A Novel Caffeic Acid Derivative and Other Constituents of *Populus* Bud Excretion and Propolis (Bee-Glue)

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The bud exudates of *Populus nigra, P. trichocarpa,* and *P. grandidentata* × *tremuloides* were analyzed for the presence of phenolics and flavonoids. A novel natural product, caffeic acid γ,γ-dimethylallyl ester, was identified from *P. nigra,* along with further phenolics such as *p*-hydroxyacetophenone, dimethyl caffeic acid, cinnamoyl cinnamata and vanillin. The flavonoid aglycones correspond to those reported earlier. Propolis samples from the Sonoran Desert were shown to exhibit the flavonoid pattern that is typical for poplar bud exudates, thus confirming this material as the major source for bee-glue. Only a single flavonoid might originate from the leaf exudate of a desert shrub.

Introduction

Bud exudates of poplars were reported earlier to contain a great variety of flavonoid aglycones, depending on the species studied [1] and they also contain other phenolics, especially cinnamic acid derivatives. We want to report on the detection of further constituents and in particular on the structure identification of a novel caffeic acid ester. Propolis or bee-glue was included in this study as it is well known that poplar bud exudates are the most important source of this material for bees. We were particularly interested in using flavonoid analyses of propolis to confirm the precise source used by bees in the Sonoran Desert, Arizona.

Materials and Methods

Bud material of *Populus nigra* L. and *P. grandidentata* × *tremuloides* was collected from trees cultivated in the Botanischer Garten der TH Darmstadt; twigs of *P. trichocarpa* (Klon 608/52) were obtained from the Forschungsinstitut für Pappelwirtschaft, D-3510 Hann. Münden. The buds were briefly extracted with acetone to dissolve the lipophilic exudate material and the concentrated solutions were further analyzed by chromatographic methods as usual with silica, polyamide, and Sephadex LH-20 as adsorbents (*c.f.* [1, 2]). Most flavonoids were identified by direct comparisons with markers (*c.f.* [2]).

Propolis samples from the Sonoran Desert were obtained from the Carl Hayden Bee Research Center in Tucson, AZ, with the following information on their origins: 1) Silverbell Mts., Pima Co. (I-7-83), 2) Page Ranch, Pinal Co. (I-13-83), 3) Pima Canyon, Pima Co. (I-13-83), 4) Canyon del Obo Ranch, Pima Co. (I-17-83) and 5) Pima Co., site and date unknown. Samples 4 and 5 showed the most interesting flavonoid patterns and were, therefore, analyzed in detail. Portions of 5 g were cut in pieces, deep frozen overnight at −70 °C, ground in a coffee-mill and macerated at room temperature with 100 ml ethanol for 3 h while stirring. The ethanolic solution was separated from undissolved waxy material and insect carcasses by centrifugation and analyzed in the same way as the poplar bud excretions. Demethylation of 5-methyl galangin was with anilin-HCl according to Klemenc [3].

A compound isolated from the bud exudate of *Populus nigra* and designated LB-1 crystallized from benzene after addition of some petrol in whitish, glittering platelets, m.p. 119–120 °C. For spectral data of LB-1 (Fig. 1, **1**) and its derivatives (**2**–**4**), see Table I. 100 mg of LB-1 (**1**) in dry acetone were methylated with dimethyl sulphate in the presence of K_2CO_3 to yield 106 mg of the dimethyl ether (**2**). 30 mg of **1** were acetylated with acetic anhydride in the presence of pyridine to give 22 mg of the diacetate (**3**). 30 mg of the methylderivative **2**, dissolved in ethyl acetate, were hydrogenated in the presence

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of pre-reduced PtO_2 to yield a viscous yellow oil which on TLC exhibited two spots. 15 mg of the tetradihydro derivative (**4**) and 8 mg of 3,4-dimethyl caffeic acid were isolated by prep. TLC.

Mass spectra were recorded at 70 eV either on a Varian MAT 311A or on a Shimazu LKB 9000B equipped with computer; ^1H NMR spectra ($CDCl_3$/TMS) were recorded on a JEOL GX 400.

Results

Isolated Products

Product LB-1, isolated from the bud exudate of *Populus nigra*, formed a conspicuous spot with light blue fluorescence on polyamide TLC. This fluorescence as well as the base peak in the MS at *m/z* 180 provided evidence that it is a derivative of caffeic acid. The IR spectrum indicated the presence of OH-groups, an aromatic group, and a α,β-unsaturated ester group. Methylation of LB-1 gave a dimethyl ether (**2**) and acetylation yielded a diacetate (**3**), thus confirming that LB-1 has two OH-groups. The ^1H NMR spectrum exhibited the signals of a γ,γ-dimethylallyl group located between the benzene ring and carbonyl group, and three protons on the benzene ring. The UV-spectrum and the signal pattern of the aromatic region in the NMR spectrum of methylated LB-1 were strikingly similar to those of dimethyl caffeic acid (LB-2). Hydrogenation of methylated LB-1 gave the tetrahydroderivative $C_{16}H_{24}O_4$ (M^+ 280) and 3,4-dimethoxydihydrocinnamic acid, indicating LB-1 to be an allyl ester of caffeic acid. Alkaline hydrolysis of methylated LB-1 afforded γ,γ-dimethyl-allyl alcohol and 3,4-dimethoxy cinnamic acid (dimethyl caffeic acid). On

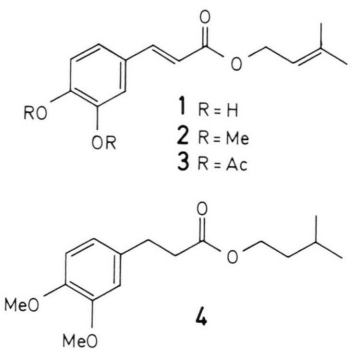

Fig. 1. Structural formulae of γ,γ-dimethylallyl caffeic acid (LB-1) and its derivatives.

Table I. Spectral data of γ,γ-dimethylallyl caffeic acid (LB-1) and its derivatives.

	LB-1 (**1**)	Methyl-LB-1 (**2**)	Acetyl-LB-1 (**3**)	Hydrogenated LB-1 (**4**)
UV λ_{max}^{MeOH}	330, 302, 245, 235 sh, 218	323, 294, 240 sh, 234, 217, 203	277, 218, 205	287, 280, 230, 205
IR ν_{max}^{nujol} [cm^{-1}]	3480, 3320, (OH), 1687 (C=C−COO−), 1640, (C=C), 1608, 1541, 1308, 1286, 1190, 1112, 982, 852, 818, 796	1710 (ester), 1635 (C=C), 1600, 1586, 1510 (Ph), 1422, 1338, 1308, 1260, 1209, 1160, 1140, 1025, 1010, 982, 845, 828, 810, 760	1770 (OAc), 1710 (ester), 1640 (C=C), 1610, 1590, 1510 (Ph), 1320, 1260, 1215, 1188, 1153, 1112, 1018, 997, 908, 875, 845, 810	1735 (ester), 1592, 1515, 1420, 1263, 1240, 1160, 1140, 1030, 850, 807, 770, 703
MS *m/z* [rel.int.]	248 (22%, M^+), 180 (100), 163 (77), 69 (20)	276 (29%, $C_{16}H_{29}O_4$), 208 (100), 193 (21), 191 (62), 69 (21), 41 (21)	332 (1%, $C_{18}H_{20}O_6$), 290 (13), 248 (44), 180 (100), 163 (44), 69 (47), 41 (42)	280 (25%, $C_{16}H_{24}O_4$), 209 (11), 167 (19), 165 (37), 151 (100)
^1H NMR [δ ppm]	7.59 (J=16, 1H), 6.93−7.30 (m, 3H), 6.23 (d, J=16, 1H), 5.41 (bt, J=8, 1H), 4.47 (d, J=8, 2H), 1.78 (bs, 6H)	7.75 (d, J=16, 1H), 7.22 (dd, J=9, 1H), 7.16 (bs, 1H), 6.59 (d, J=9, 1H), 6.14 (d, J=16, 1H), 5.50 (bt, J=8, 1H), 4.78 (bd, J=8, 2H), 3.98 (s, 6H, 2×OMe), 1.81 (bs, 6H)	7.70 (d, J=16, 1H), 7.33−7.4 (overl., 3H), 6.41 (d, J=16, 1H), 5.46 (bt, J=7, 1H), 4.74 (bd, J=7, 2H), 2.33 (s, 6H, 2×OAc), 1.80 (bs, 6H, −C=C $(CH_3)_2$)	6.81 (bs, 3H), 4.15 (t, J=7, 2H), 3.93 (s, 6H, 2×OMe), 2.80 (m, 4H, Ph−CH_2−CH_2−CO), 1.53 (m, 3H), 0.91 (d, J=7, 6H)

this spectral and chemical evidence the structure of LB-1 was established to be the γ,γ-dimethylallyl ester of caffeic acid.

A compound designated LB-2 forms whitish glittering platelets, m.p. 178–179 °C. UV λ_{max}^{MeOH} 312, 286, 229 nm. MS: *m/z* (rel.-int.) 280 (M^+, 100%), 193 (34), 161 (8), 147 (14), 133 (15), 121 (19), 119 (16), 105 (12), 103 (16), 91 (26). This product shows the same blue fluorescence as caffeic acid, but higher R_f. It was readily identified as dimethyl caffeic acid.

From *P. trichocarpa*, compd. LD-2 formed slightly impure crystals, m.p. 125–127 °C. UV λ_{max}^{MeOH} 276 (226) nm. M^+ at *m/z* 148 ($C_9H_8O_2$, base peak). This product was identified as cinnamic acid.

Compound LD-1 formed glittering colourless crystals, m.p. 108 °C, UV λ_{max}^{MeOH} 281 (220) nm. M^+ 136 ($C_8H_8O_2$), base peak at *m/z* 121 ($M-CH_3$). LD-1 is identical with *p*-hydroxy acetophenone.

A non-polar product Pt-1 that appeared as a light blue fluorescent spot on TLC was identified by direct comparisons as the benzyl ester of salicylic acid.

Product Pt-2, another non-polar constituent, was visible in UV_{366} as a faint yellowish spot. After spraying with Naturstoffreagenz A and exposure to UV-light it turned ochre. The product was isolated as a colourless oil with aromatic fragrance, M^+ 264. By MS, GC and TLC it was shown to be identical with cinnamoyl cinnamate (styracin), a compound that had been isolated earlier from the bud oil of *P. tristis* in a mixture with cinnamoyl benzoate (Wollenweber, unpubl.) and was identified by GC and MS comparisons with a synthetic sample (J. Favre-Bonvin, pers. comm.).

A flavonoid Az-4H, never found before in any *Populus* species, crystallized from some fractions of propolis sample #4 in yellow needles, m.p. 229 °C. UV λ_{max}^{MeOH} 334, 285; + $AlCl_3$ 361, 312, 286; $AlCl_3$ + HCl 355, 319, 286; + NaOH 393, 277; + NaOAc 386, 272; NaOAc + H_3BO_3 330, 278 nm. MS (rel. int.) 344 (59%, $C_{18}H_{16}O_7$, M^+), 329 (100), 211 (16), 183 (15), 69 (35). ^1H NMR (ppm) 10.28 (s, chelated OH), 7.80 and 6.93 (d each, A_2B_2-system), 4.04, 3.90, 3.88 (s, each 3H, $3\times OCH_3$). All these data indicated the structure to be 5,4′-dihydroxy-6,7,8-trimethoxy flavone. Direct comparisons with a sample of authentic xanthomicrol confirmed this identification.

A product AZ-5G, that appeared on TLC as a brilliant yellow spot, was isolated from propolis sample #5. It formed yellow crystals, m.p. 218 °C. UV λ_{max}^{MeOH} 350, 266; + $AlCl_3$ 408, 271, unchanged with HCl; + NaOH 400, 322, 277; + NaOAc 376, 325, 276 n. MS *m/z* (rel.int.) 284 (11%, M^+ for $C_{16}H_{12}O_5$), 270 (36), 193 (18), 167 (100), 138 (32). ^1H NMR (ppm) 8.26 and 7.64 (m, 5H, unsubstituted B-ring), 6.66 and 6.51 (d, 2H, each; H-6 and H-8), 3.96 (s, 3H; $-OCH_3$). The brilliant yellow fluorescence of the spot indicates that this compound is a 5-deoxy- or 5-methoxy flavone or -flavonol, and the NMR spectrum showed the latter possibility to be the correct one. Demethylation yielded galangin, thus the product is identified as galangin-5-methyl ether.

Product distribution

From the bud exudate of *Populus nigra* we identified the following flavonoids: chrysin, chrysin-7-methyl ether (tectochrysin), apigenin, galangin, galangin-7-Me, kaempferol-7-Me, quercetin, quercetin-7-Me and quercetin-3,7-diMe, pinocembrin, pinocembrin-7-Me, 2,5-dihydroxy-7-methoxy flavanone and 2′,6′-dihydroxy-4′-methoxy chalcone. The most interesting constituent is LB-1, the γ,γ-dimethylallyl ester of caffeic acid. As far as we know it has not been reported before from any natural source. Compound LB-2 was identified as dimethyl caffeic acid, while LB-3, the third spot with blue fluorescence, is a mixture of four cinnamic acid derivatives. According to preliminary MS and NMR studies it is assumed that two of these are the dimethylallyl esters of ferulic acid and of isoferulic acid, respectively. Caffeic acid was also present in this species' bud exudate.

In the bud exudate of *P. trichocarpa* we identified apigenin-7,4′-dimethyl ether, galangin, galangin-7-Me, 2′,6′-dihydroxy-4′-methoxy chalcone and 2′,6′-dihydroxy-4′-methoxy dihydrochalcone as well as the benzyl ester of salicylic acid by TLC comparisons with markers. *p*-Hydroxy-acetophenone, cinnamic acid and cinnamoyl cinnamate (styracin) were isolated and confirmed by their respective m.p., MS, and GC data.

Flavonoids identified in *P. grandidentata* × *P. tremuloides* are apigenin, apigenin-7-Me, and apigenin-4′-Me. Salicyl benzoate was also identified by chromatographic comparisons, while two further aromatic compounds were obtained in crystalline form and identified as benzoic acid and vanillin, respectively.

The flavonoid patterns observed with TLC of the five propolis samples from Arizona are very similar to those exhibited by bud excretions of certain *Populus* species [1]. The two samples that were analyzed extensively both contain the following flavonoid aglycones: chrysin, chrysin-7-Me, galangin, galangin-3-Me, galangin-5-Me, galangin-7-Me, kaempferol, kaempferol-7-Me, kaempferol-3,7-diMe, quercetin-3-Me, quercetin-7-Me, quercetin-3′-Me, quercetin-3,7-diMe, quercetin-3,3′-diMe and quercetin-7,3′-diMe. 5,4′-Dihydroxy-6,7,8-trimethoxy flavone (xanthomicrol) was only found in sample # 4, and galangin-3,7-diMe, kaempferol-5-Me and quercetin-3,7,3′-triMe were found in sample # 5 only.

Discussion

The results presented here originate from students' work executed in the senior author's laboratory in 1978 (U.L., *P. nigra, P. trichocarpa*), 1980 (D.S., *P. grandidentata* × *tremuloides*) and 1984 (H.W., propolis). Publication in particular of the structure of product LB-1, already determined by one of us (Y.A.) in 1978, is deemed necessary as this product is now gaining considerable interest. It is most likely responsable for an increasing number of contact allergies in bee-keepers and in persons using propolis preparations externally. Sensitizing experiments with guinea pigs confirmed these findings (4). The γ,γ-dimethyl ester of caffeic acid (LB-1) (**1**), isolated from bud exudate of *Populus nigra* and reported here for the first time as a natural product, is present in propolis samples from central Europe, while we did not observe it in the samples from Arizona. In a just published GC/MS study on phenolic propolis constituents, Bankova *et al.* [5] mention that they assume esters of disubstituted cinnamic acids with monosubstituted C_5-alcohols to be present in Bulgarian propolis.

With LB-1, identified as γ,γ-dimethylallyl ester of caffeic acid and LB-2 as dimethyl caffeic acid we now know two more of the blue fluorescent spots observed in the earlier work on *Popolus* section *Leuce* and also in *P. nigra* and others [1], in addition to caffeic acid itself and to the previously reported lasiocarpins from *P. lasiocarpa* [6]. 2-Acetyl-1,3-diferuloylglycerol and 2-acetyl-3-*p*-coumaroyl-1-feruloylglycerol, reported by Popravko *et al.* [7] to occur in buds of *P. tremula* as well as in propolis, must also be mentioned in this context. Caffeic acid, dimethyl caffeic acid, ferulic acid and isoferulic acid were reported to occur in *Populi gemma* (= poplar buds as drug) and in propolis by Papay *et al.* [8, 9]. These authors also found the benzyl ester and the cinnamoyl ester of caffeic acid and, for the first time, the phenylethyl ester of caffeic acid in both materials. Such aromatic products contribute to the fragrance of some *Populus* bud oils and of warm propolis, as do vanillin, salicyclic acid benzyl ester, and cinnamoyl cinnamate. Popravko *et al.* [10] reported coniferyl benzoate and *p*-cumaroyl benzoate as propolis constituents. Vanillin and isovanillin were found as propolis constituents already in 1911 [11]. *p*-Hydroxy-acetophenone and salicylic acid benzyl ester were reported some decades ago from buds of *P. trichocarpa* [12]. Cinnamic acid and cinnamyl alcohol were reported in the same period from hydrolyzed etherical extract of *P. balsamifera* buds [13]. The cinnamyl alcohol probably originated from cinnamoyl cinnamate (styracin), a product we now report for *P. trichocarpa*. We assume that it is a major component of the characteristic fragrance of *P. balsamifera* and other species with aromatic scent. In the earlier chemotaxonomic studies on eighty taxa of *Populus* [1] emphasis was on TLC analysis of flavonoid aglycones which are visible in UV_{366} before and after spraying with Nat. A. Some of the now identified phenolics are visible only in UV_{254} and mostly concealed by the many flavonoid aglycones present in poplar bud exudates and had, therefore, not been considered.

A spot with brilliant yellow fluorescence, on the other hand, had raised our attention earlier and was noted as unknown "G" in 1975 [1]. We never had been able to isolate it for analysis. Strangely enough this was now possible from propolis sample # 5 from Arizona. The bees probably collected their material from a poplar species that produces this flavonol in better amount than the species we had studied. Galangin-5-methyl ether was reported recently by Papay *et al.* for *Populus gemma* and for propolis [8]. Unfortunately the authors do not cite the *Populus* species from which the "gemmae" were collected. We had noted its presence earlier – marked by "G" – in *P. deltoides, P.* × *euramericana, P. candicans,* and the intersectional hybrids *P. deltoides* × *P. Simonii, P. jackii* and *P.* × *generosa* [1]. The isolation of galangin-5-methyl ether from propolis sample # 5 did not only allow us to precisely identify the "G".

This flavonol is also identical with a previously unidentified constituent of the farinose frond exudate of the fern, *P. triangularis* var. semipallida. It should also be mentioned that naringenin-7-methyl ether, now isolated from *P. grandidentata* × *tremuloides*, corresponds to the product cited as "U" (unknown) for *P. tremuloides* in [1].

In his 1979 review on propolis, Ghisalberti [14] had listed the flavonoids and other phenolics so far known to occur in propolis. From this survey it became quite clear that the major source for these compounds and hence for propolis itself must be poplar bud exudate, a fact that was known already to *Plinius sec.* (Historia naturalis). The flavonoid pattern in both materials is confusingly alike, even more so when viewed on TLC than when presented in tables. Also several compounds that have so far only been found in poplar buds (*e.g.* pinobanksin-3-acetate, ref. [15, 16]) offer a good proof. Other flavonoids reported by several authors, *e.g.* pectolinarigenin, kaempferol-3,4′-dimethyl ether, and naringenin-4′-methyl ether, seem to indicate that bud exudates of *Alnus* and *Betula* species [17] may be collected in addition, as are further resinous or waxy plant materials (*c.f.* [14]). An increasing number of recent publications, particularly from socialist countries, confirm these findings (see *e.g.* [5, 8]), whereas reports on propolis flavonoids and coumarins that would prove the use of *Prunus* and *Fraxinus* bud exudates, for example, are still missing. Asteraceae from arid and semiarid regions were shown recently to exhibit a variety of flavonoid aglycones in their leaf resins (see *e.g.* [18, 19]). Therefore we had expected to find in our propolis samples from the Sonoran Desert, where *Populus* is rare, flavonoid aglycones from Asteraceae or other desert shrubs. Surprisingly enough we also found in the desert samples the typical flavonoid patterns of poplars as characterized by the presence of chrysin, tectochrysin, galanging and its 3-, 5-, and 7-methyl ethers. Only one flavonoid was encountered that can by no means be derived from *Populus*, namely xanthomicrol (5,4′-dihydroxy-6,7,8-trimethoxy flavone). This flavone was found recently in the leaf resin *e.g.* of two *Baccharis* species (*c.f.* [2]) and of *Ambrosia deltoidea* [20] and its presence in propolis might, therefore, point to such a source.

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