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ANTHRAQUINONES FROM *OPHIORRHIZA PUMILA* TISSUE AND CELL CULTURES

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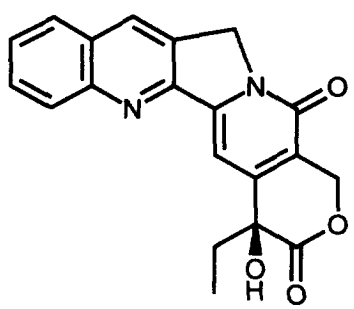
Key Word Index—*Ophiorrhiza pumila*; Rubiaceae; callus culture; anthraquinones.

Abstract—We have succeeded in initiating and establishing systems of tissue and cell cultures of *Ophiorrhiza pumila*. Examination of the constituents of the methanol extract of the cultured calli revealed the presence of 11 anthraquinones including two new ones whose structures have been rigorously proved using advanced spectroscopic methods. These findings demonstrated a remarkable difference in the constituents between the wild plants and the callus tissue or cultured cells; the former is devoid of anthraquinones and contains a variety of camptothecin-related alkaloids whereas the latter contains a significant amount of anthraquinones and shows no indication of the presence of alkaloids after several sub-culturings or final establishment of well growing cell suspensions.

INTRODUCTION

Camptothecin (**1**) is well known for its remarkable inhibitory activities against tumour cells by blocking the eukaryotic topoisomerase I [1] as well as for its activity against HIV-I [2]. Recently, a camptothecin derivative, topotecan® [3], began to be used as a clinical anti-tumour agent. Synthetic and biological studies on camptothecin and related compounds have been extensively carried out [4–6]. We have already found that *Ophiorrhiza pumila* Champ. produces not only camptothecin (**1**) but also a variety of related alkaloids. Among them, chaboside [7, 8] was the first natural camptothecinoid glucoside, and pumiloside [9, 10] and deoxypumilosides [9, 11] were plausible biogenetic intermediates in camptothecin formation from the established intermediate strictosamide [12]. During our studies on plant cell cultures [13–17], we succeeded in inducing callus and cell culture systems of *O. pumila* and investigated the chemical constituents of the calli and cell suspensions.



1 Camptothecin

RESULTS AND DISCUSSION

We succeeded in initiating callus cultures from the leaves and shoots of *O. pumila* on Linsmaier–Skoog (LS) medium supplemented with 2,4-D (0.22 mg l^{-1}), and NAA (0.186 mg l^{-1}). Callus cultures were maintained in the dark at 25° by frequent sub-culturing on fresh LS medium at intervals of 5–7 weeks. Moreover, we were able to generate efficiently growing cell suspensions from the callus stage, which provided enough cell material (*ca* 12 g dry weight l^{-1} medium) for phytochemical investigations. It was our intention to establish a new source for the pharmacological interesting and rare alkaloid camptothecin (**1**) and related compounds. Similar attempts have been made during the last two decades to isolate **1** from cultivated cell systems of *Camptotheca acuminata*, but the amounts of alkaloid produced were extremely low (*ca* 20 times lower than in the original plants) [18, 19]. A recently published work describes the detection of only traces of **1** in *Camptotheca* tissue systems [20]. These obser-

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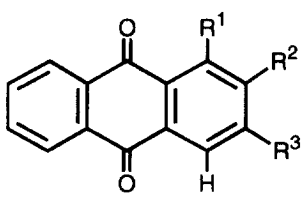
vations obviously parallel our results obtained with *Ophiorrhiza* cultures. When we analysed the callus tissue in an early stage of cultivation (after 3 or 4 transfers to fresh medium), the alkaloids **1**, pumiloside and 3(*R*)-deoxypumiloside, were present. Calli, however, which had been sub-cultured 10 times, did not show any formation of camptothecin-type alkaloids. This was the same with well established cell suspensions. Therefore, it seems to be very clear that the process of increasing cell de-differentiation leads to repression of camptothecin (**1**) biosynthesis. Such observations have been made many times for a variety of natural products and cell cultures [21, 22]. Prominent examples in the alkaloid field are those of vinblastine formation [23] or vindoline biosynthesis in *Catharanthus roseus* cultures. In the case of vindoline, its biosynthetic pathway is repressed in cell suspensions only during the late stages [24, 25]. The situation described here for *Ophiorrhiza* cell systems might be similar, because the first enzyme of the camptothecinoid pathway, strictosidine synthase, is present in calli and cell suspensions. Therefore, one can expect that the biosynthesis of the camptothecin-type alkaloids is blocked during the later stages, which presently remain unknown.

Despite these results, it was interesting to note that all the *Ophiorrhiza* tissues developed light yellow to brown colours during cultivation. In order to identify these pigments, freshly harvested, six-month-old cultures were extracted with methanol. The methanol extract was partitioned with ethyl acetate–water to give an ethyl acetate extract containing the less polar compounds. The aqueous layer was extracted with *n*-butanol resulting in a *n*-butanol extract which contained more the polar constituents. The extracts were subjected to silica gel column chromatography and further purification, respectively.

Six known anthraquinones, 2-methylanthraquinone (**2**) [26, 27], 1-hydroxy-2-methylanthraquinone (**3**) [26], 3-hydroxy-2-methylanthraquinone (**4**), 3-hydroxyanthraquinone-2-carbaldehyde (**5**) [26], and 1-hydroxy-2-hydroxymethylanthraquinone (**6**) [26], 3-hydroxy-2-hydroxymethylanthraquinone (**7**) [28, 29], and cholesterol along with a new anthraquinone (**8**) were obtained from the EtOAc extract. A further five known anthraquinones, 3-hydroxyanthraquinone-2-carbaldehyde (**5**), 1-hydroxy-2-hydroxymethylanthraquinone (**6**), 1,3-dihydroxy-2-methylanthraquinone (**9**) [26, 27] and 1,3-dihydroxy-2-methoxymethylanthraquinone (**10**) [30], 1,3-dihydroxy-2-hydroxymethylanthraquinone (**11**) [26, 27], and a new anthraquinone (**12**) were isolated from the *n*-butanol extract. The known compounds were identified by spectroscopic comparison with literature data. The structures of the new anthraquinones were confirmed from their spectroscopic data.

The UV-VIS spectrum of **8** showed absorptions at 241, 245, 276, and 407 nm in EtOH. The high-resolution FAB mass spectrum displayed a protonated molecular ion at *m/z* 285.0763 corresponding to the molecular formula $C_{16}H_{13}O_5$. The ^1H NMR spectrum in the aromatic region showed two multiplets of four aromatic protons at δ 7.78–7.84 and δ 8.28–8.32 and a singlet proton at δ 7.45, indicating that **8** was an anthraquinone possessing a tri-substituted C-ring. Other ^1H NMR signals were a singlet of a hydrogen-bonded hydroxy group at δ 13.14, a broad 2H-singlet of hydroxymethyl protons at δ 4.89 and a 3H-singlet of methoxy protons at δ 4.06. The hydroxy group at δ 13.14 was located at the *peri* position to the C-9



2 : R^1=H, R^2=CH_3, R^3=H

3 : R^1=OH, R^2=CH_3, R^3=H

4 : R^1=H, R^2=CH_3, R^3=OH

5 : R^1=H, R^2=CHO, R^3=OH

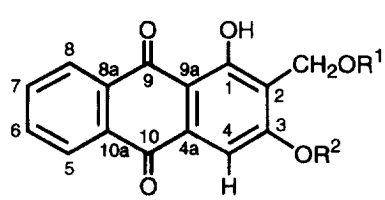
6 : R^1=OH, R^2=CH_2OH, R^3=H

7 : R^1=H, R^2= CH_2OH, R^3=OH

9 : R^1=OH, R^2=CH_3, R^3=OH

10 : R^1=OH, R^2=CH_2OCH_3, R^3=OH

11 : R^1=OH, R^2=CH_2OH, R^3=OH



8 : R^1 = H, R^2 = CH_3

12 : R^1 = $\overset{1'}{C}H_2\overset{2'}{C}H_2\overset{3'}{C}H_2\overset{4'}{C}H_3$, R^2 = H

carbonyl group. This is in good agreement with the UV-VIS spectrum in ethanol-OH^- showing the absorption maximum at 496 nm. Differential NOEs were observed between the hydroxy proton (δ 13.14) and methylene protons (δ 4.89) and between the aromatic proton (δ 7.45) and methoxy protons (δ 4.06). Irradiation of the methylene protons during the differential NOE experiment led to enhancement (0.5%) of the peak intensity of the methoxy protons. These observations suggested that the hydroxymethyl group and the methoxy group were located at the C-2 and C-3 positions, respectively. The cross-peak between δ_C 162.4 and δ_H 4.89 in the HMBC spectrum also indicated that the hydroxymethyl group was attached to the C-2 position. Consequently, **8** was determined to be 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (lucidin 3-methyl ether).

Another new compound, **12**, showed UV-VIS absorptions at 241, 246, 280, and 414 nm in ethanol. Its high-resolution FAB mass spectrum displayed a protonated molecular ion at *m/z* 327.1234 corresponding to the formula $C_{19}H_{19}O_5$. In the ^1H NMR spectrum, five aromatic protons that were similar to those of lucidin ω-methyl ether (**10**), a *peri* hydroxy proton at δ 13.30, a free hydroxy proton at δ 9.66, and methylene protons at δ 4.97 were observed, indicating that **12** was also an anthraquinone possessing a trisubstituted C-ring. The ^1H NMR signals [δ 3.68 (2H, *t*), 1.69 (2H, *m*), 1.44 (2H, *m*), 0.96 (3H, *t*)] and the appearance of a fragment ion at *m/z* 253 $[M-C_4H_9O]^+$ in the FAB mass spectrum indicated the existence of a *n*-butoxy group. In a differential NOE experiment, irradiation of methylene protons at δ 4.97 led to enhancements of the intensity of the hydroxy proton at δ 13.30 and methylene protons at δ 3.68, suggesting that a butoxy group was located at the C-2 position. On the basis of the above observations, **12** was deduced to be 2-*n*-butoxymethyl-1, 3-dihydroxyanthraquinone (lucidin ω-butyl ether). We cannot exclude the possibility that **12** was an artefact formed from lucidin upon extraction of the callus cultures with *n*-butanol.

It is well known that anthraquinones of shikimate origin are widely distributed in Rubiaceae plants and a great number of cell suspension cultures are known to produce such pigments [27, 31, 32]. In our previous studies on the constituents of *O. pumila*, we never found anthraquinones in the wild plants, not even in trace amounts. We now find that different cells and tissues of the same plant produce this group of metabolites when they are cultured as calli or cell suspensions.

Our present observations on *O. pumila* are quite similar to those from *Cinchona* [33, 34]. *Cinchona* plants do not contain anthraquinones but start to produce these as dominant metabolites when their cells are cultured as tissue or cell suspension culture. These authors regard these metabolites as phytoalexins, the formation of which is initiated with 'elicitor molecules of mechanical stresses' given to the original cells [33, 34]. Unfortunately, there are no available reports on the nature of these anthraquinone-forming elicitor molecules, so that a direct proof with the *O. pumila* system is not yet possible. However, since *Ophiorrhiza* calli cultured on solid media under exactly the same conditions show a different pigmentation, it is doubtful that 'mechanical stress' is the reason for anthraquinone biosynthesis. Future elicitation experiments might help to explain pigment formation in our cell systems.

EXPERIMENTAL

General. UV-VIS: EtOH; ^1H and ^{13}C NMR: 500 and 125.65 MHz, respectively (ppm, *J* in Hz with TMS as int. standard); EIMS: direct probe insertion at 70 eV; FAB MS: JEOL JMS-HX110; TLC: precoated Kieselgel 60 F_{254} plates (Merck, 0.25 mm thick); CC: Kieselgel 60 [Merck, 70–230 and 230–400 mesh (for flash chromatography)]; MPLC: silica gel prepacked column Kusano CPS-HS-221-05; Prep. TLC: silica gel 60 GF_{254} (Merck 7730, 0.5 mm thick).

Plant material and culture methods. Callus cultures were induced from leaf segments and 2 to 4-week-old shoots of *Ophiorrhiza pumila* Champ. on LS medium supplemented with 2,4-D (0.22 mg l^{-1}), NAA (0.186 mg l^{-1}), and sucrose (30 g l^{-1}) in the dark. Calli were maintained in the dark at 25° by frequent subculturing on fresh LS medium at intervals of 5–7 weeks. Cell suspensions were established by transferring 15–18 g fresh callus tissue to 40 ml LS medium in 300 ml Erlenmeyer flasks. After 20 sub-culturings (each 7 days), a good growing cell suspension (*ca* 12 g dry wt l^{-1} medium) was established which was further cultivated in 1 l flasks for cell production (25 ± 2°, 100 rpm, 750 lux).

Chromatographic analyses of shoot culture. Callus tissue derived from shoots and sub-cultured 3–4 times was freeze-dried and crushed. The resulting powder was extracted with MeOH (48 hr) at 4° and after filtration, the solvent was evapd. The residue was taken up in 2% H_2SO_4, and the H_2O layer was extracted with EtOAc (first extract). The aq. soln was adjusted to pH 10 (NaOH) and extracted with EtOAc and *n*-BuOH (second extract). First extract: EtOAc soln was evapd and chromatographed on TLC (EtOAc–MeOH–H_2O–25% NH_3 = 7:2:1:0:1). It showed a blue-fluorescent band with the same R_f (0.66) as the standard camptothecin (**1**). MeOH-extraction of this unknown band and UV measurement showed identical UV compared to **1**. HPLC analyses: (a) column RP-18 Superspher 100 (0.4 × 12.5 cm, 5 μm), solvent (1 ml min^{-1}) MeCN–H_2O: 0 min 0:100, 10 min 100:0, 15 min 100:0, 15.5 min 0:100, 20 min 0:100; R_t = 9.02 min (unknown and **1**, resp.); (b) column RP-Select B LiChroCART®, LiChrospher 60 (0.4 × 20 cm, 5 μm), solvent (1 ml min^{-1}) MeCN–50 mM KPi pH 2.3–MeOH: 0 min 1:7:2, 15 min 3:3:4, 17 min 3:3:4, 18 min 1:7:2, 25 min 1:7:2; R_t = 13.08 min (unknown and **1**, resp.). On EIMS the unknown

was identified as **1**; concn of **1** in callus was 1/300 compared to plant material. Second extract: In the second extract, traces of pumiloside and 3(*R*)-deoxypumiloside were detected by TLC, UV and HPLC. After sub-culturing of the calli more than × 10 TLC and HPLC analyses did not show the presence of these alkaloids. TLC-analysis in the above TLC solvent system revealed the presence of strictosamide, as proven by the R_f value (0.71), UV and EIMS data. The ^{1}H NMR (CD_3OD) data were identical with those in the literature [35].

Isolation of metabolites. Freshly harvested cultures (810 g) were homogenized and extracted with MeOH (1 l × 3) to give the MeOH extract (27 g). The MeOH extract was partitioned with EtOAc–H_2O to give the EtOAc extract (1.82 g). The aq. layer was extracted with *n*-BuOH to give the *n*-BuOH extract (2.80 g). The EtOAc extract was sepd by silica gel flash CC eluted with 0–100% MeOH–$CHCl_3$. From the 3% MeOH–$CHCl_3$ eluent, 2-methylanthraquinone (**2**) (1.4 mg), 1-hydroxy-2-methylanthraquinone (**3**) (6.5 mg) and 3-hydroxy-2-methylanthraquinone (**4**) were isolated. Further purification of the 3% MeOH–$CHCl_3$ eluent gave 3-hydroxyanthraquinone-2-carbaldehyde (**5**) (5.5 mg), 1-hydroxy-2-hydroxymethylanthraquinone (**6**) (4.4 mg), 3-hydroxy-2-hydroxymethylanthraquinone (**7**), cholesterol (32 mg) and the new compound (**8**) which was purified as follows. The 3% MeOH–$CHCl_3$ eluent was subjected to silica gel flash CC eluted with $CHCl_3$ to give five frs. The $CHCl_3$ eluent was purified by silica gel flash CC eluted with 10–80% EtOAc–hexane and MeOH. The 30–40% EtOAc–hexane was further purified by silica gel CC eluted with hexane–$CHCl_3$ (1:1) to give (**8**) (1.3 mg). From the *n*-BuOH extract, five known anthraquinones (**5**, **6**, **9**–**11**) and one new anthraquinone (**12**) were isolated. The *n*-BuOH extract was sepd by silica gel flash CC eluted with 0–100% MeOH–$CHCl_3$. The 0–30% MeOH–$CHCl_3$ eluent was purified by silica gel CC and MPLC eluted with $CHCl_3$. By further sepn of the $CHCl_3$ eluent, 3-hydroxyanthraquinone-2-carbaldehyde (**5**) (3.0 mg), 1-hydroxy-2-hydroxymethylanthraquinone (**6**) (2.8 mg), 1,3-dihydroxy-2-methylanthraquinone (**9**) (0.4 mg), 1,3-dihydroxy-2-methoxymethylanthraquinone (**10**) (1.5 mg), 1,3-dihydroxy-2-hydroxymethylanthraquinone (**11**) and a new anthraquinone (**12**) (1.2 mg) were obtained.

1-Hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (**8**). Amorphous powder. HR FAB MS: $[M+H]^+$ *m/z*: 285.0763 (calcd for $C_{16}H_{13}O_5$ 285.0763); EIMS *m/z* (rel. int.): 284 $[M]^+$ (62), 269 (48), 255 (100), 139 (63); UV-VIS λ_{max}^{EtOH} nm: 241, 245, 276, 407; UV-VIS $\lambda_{max}^{EtOH-OH^-}$ nm: 257, 283 (sh), 318 (sh), 383, 496; ^{1}H NMR (500 MHz, $CDCl_3$); δ 13.14 (1H, *s*, OH-1), 8.28–8.32 (2H, *m*, H-5, 8), 7.78–7.84 (2H, *m*, H-6, 7), 7.45 (1H, *s*, H-4), 4.89 (2H, *br s*, CH_2O-2), 4.06 (3H, *s*, OCH_3-3), 2.58 (1H, *br s*, OH); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ: 162.45 (C-1), 121.97 (C-2), 163.59 (C-3), 102.78 (C-4), 126.89 (C-5)*, 134.33 (C-6)†, 134.30 (C-7)†, 127.40 (C-8)*, 187.35 (C-9), 182.36 (C-10), 133.36 (C-10a)‡, 133.35 (C-8a)‡, 111.34 (C-9a), 134.34 (C-4a), 54.04 (2-CH_2O), 56.46 (3-OMe).

2-n-Butoxymethyl-1,3-dihydroxyanthraquinone (**12**). Amorphous powder. HR FAB MS $[M+H]^+$ *m/z* 327.1234 (calcd for $C_{19}H_{19}O_5$ 327.1233); FAB MS *m/z* (rel. int.): 327 $[M+H]^+$ (44), 253 (100); UV-VIS λ_{max}^{EtOH} nm: 241, 246, 280, 414; ^{1}H NMR (500 MHz, $CDCl_3$); δ 13.30 (1H, *s*, OH-1), 9.66 (1H, *s*, OH-3), 8.26–8.29 (2H, *m*, H-5, 8), 7.75–7.81 (2H, *m*, H-6, 7), 7.32 (1H, *s*, H-4), 4.97 (2H, *s*, CH_2O-2), 3.68 (2H, *t*, $J = 6.6$ Hz, H_2-1′), 1.69 (2H, *m*, H_2-2′), 1.44 (2H, *m*, H_2-3′), 0.96 (3H, *t*, $J = 7.5$ Hz, H_3-4′); ^{13}C NMR (125.65 MHz, $CDCl_3$); δ 161.81 (C-1), 114.69 (C-2), 164.22 (C-3), 109.78 (C-4), 126.71 (C-5)*, 134.10 (C-6)†, 134.09 (C-7)†, 127.35 (C-8)*, 186.93 (C-9), 182.27 (C-10), 133.60 (C-10a)‡, 133.56 (C-8a)‡, 109.62 (C-9a), 134.03 (C-4a); 67.30 (2-$\underline{C}H_2O$), 72.02 (C-1′), 31.38 (C-2′), 19.2 (C-3′), 13.77 (C-4′).

Determination of strictosidine synthase (SS). Protein isolation was done using a standard procedure. Frozen tissue or cell material was homogenized with KPi buffer (100 mM, pH 7.0, 10 mM β-mercaptoethanol), filtered and centrifuged (11 000 *g*, 25 min). The supernatant was subjected to Amberlite XAD-4 filtration followed by 30–70% $AmSO_4$ pptn. Protein was solubilized in 50 mM KPi buffer and dialysed overnight. A protein determination was performed as described elsewhere [36].

For the determination of SS, the previous assay system [37] with modification was applied; a total vol. of 0.1 ml of a mixt. containing tryptamine (1 μmol), secologanin (2 μmol) and various amounts of gluconolactone and crude enzyme were incubated for 60 min at 35°. After the addition of 200 μl MeOH and centrifugation, the formed strictosidine in the supernatant was measured by HPLC at 240 nm (with external standard (strictosidine)).

HPLC: RP-select B column (see above), solvent MeCN–H_2O, pH 2.5, containing 5% MeOH (1 ml min^{-1} flow); gradient: 0 min 3:7, 8 min 9:1, 9 min 100:0, 11 min 100:0, 11.5 min 3:7, 20 min 3:7; R_t strictosidine 6.19 min.

The following SS activities were measured: *O. pumila* callus (LS medium), 2.8 nkat g^{-1} dry wt; suspension culture, 2.26 nkat g^{-1} dry wt; cells grown in Murashige–Skoog (MS) medium [38], 8.89 nkat g^{-1} dry wt; *Rauwolfia serpentina* cell culture (LS medium), 7.7 nkat g^{-1} dry wt, and *O. pumila* leaves, 0.09 nkat g^{-1} dry wt.

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*,†,‡ Assignments may be interchanged.

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