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FORMATION OF β-PELTATIN-A METHYL ETHER AND CONIFERIN BY ROOT CULTURES OF *LINUM FLAVUM*

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ABSTRACT.—Extracts of root cultures of *Linum flavum* contained high cytotoxic activity due to the presence of 1% β-peltatin-A methyl ether of the dry mass. During chromatographic analysis of the cell extracts, coniferin was identified as the major uv-absorbing but noncytotoxic constituent with levels of up to 3% of the dry mass. Growth, culture appearance, and product accumulation varied greatly with the 2,4-D concentration in the medium.

Sensitive biological assays are necessary for screening of plants or plant cell cultures for active principles (1-4). In order to compare the likelihood of detecting such compounds in extracts of differentiated plants and in the corresponding tissue cultures, we have recently measured the cytotoxicity of extracts from 50 randomly chosen plants and the tissue cultures derived therefrom (5). It was concluded that the extracts of field-grown plants usually contained distinctly higher cytotoxic activities than the corresponding cell culture extracts (5). Often, only the plant extracts (e.g., lipophilic extracts) showed cytotoxic activity while the cell culture extracts were inactive (5). In no case was the biological activity exclusively present in a cell culture extract. The results clearly showed that the differentiated plants give positive responses more often in screening programs than cell cultures. Sometimes, however, cell culture extracts may contain very high biological activity (3-5).

Among the extracts tested for cytotoxicity (5), only those of the plants and root cultures of *Linum flavum* L. exhibited very high cytotoxic activity (corresponding to 0.1 mg vinblastine/ml). As initially differentiated tissue cultures often lose their original characteristics when maintained continuously in liquid medium, the cytotoxic principle was only investigated when a stable productive root culture was obtained.

RESULTS AND DISCUSSION

IDENTIFICATION OF β-PELTATIN-A METHYL ETHER AS THE CYTOTOXIC PRINCIPLE OF ROOT CULTURE EXTRACTS.—The cytotoxic activity of root cultures of *L. flavum*, measured by their growth inhibitory effect on the mouse fibroblast line L929, was present in the CH_2Cl_2 and the H_2O phase of methanolic cell extracts. Chromatograms of the CH_2Cl_2 extracts on silica gel were divided into seven zones which were eluted with MeOH and tested for cytotoxicity. While a zone containing the only major uv-absorbing band (Rf=0.6) had to be diluted 3^{11} times to allow growth of L929 cells, dilutions of 3^5 of the adjacent zones and 3^3 of the other four zones permitted growth. The major aromatic compound of the CH_2Cl_2 extract was the active principle. This compound was β-peltatin-A methyl ether (**1**), a compound of known high cytotoxicity (6,7).

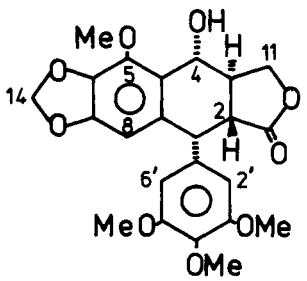
The identity of **1** was unambiguously determined from its uv spectrum, mass spectrum, and high-field ^{1}H-nmr spectrum. Comparison of the 400 MHz ^{1}H-nmr spectrum of **1** with that of an authentic sample of podophyllotoxin showed the presence of an additional aromatic methoxyl group (4.122 ppm) and the absence of one ring proton signal, which was compatible with the molecular ion at *m/z* 444 in the mass spectrum. The other ^{1}H signals had the same multiplicities and coupling constants as those of the reference, indicating the identical geometry of the molecules. The position of the methoxyl group was established from ^{1}H nOe difference spectra. Irradiation of H-4 caused a positive enhancement of the additional methoxyl signal confirming its position

at C-5. The alternative position at C-8 was excluded by the observation of positive nOe's to H-8 upon irradiation of H-1. The same experiments confirmed the molecular geometry.

The cytotoxicity of the H_2O phase was also caused by **1** which was difficult to extract completely into CH_2Cl_2. Under our standard conditions 80-85% of **1** was found in the lipophilic phase. Despite this distribution, the H_2O phase contained as much cytotoxic activity as the CH_2Cl_2 phase according to the biological assay. As no other zone with distinct cytotoxic activity was detected on chromatograms of the H_2O phase, one has to assume that not only the actual concentration but also the dispersion of the active compound in the test solution affects the quantitative result of the biological assay. Even if the organic solvent was removed in the presence of H_2O, serum albumin formation of different sized lipophilic droplets was sometimes noticed which prevented a proportional distribution upon dilution. Thus quantitative determinations of cytotoxic compounds in crude extracts, especially of cell cultures, by biological assays or by other techniques may not necessarily give identical results.

To our knowledge, *L. flavum* has not been analyzed for podophyllotoxins. However, the identification of a podophyllotoxin as the cytotoxic principle in root cultures of *L. flavum* may not be that surprising as peltatin A and B as well as other podophyllotoxins have been found in the closely related species *Linum album* (8). As the plant extracts of *L. flavum* also showed very high cytotoxicity, its aerial parts were analyzed for podophyllotoxins. Again **1** was the main podophyllotoxin of the plant extract. Its level on a dry mass basis was only ⅕ to 1/10 of that found in the root cultures. However, as roots of field-grown plants were not available for this study, the superior productivity of root cultures compared to the intact plant cannot be claimed from the results presented here. *L. flavum* is, to our knowledge, the third plant species known to accumulate β-peltatin-A methyl ether, the others being *Bursera* and *Juniperus* (6,7).

During chromatographic analysis of the H_2O phase of root culture extracts for further cytotoxic compounds, the major uv-absorbing compound was isolated. This compound showing no cytotoxicity was identified as coniferin (**2**) with levels of 3% of dry mass.



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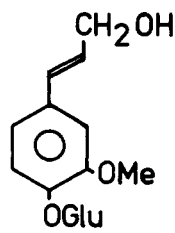

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SOME CHARACTERISTICS OF THE ROOT CULTURE.—The cultures have been maintained on MS-medium (9) with 2 μM 2,4-D for over 3 years. When this medium was used, the flasks usually contained several balls (10-30 mm in diameter depending on the size of the shake flask) or a flat cake of interlacing roots of up to 40 mm in length at the end of a growth cycle. Callus-like material represented only 5-10% of these cultures. The growth and production characteristics of the root cultures are given in Figures 1 and 2. The cells regularly accumulated more than 1% β-peltatin-A methyl ether and 3% coniferin on a dry mass basis, corresponding to 150-200 mg and 400-500 mg/liter, respectively, without optimization. Thus, the higher yields, as well as the better

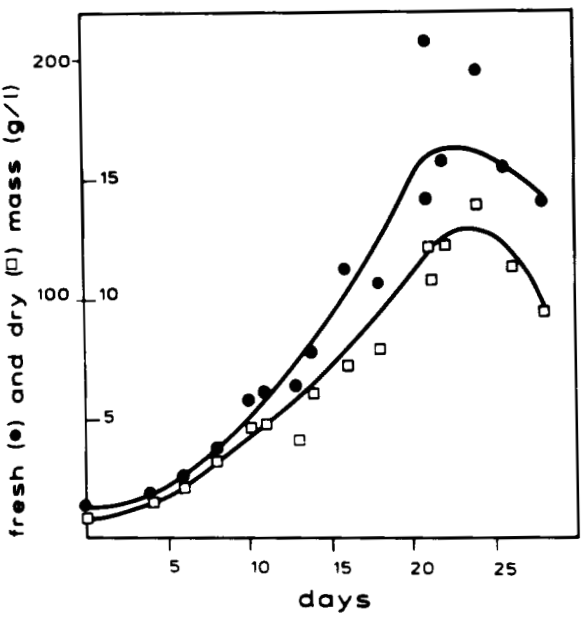

FIGURE 1. Fresh and dry mass formation of root cultures of *Linum flavum* grown on MS-medium plus 2 μM 2,4-D (70 ml/200 ml flask).

growth characteristics, would make the *Linum* culture a better source for podophyllotoxins than the recently reported callus cultures of *odophyllum peltatum* (10).

By increasing the 2,4-D concentration or by reducing the inoculum size, the percentage of callus-like material with shorter but thicker roots can be increased. Root cultures, maintained for 2 years on a medium with 5 μM 2,4-D, contained a 50% reduced specific yield of **1**. However, when transferred back to the low level 2,4-D medium, the

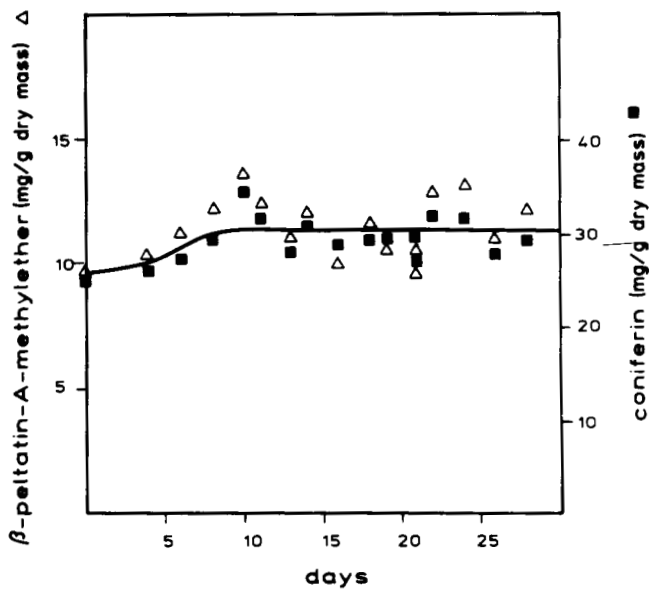

FIGURE 2. Specific yields of β-peltatin-A methyl ether and coniferin in root cultures grown on MS-medium plus 2 μM 2,4-D.

long, thin, and interlacing roots reappeared after one growth cycle, and higher production resumed. The different growth behavior of the cells on the different 2,4-D supplemented media is shown in Table 1. Complete depletion of 2,4-D greatly reduced growth of the root cultures without increasing specific yields of **1**. High concentrations of 2,4-D had also a negative effect on growth and also on the specific yields. At 10 μM some calluses without root structures and freely suspended cells were found. To date, however, we have failed to establish suspension cultures producing **1**. It appears that a certain degree of differentiation may be necessary for distinct expression of podophyllotoxin biosynthesis in *Linum* cultures. The percent alterations of coniferin levels by the different 2,4-D media paralleled those of **1**.

TABLE 1. Effect of Various 2,4-D Concentrations on Growth, Appearance, and β-Peltatin-A Methyl Ether (**1**) Production of the Root Cultures[a]

Concentration 2,4-D (μM)	Dry Mass/Flask (mg/70 ml)			
	after 7	14	21	28 days
0	131[b]	192[b]	290[b] (0.94)[e]	393[b] (1.02)
2	160[b,c]	230[b,c]	475[b] (1.27)	825[b] (1.18)
5	135[c]	251[c]	450[c] (1.31)	873[c] (1.12)[f]
10	121[c]	225[c]	395[c,d] (0.63)	383[c,d] (0.52)

[a]Initial inoculum: 1 g fresh weight (80 mg dry weight) of 14-day-old cells grown on 2 μM 2,4-D medium.

[b]Long, thin, interlacing roots.

[c]Calluses with short, thicker roots.

[d]Small calluses with reduced or no root formation and some single cells.

[e]Podophyllotoxin levels as % of dry mass. In parallel experiments levels of **1** varied by 10-15%.

[f]When grown for two years with 5 μM 2,-D specific yields decrease to about 50%.

EXPERIMENTAL

PLANT MATERIAL.—Callus cultures were initiated from surface sterilized leaf pieces of *L. flavum* (from the Botanical Garden of the University Münster, FRG) on MS-medium (9) plus 2 μM 2,4-D in June 1982. After 7 weeks, pieces of the slowly growing callus material were transferred to the same liquid medium where roots soon developed. (Initial experiments to establish a rapidly growing suspension culture without roots by using different phytohormone regimes failed.) For routine subcultivation, 2 g of fresh weight root cultures were grown in 70 ml of medium for 3 weeks.

PREPARATION OF EXTRACTS FOR ANALYSIS.—100 mg of dry mass were extracted twice with 10 ml 80% MeOH with an Ultraturrax. The methanolic extract was fractionated into a CH_2Cl_2 and a H_2O phase. For the cytotoxic tests, the organic solvent was removed in the presence of 2 mg of serum albumin/2 ml H_2O pH 7 to give better dispersion of the lipophilic compounds.

a) *Test for cytotoxicity*.—Defined amounts of cell extracts or eluates of chromatograms were dissolved in 2 ml of H_2O pH 7 containing 2 mg of serum albumin. These solutions were added in 1:3 dilutions to a suspension of mouse fibroblast cells L929, grown on DMEM (Flow Laboratories) with 0.45% glucose, 10% foetal calf serum, 10^3-3×10^3 cells/ml. After 5-6 days of growth in microtiter plates (10% CO_2, 37°), the dilution was determined at which first colony formation occurred. The negative control was a phosphate saline buffer (dilution 3^1), and the positive control contained 0.1 mg vinblastine/ml (3^{11}).

b) *Chromatography*.—The CH_2Cl_2 extracts were chromatographed on silical gel G 60_{F254} plates with toluene-formic acid ethyl ester-HOAc (5:4:1), the H_2O phase in BuOH-HOAc-H_2O (4:1:1). The absorbance was measured with a Shimadzu-TLC scanner at 278 nm (β-peltatin-A methyl ether) and 260 nm (coniferin), and concentrations were calculated from calibration curves of authentic podophyllotoxin and coniferin.

c) *Structural identification*.—The chromatographed zones were scraped off from the tlc-plates and eluted with MeOH or 80% MeOH; the solvents were evaporated to dryness, taken up again in organic solvent, filtered, and evaporated for spectroscopic analyses. ^{1}H-nmr spectra and nOe difference spectra were

recorded at ambient temperature on a Bruker WM-400 nmr spectrometer in Me_2CO-d_6 with internal TMS as reference. A mass spectrum was taken on an AEI MS 902S spectrometer at 70 eV. β-Peltatin-A methyl ether (**1**): ^{1}H nmr (Me_2CO-d_6) δ=6.501 (2H, s, H-2′, H-6′), 6.320 (1H, s, H-8), 6.006 [1H, d, H-14A, *J* (14A-14B) 1.1], 5.991 (1H, d, H-14B), 5.102 [1H, d, H-4, *J* (4-3) 8.9], 4.537 [1H, d, H-1, *J* (1-2) 4.5], 4.505 [1H, d, d, H-11A, *J* (11A-3) 7.2, *J* (11A-11B) 8.4], 4.122 (3H, s, 5-OCH_3), 4.122 [1H, d, d, H-11B, *J* (11B-3) 10.6], 3.700 (6H, s, 3′-OCH_3, 5′-OCH_3), 3.679 (3H, s, 4′-OCH_3), 2.960 [1H, d, d, H-2, *J* (2-3) 14.9], 2.767 (1H, d, d, d, d, H-3). Irradiation at H-8 caused signal enhancement (nOe) to H-4 and H-1, at H-4 to 5-OCH_3, H-2 and H-3, at H-1 to H-8, H-2′ (H-6′) and H-2, at H-11A to H-11B and H-3, at 5-OCH_3 to H-4, and at H-11B to H-11A and H-2; ms *m/z* 444 (M^+), 426 $(M-18)^+$.

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