External Accumulation of Biflavonoids on Gymnosperm Leaves

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Biflavonoids are well known to occur in many Gymnospermae. Here we report on their occurrence in epicuticular material of a number of species. Several compounds are characterized by detailed NMR studies and FAB-MS. They are identified as amentoflavone, bilobetin, podocarpus-flavone A, sciadopitysin, dihydrosciadopitysin and cupressuflavone.

Introduction

Monomeric flavonoids normally occur as glycosides in plant tissues, dissolved in the cell sap, hence localized in the vacuoles. When flavonoid aglycones accur as such, they are mostly accumulated externally, on plant surfaces. They are produced preferentially by plants exhibiting secretory structures, and they are encountered in many plants living in or originating from arid and semiarid habitats (Wollenweber, 1994). Such occurrences are found more and more often, provided the localisation of aglycones is considered in phytochemical studies (Wollenweber, 1996). Biflavonoids, on the other hand, have hardly ever been encountered as glycosides (*Psilotum;* Markham, 1984), they normally occur as aglycones (Geiger and Quinn, 1988; Geiger, 1994). These were so far regarded as being constituents of plant tissues. The question arose, however, if they can also be eliminated from the tissue and deposited externally on leaves and stems. Biflavonoids are particularly abundant in Gymnospermae, but so far only one report dealt with the occurrence of biflavonoids in the outer epidermal cell wall and in the cuticle of *Agathis robusta* (Gadek *et al.,* 1984). We have, therefore, studied fresh leaves of several species of conifers for the presence of biflavonoids in epicuticular materials.

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Material and Methods

Twigs of *Cupressus torulosa* (Acc.No. 2137), *Gingko biloba* (Acc.No. 1446), *Sciadopitys verticillata* (Acc.No. 2053), *Sequoiadendron giganteum* (Acc.No.1578), *Taxus baccata* (Acc.No. 2481) and *Thuja plicata* (Acc.No. 1683) were taken from trees cultivated in the Botanischer Garten der TU Darmstadt. Vouchers are kept in the Herbarium of this institution.

Fresh leaves (on twigs) were very briefly rinsed with acetone to dissolve epicuticular material. The solutions were evaporated to dryness under reduced pressure and the residues were redissolved in a small amount of boiling MeOH, cooled to – 18° and centrifugated to eliminate the major part of "fatty" constituents. The supernatant was chromatographed over Sephadex LH-20, eluted with MeOH, to separate the phenolic portion from terpenoid material. Flavonoids were fractionated by chromatography on acetylated polyamide (SC-6 Ac, Macherey-Nagel), eluted with toluene and increasing amounts of MeCOEt and MeOH. Fractions were monitored by TLC on polyamide (DC-11, Macherey-Nagel) with the solvents: A) toluene – petrol$_{100-140}$ – MeCOEt – MeOH 12:6:2:1, v/v/v/v B) toluene – dioxane – MeOH 8:1:1 and C) toluene – MeCOEt – MeOH 12:5:3 v/v/v on silica (Polygram SIL G/UV$_{254}$, Macherey-Nagel) with the solvents D) toluene – MeCOEt 9:1 and E) toluene – dioxane – HOAc 18:5:1 v/v/v. Chromatograms were viewed under UV$_{366}$ before and after spraying with Naturstoffreagenz A (NA). Monomeric flavonoids as well as the biflavones ginkgetin and isoginkgetin were iden-

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tified by direct comparison with authentic samples. Several fractions were further chromatographed using prep. TLC (SIL-G-25 $UV_{254+366}$). Others were subjected to prep. HPLC (Lichrospher 100 RP 18, 5 μm, Merck; 250 x 4 mm; Nucleosil 100 RP 18, 7 μm, Macherey-Nagel; 250 x 10 mm; Bondapak RP 18, 10 μm, Waters, 300 x 19 mm. Elution was with MeOH / 2% aq. HOAc-mixtures). Final purification was achieved by CC on Sephadex LH-20 using 80% aq. MeOH or MeOH as solvent.

NMR spectra were recorded at 400 MHz (1D) and at 500 Mhz, repsectively (2D: NOESY and ROESY; using NOESY we did not observe a reciprocal enhancement on irradiation of H-5' and 4'-OMe as expected for **2**, **4** and **5**; these NOE's were only visible in the respective ROESY spectrum). Mass spectra were recorded using FAB-MS (3–7 keV, glycerol as matrix). In the case of **2** and **3** the $[M-H]^-$-peak at *m/z* 551 corresponded to an ion of the glycerol matrix ($[(glycerol)_6-H]^-$). To our experience, however, the glycerol matrix peaks (e.g. *m/z* 275, 367, 459, 551 etc.) permanently decrease from lower to higher masses in a FAB mass spectrum, i.e. the respective parent ions at *m/z* 551 of **2** and **3** which represented the base peaks during some scans really indicated the respective compound.

Compound **1**: UV (λ_{max} nm, MeOH): 269, 335. ^{13}C NMR (DMSO-d_6): δ 181.7, 181.5, 164.1, 163.9, 163.1, 162.1, 161.3, 160.7, 160.4, 157.2, 154.5, 131.2, 128.0, 126.9, 121.7, 121.5, 119.2, 117.7, 115.5, 105.4, 103.5, 102.5, 102.4, 102.3, 100.1, 98.7, 93.8.

Compound **2**: UV (λ_{max} nm, MeOH): 270, 333. ^{13}C NMR (DMSO-d_6): δ 181.8, 181.6, 164.6, 163.3, 162.6, 161.4, 161.0, 160.5, 160.4, 157.4, 154.3, 130.9, 127.9, 122.4, 121.9, 121.2, 115.7, 111.6, 103.6, 103.2 102.4, 98.9, 98.7, 94.1, 55.8.

Compound **3**: UV (λ_{max} nm, MeOH): 270, 332. No ^{13}C NMR data obtained.

Compound **4**: UV (l_{max} nm, MeOH): 272, 330. ^{13}C NMR (DMSO-d_6): δ 181.9, 181.8, 165.1, 163.6, 162.8, 162.1, 161.1, 160.5, 157.3, 154.3, 130.8, 128.1, 127.6, 122.8, 122.3, 121.9, 114.4, 111.7, 104.7, 103.7, 103.6, 103.1, 98.8, 98.0, 92.6, 55.9, 55.8, 55.4.

Compound **5**: UV (λ_{max} nm, MeOH): 278, 325. ^{13}C NMR (DMSO-d_6; doubling of many signals observed): δ 196.7, 181.8, 167.3, 163.1, 162.8, 162.1, 160.3, 157.6, 154.2, 131.4, 131.0, 129.9, 129.8, 127.8, 122.8, 121.0, 114.4, 111.1, 111.0, 104.4, 103.2, 102.9, 102.5, 98.7, 94.6, 93.7, 78.4, 55.7, 55.4, 42.2, 42.0.

Compound **6**: UV (λ_{max} nm, MeOH): 274, 329. ^{13}C NMR (DMSO-d_6): δ 181.9, 163.3, 160.9, 160.8, 154.7, 127.8, 121.2, 115.7, 103.4, 102.5, 99.1.

Results and Discussion

Flavonoid identification

Structure elucidation of the biflavonoids **1–6** could readily be performed with the help of their ^1H NMR (Table I) and NOESY or ROESY spectra, together with the results from FAB-MS. Although they all are known products, we report their ^1H NMR data in Table I because literature data, if existant at all, differ significantly from ours (Δ 0.1–0.3 ppm; probably due to different amounts of water in the samples) or are incomplete. Moreover we present exact assignments for the OMe groups of the polymethoxylated derivatives **4** and **5**. Compounds **1–5** all exhibited the same spin patterns with regard to rings A and B of the flavonoid monomers: two *meta*-coupled protons for H-6 and H-8 of an A-ring (not resolved with **2**), and a singlet for H-6 or H-8, respectively, of the second A-ring. The B-rings could be characterized by a 1,3,4- and a 1,4-substitution pattern. Except for **5**, the C-rings were of the flavone type (singlet for H-3 and H-3'' respectively). One C-ring of **5** exhibited the NMR spectroscopic features of a 2,3-dihydroflavone moiety. Moreover we observed signals for a H-bonded 5-hydroxyl in each flavonoid monomer. In addition, four biflavonoids were also substituted with one (**2** and **3**) or three methoxyl groups (**4** and **5**), respectively.

These features together with the mass spectra of **1–5** suggested biflavonoids of the 3',6''- (= robustaflavone type) or 3',8''-biapigenin type (= amentoflavone type). The chemical shifts of the II B-ring resonances of the isolated compounds were consistent with the second alternative (amentoflavone type) according to Markham and Geiger (1994).

Biflavonoid **1** was identified as amentoflavone ($[M-H]^-$ at *m/z* 537, $C_{30}H_{18}O_{10}$), also with the help of literature data (Markham and Geiger, 1994; Markham *et al.*, 1990) and co-chromatography with an authentic marker.

Table I. ^{1}H-NMR data of **1–6** (400 MHz, DMSO-d_6). Assignments according to (Markham and Geiger, 1994) and by results from NOESY and/or ROESY; the assignments for H-3/H-3″ and OH-5/OH-5″ may be exchangeable.

H	1	2	3	4	5	6
2	–	–	–	–	5.61 dd*	–
3	6.78 s	6.88 s	6.85 s	7.00 s	3.44 dd* 2.78 dd*	6.76 s
6	6.15 d (2.1)	6.17 s	6.16 d (2.0)	6.35 d (2.2)	6.06 d*	6.40 s
8	6.36 d (2.1)	6.46 s	6.37 d (1.8)	6.79 d (2.2)	6.10 d*	–
2′	8.12 d (2.4)	8.04 brs	8.05 d (2.2)	8.07 d (2.4)	7.49 d*	7.45 d (8.7)
3′	–	–	–	–	–	6.72 d (8.8)
5′	6.99 d (8.6)	7.32 d (8.8)	7.07 d (8.5)	7.35 d (8.9)	7.20 d (8.6)	6.72 d (8.8)
6′	7.93 dd (2.4/8.7)	8.15 brd (8.6)	7.96 dd (2.4/8.6)	8.21 dd (2.5/8.8)	7.56 dd*	7.45 d (8.7)
3″	6.72 s	6.76 s	6.80 s	6.87 s	6.86 s	6.76 s
6″	6.19 s	6.35 s	6.29 s	6.37 s	6.34 s	6.40 s
2‴, 6‴	7.61 d (8.8)	7.49 d (8.2)	7.69 d (9.0)	7.59 d (9.0)	7.62 d*	7.45 d (8.7)
3‴, 5‴	6.62 d (8.8)	6.70 d (8.2)	6.85 d (8.6)	6.92 d (9.0)	6.98 d*	6.72 d (8.8)
OH-5	13.02 s	12.91 s	12.99 s	12.91 s	12.09 s	13.15 s
OH-5″	13.14 s	13.07 s	13.08 s	13.03 s	13.01	13.15 s
OMe-7	–	–	–	3.82 s	3.75 s	–
OMe-4′	–	3.77 s	–	3.78 s	3.71 s	–
OMe-4‴	–	–	3.70 s	3.75 s	3.80 s	–

* Coupling constants not exactly determinable due to doubling of signals.

Compound **2** showed an additional OMe-group (d_H 3.77, d_C 55.8; $[M-H]^-$ at *m/z* 551, $C_{31}H_{20}O_{10}$) which could be located at C-4'. This was proven by irradiation of the OMe-group, thus enhancing the doublet at *d* 7.32 (H-5') and *vice versa*. Compared to **1** ($d_{H\text{-}5'}$ 6.99), H-5' of **2** was shifted downfield by ca. 0.3 ppm which was also a hint for a monomethyl formulation at 4'-OH. Thus **2** represents bilobetin (4'-OMe-amentoflavone).

The structure of **3** was elucidated as podocarpusflavone A (4‴-OMe-amentoflavone). The $[M-H]^-$-peak also appeared at *m/z* 551 as with **2**. The methoxyl group (d_H 3.70) was assigned to C-4‴ because irradiation of the signal at *d* 3.70 raised the doublet at *d* 6.85 (H-3‴,5‴) and *vice versa*. Moreover our ^{1}H NMR data are in good agreement with those in (Hiermann *et al.,* 1996).

In the ^{1}H NMR spectrum of **4**, three methoxy groups were visible. The ROESY spectrum was useful in locating these substituents at C-7, C-4' and C-4‴. This also coincided with downfield shifts of the respective *ortho*-positioned protons compared to the ^{1}H NMR data of non-methoxylated **1**. Compound **4** is thus identical with sciadopitysin, which is also supported by FAB-MS ($[M-H]^-$ at *m/z* 579, $C_{33}H_{24}O_{10}$).

Biflavonoid **5** turned out to be 2,3-dihydrosciadopitysin ($[M-H]^-$ at *m/z* 581, $C_{33}H_{26}O_{10}$). Location of the 2,3-dihydro moiety in the I C-ring was evident from the presence of two *meta*-coupled resonances with nearly the same chemical shift (*d* 6.06 = H-6 and *d* 6.10 = H-8), being typical for a phloroglucinol type A-ring (Markham and Geiger, 1994). Hence the monomer with the 2,3-dihydro part could be linked to the second monomer only via its B-ring. As in the case of **4**, the ROESY spectrum allowed the assignments of the three methoxyl groups at C-7, C-4' and C-4‴. This was again paralleled by significant downfield shifts of H-6 (*d* 6.06), H-8 (*d* 6.10), H-5' (*d* 7.20) and H-3‴,5‴ (*d* 6.98) compared to non-methoxylated 2,3-dihydroamentoflavone ($d_{H\text{-}6}$ 5.89, $d_{H\text{-}8}$ 5.89, $d_{H\text{-}5'}$ 7.02, $d_{H\text{-}3''',5'''}$ 6.78; data from Markham and Geiger, 1994).

Compound **6** with $[M]^-$ at *m/z* 538 showed only a single set of signals in its ^{1}H and ^{13}C NMR spectra. The molecular mass and the NMR data (see Table 1 and Experimental) allowed us to identify **6** as cupressuflavone (8,8"-biapigenin). The interflavonyl linkage between C-8 and C-8" of the two apigenin monomers (and not between C-6 and C-6") was evident from the ^{13}C NMR spectrum. The resonance visible at δ 99.1 was typical for a tertiary C-6 and C-6" respectively, whereas C-8 and C-8" were shifted downfield from about δ 93 (typical chemical shift for C-8 of apigenin) to more than 100 ppm (δ 102.5 or 103.4, see Experimental; exact assignment unclear as no HETCOR spectra were recorded) due to the interflavonyl bond.

Flavonoid distribution

So far bulk material of six species has been worked up to isolate the above biflavonoids for analysis.

In *Cupressus torulosa* D. Don., the leaf exudate shows a big portion of terpenoid material. From the phenolic portion we isolated amentoflavone, cupressuflavone and podocarpusflavone, and we noticed traces of apigenin and luteolin. Hinokiflavone seems also to be present, although its identification is somewhat questionable. A very unpolar flavonoid constituent is observed in trace amount only, thus far not allowing its isolation and identification.

From the acetone wash of *Ginkgo biloba* L. leaves we isolated ginkgetin, isoginkgetin, bilobetin, sciadopitysin and dihyrosciadopitysin. Polar fractions contained apigenin, kaempferol, quercetin, isorhamnetin, and traces of kaempferol- and quercetin-3-O-glycosides. Bilobetin, ginkgetin, isoginkgetin and sciadopitysin are well known constituents of *Ginkgo* leaves, and so is amentoflavone, which was not detected in this study. Dihydrosciadopitysin was so far known as a constituent of *Podocarpus* leaves (Roy *et al.,* 1987). To our knowledge this is the first report on its occurrence in *Ginkgo biloba*. It should be mentioned here that exudate biflavonoids can be obtained from fresh green leaves of the maidenhair-tree as well as from its yellow automn leaves, even from those already fallen to the ground.

Sciadopitys verticillata (Thunb.) Sieb. et Zucc. exhibits isoginkgetin, podocarpusflavone A, and traces of amentoflavone and cupressuflavone.

The epicuticular material of *Sequoiadendron giganteum* (Lindl.) Buchholz exhibits amentoflavone, cupressuflavone, isoginkgetin and podocarpusflavone A. For three or four unknown components we cannot say with certainty whether they are biflavonoids or not.

In the acetone wash obtained from *Taxus baccata* L. needles the terpenoid portion is rather low. We found ginkgetin and sciadopitysin as major biflavonoids, along with some amentoflavone and bilobetin. A further flavonoid spot with similar Rf as bilobetin has not yet been identified.

Thuja plicata Donn ex D.Don. exhibits amentoflavone as the major biflavone, accompanied by hinokiflavone and several so far unidentified flavonoids.

Some 30 further species from various genera cultivated in the Botanical Garden of the TU Darmstadt have been checked for the presence of externally accumulated biflavonoids by TLC. Since we are aware of the fact that the results have to be taken with a pinch of salt, as the possible presence of isomeric products must be taken into account, we here renounce to the report of specific compounds. We want to state, however, that biflavonoids are unquestionably present on the leaf surfaces of *Agathis robusta, Calocedrus decurrens, Cephalotaxus harringtonia, Chamaecyparis spp., Cupressocyparis leylandii, Cupressus spp., Sequoia sempervirens, Taiwania cryptomerioides,* and *Thuja spp.* Traces of biflavonoids were also detected on *Araucaria bidwillii* and on *Cunninghamia lanceolata*, whereas acetone washs of *Juniperus chinensis, J. communis, J. sabina* and *J. squamata* did not show any traces of biflavonoids. For *Juniperus* this is surprising since amentoflavone, cupressuflavone and hinokiflavone were reported as constituents of *Juniperus drupacea* leaves (in extracts made with chloroform and with ethylacetate) (Sakar and Friedrich, 1984), and hinokiflavone, podocarpusflavone A and robustaflavone were isolated from the pseudoberries of *Juniperus communis* (methanolic extract), along with several flavonoid glycosides (Hiermann *et al.,* 1996).

To our knowledge there exists only one paper so far that deals with the localisation of biflavonoids in Gymnosperm leaves. Gadek *et al.* (1984) analyzed the localisation of biflavonoids in leaf sections of *Agathis rubusta,* using aluminium-chloride induced fluorescence. Representatives of all orders of Gymnosperms were also checked. According to these authors' findings, the biflavonoids are confined to the outer periclinal wall and anticlinal walls of the epidermal cells, and to the cuticle in particular. They confirmed this result by demonstrating the presence of biflavonoids in cuticular scrapings. It is very unlikely, however, that biflavonoids should be extracted from the cuticle or even from cell walls when leaves are rinsed with acetone for only a few seconds. Our present results indicate, therefore, that at least part of the biflavonoids are excreted by the epidermis and deposited on the leaf surface. The fact that acetone rinses of *Juniperus* species did not exhibit any biflavonoids, whereas such have been reported from extracts (Sakar and Friedrich, 1984), also is in favour of

the external localization in those species where we found them. Also these acetone rinses do not exhibit traces of chlorophylls and carotenoids, thus proving that they do not contain tissue constituents. On the other hand, exctracts obtained with acetone/methanol e.g. from *Ginkgo biloba* leaves that have been rinsed with acetone beforehand, still yield biflavonoids. We conclude that these compounds, due to their partly hydrophilic, partly lipophilic nature, can be localized in the leaf tissue (including outer cell walls and cuticle) as well as on the leaf surface.

A recent paper by Wilhelmi and Barthlott (1997) deals with the micromorphology of epicuticular waxes in Gymnosperms. The authors report various tubular fine structures, sometimes restricted to stomata lines, for the epicuticular material on *Ginkgo* (Ginkgoaceae), *Cupressus* and *Thuja* (Cupressaceae), *Sciadopitys* (Sciadopityaceae), and *Sequoiadendron* (Taxodiaceae), in the two latter genera combined with scale-like structures. These observations further support the above assumption that the biflavonoids we detected in acetone rinses form part of the epicuticular material. They seem to have no influence, though, on the micromorphology of the epicuticular waxes.

The observation that considerable amounts of biflavonoids are deposited externally on Gymnosperm leaves and can hence readily be obtained by rinsing leave material with organic solvents, might be of practical interest: the biologically active biflavonoids of *Ginkgo biloba* could thus probably be isolated easier and, therefore, at lower costs than from leaf extracts.

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