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Droserone from cell cultures of *Triphyophyllum peltatum* (Dioncophyllaceae) and its biosynthetic origin ☆

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Abstract

The growth and droserone content of callus cultures of *Triphyophyllum peltatum* grown in liquid 1/5 Linsmaier and Skoog medium was studied. During a lag phase in growth, droserone concentrations in the medium reached a value of 2.1 mg g^{-1} fr. wt. After this maximum value the concentration decreased slightly to 1.8 mg g^{-1} fr. wt., while the growth of the calli was enhanced (25% increase in fr. wt. within 7 days). Plumbagin and isoshinanolone were likewise present in the medium. By feeding $^{13}C_2$-labelled acetate to the cultures the biosynthesis of droserone was elucidated. The incorporation of whole C_2-units unambiguously shows its acetogenic origin and fits well in the biosynthetic scheme suggested for the structurally — and biogenetically — related naphthylisoquinoline alkaloids. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Triphyophyllum peltatum*; Dioncophyllaceae; Biosynthesis; Naphthoquinones; Droserone; Secondary metabolism; Tissue culture; Feeding experiment

1. Introduction

The naphthoquinone droserone (3-hydroxy-plumbagin; 3,5-dihydroxy-2-methyl-1,4-naphthoquinone) (**3**) is found very occasionally in taxonomically not closely related groups of dicotyledonous plants. It is known from *Diospyros melanoxylon* (Sidhu, Sankaram & Ali, 1974) and *D. maritima* (Higa, Himeno, Yogi & Hokama, 1987) (Ebenaceae), from a variety of species of Nepenthales, e.g. *Plumbago indica* (synonym *P. rosea*) (Dinda, Das & Hajra, 1995) and *P. zeylanica* (Sankaram, Srinivasarao & Sidhu, 1976) (Plumbaginaceae), from *Nepenthes rafflesiana* (Cannon, Lojanapiwatna, Raston, Sinchai & White, 1980), *N. thorelii* (Likhitwitayawuid, Kaewamatawong, Ruangrungsi & Krungkrai, 1998) (Nepenthaceae), *Drosera peltata* (Asano & Hase, 1943), *D. whittakeri* (Rennie, 1877) and *Dionaea muscipula* (Kreher, Neszmélyi & Wagner, 1990) (Droseraceae), from *Ancistrocladus heyneanus* (Desai et al., 1973) and *A. robertsoniorum* (Bringmann et al., 1993) (Ancistrocladaceae). Furthermore, it was detected in two species of Dioncophyllaceae, *Habropetalum dawei* and *Dioncophyllum thollonii* (Bringmann, François, Aké Assi & Schlauer, 1998).

From *Triphyophyllum peltatum* (Dioncophyllaceae), though chemotaxonomically closely related to *H. dawei* and *D. thollonii* and intensively investigated for its alkaloids, the occurrence of droserone has not been recorded to date. Within the Dioncophyllaceae, this 'part-time carnivorous' (Bringmann, Wenzel, Bringmann, Schlauer & Aké Assi, 1996) West African liana, which occurs in the rain forests of West Africa, represents the most versatile producer of the unique

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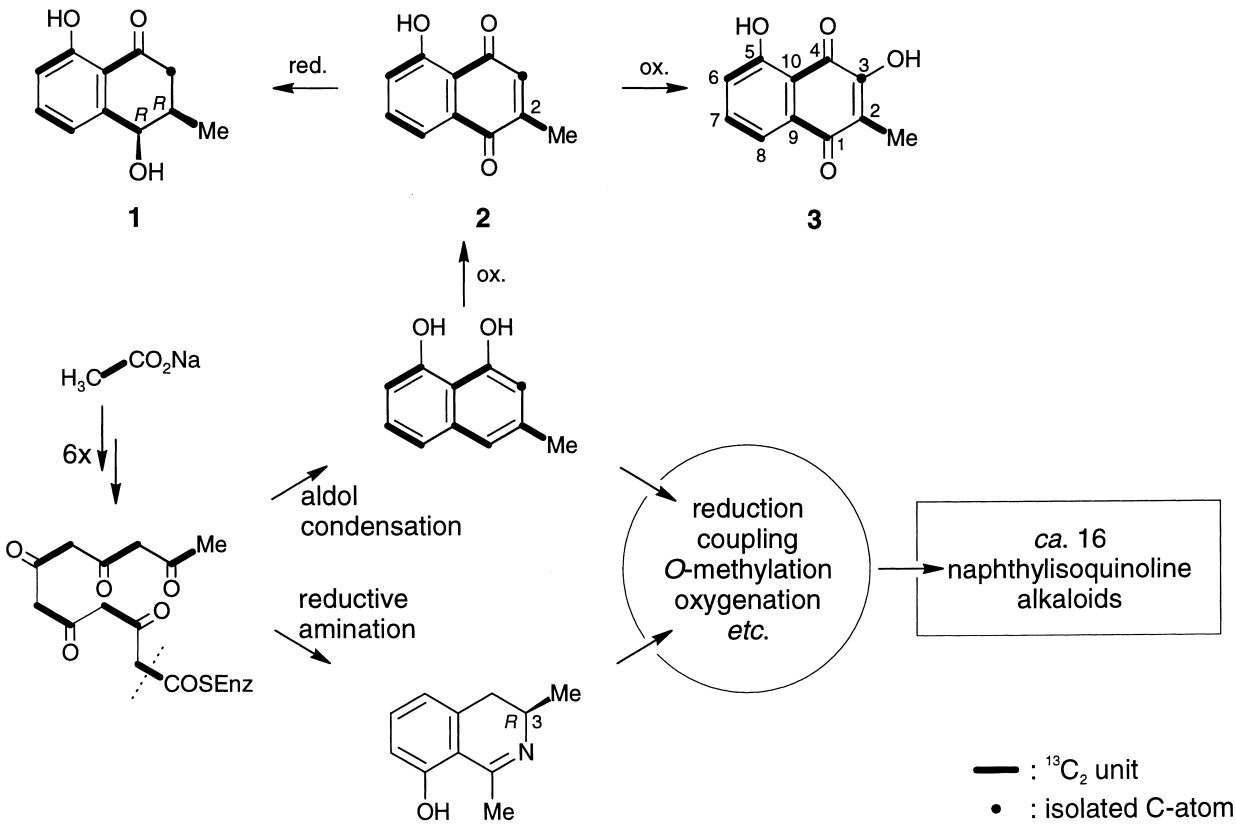

Scheme 1. Reaction sequence for the biosynthesis of **3** in callus of *T. peltatum* as deduced from feeding experiments with $^{13}C_2$-labelled acetate and suggested joint origin of the related naphthylisoquinoline alkaloids.

naphthylisoquinoline alkaloids (Bringmann & Pokorny, 1995; Bringmann et al., 1998), which are found in Ancistrocladaceae and Dioncophyllaceae, exclusively. Biosynthetically the naphthylisoquinolines are probably acetogenic (Bringmann & Pokorny, 1995). Recently we confirmed the acetogenic nature of the naphthoquinone plumbagin (**2**) (Durand & Zenk, 1971, 1974) and the related tetralone isoshinanolone (**1**), which originate from a precursor that is presumably also involved in the biosynthesis of the naphthalene moiety of naphthylisoquinoline alkaloids (Bringmann, Wohlfarth, Rischer, Rückert & Schlauer, 1998) (see Scheme 1).

In this paper we report on the production of droserone (**3**) in callus cultures of *T. peltatum*. Additionally, the acetogenic origin of **3**, including the folding mode of the poly-β-carbonyl chain, is shown unambiguously by the incorporation of whole C_2-units from ^{13}C-labelled acetate.

2. Results and discussion

Callus cultures of *T. peltatum* were initiated from axenically grown plants on 1/5 Linsmaier and Skoog solid medium (Linsmaier & Skoog, 1965) with full strength organics and supplemented with 8.88×10^{-6} M 6-benzylaminopurine, 5.37×10^{-8} M 1-naphthalene acetic acid, 3% sucrose, and 0.2% Gelrite (Roth). These cultures were maintained and propagated over a period of 1 year. Liquid cultures were established by

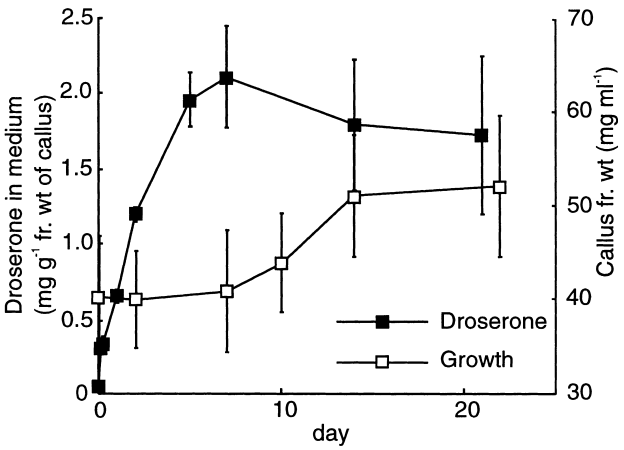

Fig. 1. Growth curve and time-course of droserone content in medium of callus cultures of *T. peltatum*. Bars represent s.d. (n = 3).

Table 1
^{13}C-NMR data of labelled **1** after application of [$^{13}C_2$]-acetate to the liquid medium

C-atom	1	2	3	4	5	6	7	8	9	10	2-CH_3
δ_C (ppm)	184.5	121.8	152.8	184.2	161.2	123.2	137.5	119.7	112.9	132.7	8.8
$^1J_{C-C}$ (Hz)	56.5[a]	44.4[b]	nd[c]	51.3[d]	66.1	66.6	54.8	54.8	57.4	52.7	44.4
I_s (%)[e]	122[a]	97[b]	nd[c]	167[d]	131	145	138	134	114	129	218

[a] Signal overlap with C-4.
[b] Line broadening of 4 Hz was applied due to weak peak intensity.
[c] nd = not detected.
[d] Signal overlap with C-1.
[e] Intensity ratio of satellite peaks compared to given central peak.

transferring the calli to the same medium without Gelrite.

Fig. 1 illustrates the profiles of growth and droserone content in the medium as a function of time in a batch culture during 3 weeks. After a lag phase of about 7 days the calli showed 25% increase in fr. wt. during the following week. Two weeks after transfer they reached a stationary phase at 52 mg ml^{-1} fr. wt.

The main metabolite in the medium was droserone (**3**), but **2** and **1** were also detected in the medium, albeit in distinctly lower concentrations. The release of **3** is probably a response to the stress caused by transfer of the calli from solid to liquid culture conditions, since neither the non-sterile nor the sterile plants contain significant droserone quantities. During the lag phase the content of **3** rose dramatically to 2.1 mg g^{-1} fr. wt. of callus. Thereafter the content dropped slightly within 7 days to 1.8 mg g^{-1} during the growth of the calli. Only a very slight decrease was observed during the stationary phase.

Based on these observations on the behavior of the calli in batch cultures, the feeding experiments were conducted.

Cultures of *T. peltatum* were incubated with [$^{13}C_2$]-acetate for 8 days. The growth characteristics of these cultures did not differ significantly from those of the unfed samples. After the isolation of **3** by reverse phase medium pressure chromatography (MPLC), its ^{13}C-NMR spectrum revealed a coupling pattern typical of the incorporation of whole $^{13}C_2$-units. Closer examination of this spectrum and the analysis of the ^{1}J(C,C)-coupling constants (see Table 1) led to the unambiguous determination of the original polyketide chain, establishing an acetogenic nature of **3** similar to that of related metabolites like plumbagin (**2**) or isoshinanolone (**1**).

The coupling constants showed pairwise coupling of the C atoms 2-CH_3/2, 1/9, 8/7, 6/5, and 10/4. C-3 remained isolated without observable coupling, confirming the proposed folding of the polyketide and decarboxylation at C-3.

In all plant species examined so far, **3** has always been found to be accompanied by its oxygen-poorer analog **2**, from which it might be derived biosynthetically. The incorporation of whole acetate units shows that the naphthoquinone is, at least in part, synthesized de novo upon stress, not from a pre-formed depot precursor such as a leucoglycoside (Kreher et al., 1990) or 5-*O*-glucosyl-droserone, as found, e.g., in *Drosera rotundifolia* (Schölly & Kapetanidis, 1989) — or by degradation of naphthylisoquinoline alkaloids, which are present in the calli as well.

In other plant families, different biogenetic pathways to naphthoquinones have been 'developed', e.g. from geranylated *p*-hydroxybenzoic acid or from prenylated homogentisic acid while others are derivatives of *o*-succinylbenzoic acid (Hegnauer, 1986).

3. Experimental

3.1. Plant material

Mature fruits of *Triphyophyllum peltatum* (Hutch. et Dalz.) Airy Shaw were collected by one of us (L. A. A.) in the Parc de Taï, Ivory Coast in May 1997. Voucher specimens are deposited in the Herbarium Bringmann, Würzburg, and in the Centre National de Floristique, Abidjan.

3.2. Tissue culture

Axenical germination and the establishment of shoot cultures was achieved as previously reported (Bringmann et al., 1999). Calli were induced by transferring stem segments (about 1 cm long, with several nodes) to 1/5 Linsmaier and Skoog medium with full strength organics and supplemented with 8.88×10^{-6} M 6-benzylaminopurine, 5.37×10^{-8} M 1-naphthalene acetic acid, 3% sucrose, and 0.2% Gelrite (Roth). The cultures were kept under fluorescent light with a 14 h photoperiod at 51 μM m^{-2} s^{-1} photosynthetically active radiation at 24 ± 2°C. Calli were subcultured in the same medium every month and were maintained over a period of 1 year. Liquid cultures were started using ca. 2 g callus as an inoculum in a 200-ml flask

containing 50 ml of the described medium devoid of Gelrite on a rotatory shaker at 100 rpm in diffuse light. The pH of the medium was adjusted to 5.8 with 1 N sodium hydroxide before autoclaving at 120°C and 120 kPa for 30 min. The growth was determined as fr. wt. For this purpose calli were dried on sterile filter paper and weighed in sterile petri dishes. After the measurement they were placed back into the original flasks.

3.3. HPLC analysis

One millilitre samples of each flask were taken under sterile conditions. They were centrifuged for 30 s at 12,000 rpm to remove the cell debris. HPLC system; two Waters liquid pumps Model 510, UV detection; Waters 484 Tunable Absorbance Detector, controlling; PC with Waters Chromatography Manager Software via Waters System Interface Module, columns; Waters μ Bondapak C18 Guard Pak HPLC Precolumn and Waters μ Bondapak C18 3.9 × 300 mm column, mobile phases; (A) acetonitrile (Merck LiChrosolv) and (B) Milipore-water with 0.1 TFA, eluting gradient; 0–2 min 20% A, 2–20 min 20–60% A (linear gradient), 20–25 min 60–20% A (linear gradient), wavelength for detection; 254 nm, flow rate; 1 ml/min, injection volume; 100 μl. Chromatographically pure droserone was used for quantitation.

3.4. Application experiments

Sodium acetate-1,2-[$^{13}C_2$] (Cambridge Isotope Laboratories) and unlabeled sodium acetate were separately dissolved in distilled water (0.05 M final concentration and 0.01 M, respectively). Each solution was sterile filtered. Prior to the application the calli were transferred to 50 ml freshly prepared medium to induce growth. 0.25 ml of the labelled acetate solution and 2 ml of the unlabeled acetate solution were then added to the medium and incubated for 8 days.

3.5. Extraction and isolation

Droserone (**3**) was isolated 8 days after the first addition of [$^{13}C_2$]-acetate. The liquid medium was decanted and filtered. After addition of 0.2 ml 0.1 N HCl to the weakly basic medium, the color of the liquid changed from dark red to yellow, a characteristic pH-dependent effect of hydroxynaphthoquinones (Bertram, 1991). The medium was exhaustively extracted with CH_2Cl_2. After evaporation of the organic solvent, the extract residue was redissolved in MeOH and purified by medium pressure liquid chromatography on RP-material (Lobar B RP-18). The chromatography was performed using MeOH/H_2O (v/v 70/30 with addition of 0.1% TFA) as the eluent, with a flow rate of 5 ml/min. Evaporation of the combined fractions yielded 0.7 mg **3** as a yellow solid, identical to reference material previously isolated (Bringmann et al., 1993).

3.6. NMR data

The NMR spectra of the isolated droserone (**3**) were measured in $CDCl_3$ with a Bruker DMX 600 using broadband decoupling. ^1J(C,C) coupling constants and patterns were determined by analysis of ^{13}C-NMR and 1D-INADEQUATE experiments. The relaxation delay for ^{13}C-NMR was set to 5 s.

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