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Naphthalene glucoside and other phenolics from the shoot and callus cultures of *Drosophyllum lusitanicum*

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Abstract

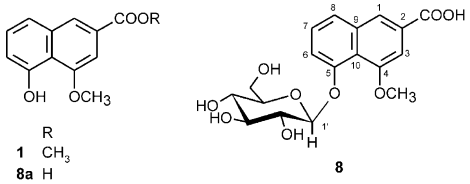
The callus and, for the first time established, shoot cultures of *Drosophyllum lusitanicum* Link. (Droseraceae) yielded new naphthalene glucoside-5-hydroxy-4-methoxy-2-naphthalenecarboxylic acid 5-*O*-β-glucoside (drosophylloside) and 5-hydroxy-4-methoxy-2-naphthalenecarboxylic acid methyl ester besides other phenolics like naphthalenes-5-hydroxy-4-methoxy-2-naphthalenecarboxylic acid (ancistronaphthoic acid B), hydroplumbagin 4-*O*-glucoside, naphthoquinones-plumbagin and 3-chloroplumbagin, *C*-glycosylflavones-vitexin, isovitexin, orientin and isoorientin. The pattern of phenolics found supports affinity of *Drosophyllum* to the families—Droseraceae, Ancistrocladaceae and Dioncophyllaceae.

Keywords: *Drosophyllum lusitanicum*; Droseraceae; In vitro cultures; Phenolic compounds; Chemotaxonomy; Naphthalene glucoside; Naphthalenes; Naphthoquinones; *C*-Glycosylflavones; Drosophylloside

1. Introduction

Drosophyllum lusitanicum Link.—a monotypic species of the family Droseraceae—is a carnivorous sub-shrub native to the southern part of the Iberian peninsula and northern Morocco (Juniper et al., 1989). The compounds found so far in this species are flavonoids—luteolin, leucocyanidin, leucodelphinidin (Juniper et al., 1989) and a naphthoquinone—plumbagin (Zenk et al., 1969); this latter is considered a taxonomic marker for the family Droseraceae (Culham and Gornall, 1994) and a compound of a broad spectrum of biological and pharmacological properties (Gujar, 1990). Previous studies on *D. lusitanicum* have been concerned with investigations of the biosynthetic pathway of plumbagin (Durand and Zenk, 1974) and with the high-yield production of this compound in the cell and organ cultures (Nahalka et al., 1996a,b, 1998).

Here we present the isolation and identification of naphthalenes (**1**, **8**, **8a**, **9**)—including a new naphthalene glucoside (**8**) and naphthalenecarboxylic acid methyl ester (**1**), naphthoquinones (**2**,**3**) and flavonoids (*C*-glycosides) (**4–7**) from the callus tissue and from the first time described shoot cultures of *D. lusitanicum*. The pattern of phenolic compounds found supports recent findings concerning the taxonomic relationships of the genus *Drosophyllum* (Bringmann et al., 2000a).



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2. Results and discussion

Seeds of *Drosophyllum lusitanicum* germinated on 1/4 MS medium after 3–6 weeks. The growth of plants with morphology typical of the species, was quick at the beginning. Reculture of seedlings to the same medium caused necrosis of 50% of the shoots and development of callus tissue at the base parts of shoots. The callus, green at the beginning, turned dark and then black after 2–3 weeks, at the point of contact with the medium. Not all cells inside such callus were necrotic, they formed, from time to time, merystematic centers that differentiated into shoot buds or globules of green callus. Nahalka et al. (1996a,b) noticed similar reactions of callus transferred to liquid medium. They described suspension cultures, which contained up to 40% of plasmolyzed cells, but the concentration of plumbagin in the cells correlated, in principle, with the amount of black aggregates in suspension. Calluses established on MS medium with 2.3×10^{-6} M of kinetin could be divided into two types: green and black. Green callus was composed of actively dividing cells which were able to regenerate shoot buds or callus. Black callus was built mainly from plasmolysed cells, but also from vital cells, that do not loose the ability to produce merystematic centers. Experiments on the formulation of a media composition that decreases the necrosis of the shoots and callus are in progress.

Preliminary analyses of pre-fractionated methanolic extracts by 1D TLC on silica gel ($CHCl_3$ fractions) and 2D TLC on cellulose (H_2O fractions) suggested similar composition of phenolic compounds in the shoots and the green callus tissue and poor phenolics content of in the black callus. Therefore, the latter was not investigated further. Subsequent preparative chromatography yielded the same compounds from each type of plant material—compounds **1**–**3** and **8a** from $CHCl_3$ fractions and compounds **4**–**9** from H_2O fractions.

Structure elucidation was mainly performed on larger samples from callus tissue, while identity with the samples isolated from shoots was assured by TLC and UV. Known compounds were identified by comparisons of their spectral data with those reported previously as: naphthoquinones–plumbagin (**2**) (co-TLC = TLC with authentic sample, UV), chloroplumbagin (**3**) (coTLC, UV, EIMS, HREIMS) (Budzianowski, 1996; Pakulski and Budzianowski, 1996); naphthalene (or hydronaphthoquinone)—hydroplumbagin 4-*O*-glucoside (**9**) (^{1}H and ^{13}C NMR in DMSO-d_6, UV, coTLC, β-glucosidase hydrolysis to plumbagin—coTLC) (Kreher et al., 1990; Budzianowski, 1996), and flavonoids (*C*-glycosylflavones)—orientin (**4**), vitexin (**5**), isoorientin (**6**), isovitexin (**7**) (UV with diagnostic shift reagents, ^{1}H and ^{13}C NMR—the latter not made for **4,** identification of which was completed by Wessely-Moser acid isomerization to **6** detected by co-TLC) (Mabry et al., 1970; Markham and Chari, 1982; Markham and Geiger, 1994; Chopin and Bouillant 1975; Budzianowski 1991; Budzianowski et al., 1991).

Compound **8** showed a dark blue spot on a TLC plate under UV light as well as a UV spectrum suggesting it to be a naphthalene derivative. The ion-negative LSI-MS spectrum showed a pseudo-molecular ion peak $[M-H]^-$ at m/z 379, whose LSI/CAD linked-scan spectrum gave the fragment at m/z 217 indicating the loss of a hexosyl moiety ($[(M-H)\text{-}162]^-$). The ion-positive HR-LSI-MS spectrum revealed an $[M+H]^+$ ion at m/z 381.11653 corresponding to the formula $C_{18}H_{20}O_9+H$ and an $[(M-H)\text{-hexosyl}]^+$ aglycone ion at m/z 219.06699 corresponding to the formula $C_{12}H_{10}O_4+H$.

The ^{13}C NMR spectrum of **8** (Table 1) exhibited 10 carbon signals in the range δ_C 111–157 ascribable to a naphthalene ring, which bore five directly attached hydrogen atoms, according to HETCOR and HMQC spectra. The signal pattern of those hydrogens in the ^{1}H NMR spectrum (Table 1)—three protons in *ortho*-related sequence and two singlets—indicated **8** to be a trisubstituted naphthalene derivative with two substituents on the one ring and third one on the other. In the NOESY spectrum, the NOE interactions observed between naphthalene protons—a singlet at δ_H 8.04 and a doublet at δ_H 7.60 ($J=8.1$ Hz) showed them to be in

Table 1
NMR data for compound **8** (in DMSO-d_6)

Position	δ_c[a]	δ_H[b]	NOESY[b]	HMBC[c]
1	122.2	8.04 *s*	H-8	C-3, C-8, C-9, C-10, COOH
2	128.5			
3	106.4	7.39 *s*	OCH_3	C-1, C-4, C-10
4	156.8			
5	154.3			
6	111.8	7.20 *d* (8.1)	H-7, H-1′	C-5, C-8, C-10
7	126.7	7.41 *t* (8.1)	H-6, H-8	C-5, C-6, C-9
8	122.8	7.60 *d* (8.1)	H-1, H-7	C-1, C-6, C-9, C-10
9	135.9			
10	118.2			
4-OCH_3	56.1	3.90 *s* (3H)	H-3	C-4
2-COOH	168.6			
1′	101.4	4.96 *d* (7.8)	H-6, OCH_3	C-5
2′	73.6	3.42 *dd* (7.8/8.7)		
3′	76.4	3.33 *t* (8.7)		
4′	69.7	3.22 *t* (8.7)		
5′	77.1	3.36 *m*		
6′	60.7	3.71 *brd* (10.5)		
		3.50 *dd* (5/10.5)		

[a] At 75 MHz. Protonated carbons are determined by HMQC and HETCOR spectra.
[b] At 300 MHz.
[c] At 600 MHz.

the *peri* position, i.e. as H-1 and H-8, respectively, and in consequence, the remaining protons could be assigned as H-6 (δ_H 7.20, *d*, $J=8.1$ Hz), H-7 (δ_H 7.41, *t*, $J=8.1$ Hz) and H-3 (δ_H 7.39, *s*). However, the placement of the latter signal in *meta* (H-3) not *para* (H-4) relation to H-1, was made in spite of the appearance of those protons (H-1, H-3) as singlets (even in 600 MHz ^{1}H NMR spectrum), which proved to be misleading of *para* relationship in view of evidence from HMBC and ^{1}H^{1}H-DQF-COSY spectra. In particular, the HMBC spectrum showed an absence of coupling between the proton singlet δ_H 7.39 and C-5 carbon of the neighbouring naphthalene ring, which would undoubtedly appear ($^3J_{HC}$) if the singlet had belonged to H-4, while the HH-COSY spectrum exhibited noticeable coupling between those singlets (i.e. H-1 and H-3). The remaining NMR signals of **8** were attributable to an aromatic methoxyl at δ_H 3.90 *s* (3H) and δ_C 56.1, a carboxylic group at δ_C 168.6, and a glucopyranosyloxy moiety due to the typical signal pattern determined by ^{1}H^{1}H-DQF-COSY and HMQC spectra and shown in Table 1. The methoxyl was placed at C-4 from its NOESY correlation with H-3 and HMBC interactions from that methoxyl group to C-4 and the latter to H-3; the carboxylic group was placed at C-2 due to the HMBC correlation of COOH carbonyl signal to H-1; the glucopyranosyloxy moiety was placed at C-5 from the HMBC interactions from H-1′ anomeric proton to C-5 and the latter to H-6 of an aglycone. The β-configuration of the glucosyl was evident from the large, diaxial, coupling $J=7.8$ Hz for H-1′ anomeric proton signal at δ_H 4.96 in ^{1}H NMR, as well as very easy hydrolysis of **8** to its aglycone (**8a**) with β-D-glucosidase (ca. 2 min). In view of proved β-anomeric configuration, the negative specific rotation of **8** means that the sugar probably belongs to the D-series (Hudson, 1909).

Hence, compound **8** is 5-hydroxy-4-methoxy-2-naphthalenecarboxylic acid 5-*O*-β-glucopyranoside, a new natural product we named drosophylloside.

However, the compound of the same structure as an aglycone of **8** was recently identified from *Ancistrocladus ealaensis* (Ancistrocladaceae) and named ancistronaphthoic acid B (Bringmann et al., 2000a). This acid could be isolated from $CHCl_3$ fractions of *Drosophyllum* with the aid of an aglycone of **8** (=**8a**) as a TLC reference and its identity was assured by comparing of its UV, EIMS and HREIMS spectra with those of **8a** and reported values (Bringmann et al., 2000a).

Compound **1** exhibited ^{1}H NMR signals of a pattern similar to those of the aglycone part of compound **8** but with evident *meta* couplings and one extra methoxyl group (see Section 3.5) suggesting it to be a dimethoxy derivative of 2-naphthalenecarboxylic acid. However, shifts differed significantly from those reported for 4,5-dimethoxy-2-naphthalenecarboxylic acid and were, with an exception of the methoxyl group at δ_H 3.96, almost identical to those reported for 5-hydroxy-4-methoxy-2-naphthalenecarboxylic acid (Bringmann et al., 2000a). The position of methoxyls was determined by NOE difference spectra. Sequential irradiation of methoxyl signals at δ_H 4.13 and 3.96 showed no interaction between them, but the former gave a spatial response to a signal at δ_H 7.39 (*d*) with *meta* coupling $J=1.2$ Hz, ascribable to H-3. From these data, the former methoxyl was placed at C-4 and the latter assigned to carboxymethyl at C-2. Hence, compound **1** is 5-hydroxy-4-methoxy-2-naphthalenecarboxylic acid methyl ester, which may be an artifact of the extraction process caused by reaction of **8a** with methanol.

All isolated compounds, with the exception of plumbagin, are new for the genus *Drosophyllum*, while the flavonoids represent the first *C*-glycosylflavones found in the family Droseraceae. Recent investigations into molecular phylogeny within the subclass Caryophyllidae suggested no close relationship of *Drosophyllum* with the family Droseraceae and strongly supported affinities outside of this family—namely to the families Ancistrocladaceae and Dioncophyllaceae (Meimberg et al., 2000). Our results seem to reflect those findings at the secondary metabolism level. Thus, naphthoquinones (plumbagin, chloroplumbagin) and hydroplumbagin glucoside link *Drosophyllum* mainly to the Droseraceae (Culham and Gornall, 1994) (plumbagin was also found in the Dioncophyllaceae (Bringmann et al., 2000b)). In turn, naphthalenes—drosophylloside (=ancistronaphthoic acid B glucoside) ancistronaphthoic acid B and its methyl ester, relate that genus to the family Ancistrocladaceae and probably also to the family Dioncophyllaceae with regards to the structural and biogenic relation of naphthoic acids to naphthylisoquinoline alkaloids, which, in turn, are restricted to those families (Meimberg et al., 2000). The significance of the *C*-glucosylflavones presence in *Drosophyllum* is difficult to evaluate as no data on the occurrence of flavonoids in the families Ancistrocladaceae and Dioncophyllaceae are yet available.

3. Experimental

3.1. General

3.1.1. Spectroscopy

Optical rotations were measured on a Perkin-Elmer 243 B digital polarimeter. UV spectra were recorded in MeOH on a Specord M-40 (Zeiss, Jena) spectrophotometer equipped with M-40 computer software using procedures described earlier (Mabry et al., 1970). NMR spectra were recorded on Varian Unity 300 (^{1}H-300 MHz, ^{13}C-75 MHz) and Bruker MDX-600 (^{1}H-600 MHz, ^{13}C-150 MHz); the HMBC spectrum was optimised for $J_{HC}=8$ Hz. LSI-MS spectra were made on

AMD 604 (Intectra) using glycerol as a matrix and conditions described previously (Frañski et al., 1999 a,b). EIMS spectra were recorded on AMD 402 (Intectra) (70 eV, probe).

3.1.2. Chromatography

Analytical TLC was carried out on pre-coated silica gel, cellulose, cellulose F plastic-backed sheets (Merck) and home-made polyamide **6** (Macherey–Nagel) plates. Detection: by viewing under UV 365 nm or 354 nm before and after spraying with 0.1% NA or 1% $AlCl_3$ in ethanol followed by warming. Preparative TLC was performed on pre-coated, plastic-backed cellulose F (0.2 mm), glass-backed silica gel (0.25 mm) (Merck) plates and home-made polyamide **6** (Macherey–Nagel) (0.5 or 1 mm) plates. Open column chromatography (CC) was performed with polyamide **6** (Roth or Macherey–Nagel) and Sephadex LH-20 (Pharmacia) eluting with MeOH of HPLC grade and neutral alumina (POCh, Poland) using EtOH–H_2O for elution.

3.2. Plant material

Seeds of *Drosophyllum lusitanicum* Link. were obtained from the Botanical and Zoological Garden in Stuttgart, in 1997, by means of co-operation and seed exchange between the Botanical Gardens.

3.3. In vitro cultures

Axenic germination of seeds was achieved on Murashige and Skoog agar medium with 1/4 of macronutrients. On the base part of shoots the appearance of callus was observed after reculture of seedlings to the same medium. The callus possessed chlorophyll and had a tendency to turn black after a short period of culture. At the same time, about 1/2 of the shoots that were grown on 1/4 MS medium, showed necrosis. With the aim of improving the growth of shoots as well as their multiplication and proliferation of calluses, kinetin in doses of 1.15, 2.3 and 4.6×10^{-6} M, was added to the MS medium.

3.3.1. Plant material for phytochemical analysis

MS medium supplemented with 2.3×10^{-6} M kinetin was chosen to maintain the growth of shoots and calluses for the purpose of plant material collection. Shoots and calluses were subcultured every 2 months for a period of 2 years. The cultures were kept under fluorescent light with 16 h photoperiod at 11.3 μM m^{-2} s^{-1} photosynthetically active radiation at 25±2 °C.

3.4. Extraction and isolation

Fresh shoots (23.3 g), green callus tissue (57.0 g) and black callus tissue (80.9 g), collected in March 2000, were plunged into separate portions of boiling MeOH (200 ml) and left for maceration at ambient temp. The maceration was repeated twice and lasted 5 days in total. The concentrated MeOH extracts (2.4, 2.4 and 0.5, respectively) were each suspended in water (30 ml) and extracted successively with $CHCl_3$ (3×30 ml) and H_2O satd. *n*-BuOH (5×30 ml). Each fraction was immediately concentrated to dryness to avoid decomposition of unstable compounds such as hydroquinone glucosides (Budzianowski, 1996).

3.4.1. Isolation from shoots

The $CHCl_3$ fraction (0.18 g) was separated by prep. TLC on silica gel in toluene–HCOOH 99:1 into four bands (1–4). From band 1, using aglycone of compound **8** (=**8a**) (see Section 3.7) as a TLC reference, the same compound was isolated by prep. TLC on polyamide in MeOH (run×3) followed by Sephadex LH-20 CC (yield: <0.5 mg). Bands 2–4 after CC on polyamide (band 2) and Sephadex LH-20 (bands 2–4), afforded compounds **1** (0.6 mg), **2** (11 mg) and **3** (1 mg). The BuOH fraction (0.35 g) was separated by prep TLC on polyamide in H_2O–*n*-BuOH–Me_2CO 16:3:3 into 11 bands (1–11). Bands 3 and 4, after prep. TLC on cellulose F in HOAc–H_2O 3:17, gave crude compounds **4**, **5** and **6–8**, respectively, which were further purified by prep. TLC on polyamide in H_2O satd. MeCOEt (compounds **4–7**) or CC on polyamide (compound **8**) followed by CC on Sephadex LH-20. Yields: **4** (<0.5 mg), **5** (<0.5 mg), **6** (1 mg), **7** (1.8 mg), **8** (1.9 mg). Band 6 was purified by CC on alumina according to (Budzianowski, 1996) to give compound **9** (17 mg).

3.4.2. Isolation from green callus

The $CHCl_3$ (0.23 g) and BuOH (0.32 g) fractions were separated in the same way as in the case of the relevant fractions from shoots (see Section 3.3.1) to yield: **1** (0.7 mg), **2** (21 mg), **3** (1.7 mg), **4** (0.4 mg), **5** (0.8 mg), **6** (1.4 mg), **7** (1.5 mg), **8** (4.5 mg), **9** (22 mg).

*3.5. 5-Hydroxy-4-methoxy-2-naphthalenecarboxylic acid methyl ester (**1**)*

Colourless solid. HR-EI-MS *m/z*: 232.07417 $[M]^+$, calc. for $C_{13}H_{12}O_4$: 232.07356. EIMS *m/z* (rel.int.): 232 $[M]^+$ (100), 217 $[M-Me]^+$ (33), 201 (12), 189 $[M-Me-CO]^+$ (21), 173 $[M-COOMe]^+$ (17), 159 (28), 102 (23); ^1H NMR (600 MHz, CD_3OD): 8.17 (1H, *d*, *J*=1.2 Hz, H-1), 7.44 (1H, *dd*, *J* =1 .8/8.1 Hz, H-8), 7.42 (1H, *t*, *J*=8.1 Hz, H-7), 7.39 (1H, *d*, *J*=1.2 Hz, H-3), 6.93 (1H, *dd*, *J*=1.8/8.1 Hz, H-6), 4.13 (3H, *s*, 4-OCH_3), 3.96 (3H, *s*, 2-$COCH_3$). DIFNOE (600 MHz): 4.13→7.39, 3.95—no response.

*3.6. 5-Hydroxy-4-methoxy-2-naphthalenecarboxylic acid 5-O-β-glucoside (**8**)*

Amorphous beige solid; $[\alpha]_D^{20}$ −81° (MeOH; *c* 0.075). UV λ_{max} MeOH nm: 237, 295, 307, 330, 334. Ion-negative

LSI-MS *m/z*: 379 [M−H]−, 217 [(M−H)−162]$^-$. Ion-positive HR-LSI-MS *m/z*: 381.11653 $[M+H]^+$, calc. for $C_{18}H_{20}O_9$ + H: 381.11856; 219.06699 [(M−H)-hexosyl]$^+$, calc. for $C_{12}H_{10}O_4$ + H: 219.06573. ^{1}H and ^{13}C NMR: see Table 1.

3.7. Enzymatic hydrolysis of **8**

0.3 mg of **8** and 0.3 mg of β-D-glucosidase from sweet almonds (No. 0395, Sigma) were dissolved in 5 drops H_2O and left for 1 h at ambient temp. The reaction mixture was sampled at known time intervals and analysed by TLC on polyamide in MeOH [**8**: R_f 0.34; aglycone of **8** (=**8a**) R_f 0.22]. The hydrolysate was extracted with toluene (3×0.5 ml) to recover the aglycone (**8a**) as a colourless solid (~0.2 mg). **8a**: HR-EI-MS *m/z*: 218.05769 $[M]^+$, calc. for $C_{12}H_{10}O_4$: 218.05791. EI–MS *m/z* (rel. int.): 218 $[M]^+$ (100), 203 $[M-Me]^+$ (47), 175 $[M-Me-CO]^+$ (21), 149 (32); UV data were similar to those reported previously (Bringmann et al., 2000a).

3.8. 5-Hydroxy-4-methoxy-2-naphthalenecarboxylic acid (**8a**) (sample from green callus)

HR-EI-MS *m/z*: 218.05808 $[M]^+$, calc. for $C_{12}H_{10}O_4$: 218.05791. EI–MS *m/z* (rel. int.): 218 $[M]^+$ (100), 203 $[M-Me]^+$ (47), 175 $[M-Me-CO]^+$ (63), 149 (35).

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