received 24 May 1982