STUDIES WITH PLANT CELL CULTURES OF PODOPHYLLUM PELTATUM L. I. PRODUCTION OF PODOPHYLLOTOXIN, DEOXYPODOPHYLLOTOXIN, PODOPHYLLOTOXONE, AND 4'-DEMETHYLPODOPHYLLOTOXIN

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Abstract - Podophyllotoxin (1) isolated from living plants of Podophyllum peltatum L., is presently the starting material for the commercial production of the clinical anti-cancer drug etoposide. A two-step chemical conversion of 1 affords the analogue, 4'-demethylepipodophyllotoxin (2) and the latter, via a five step chemical process, provides this important drug. The direct production of 1 and/or its appropriate analogues via processes which do not depend on the living plant would provide an attractive alternative route to this compound. The first successful development of a stable plant cell culture line of P. peltatum which provides 1 and its analogues, deoxypodophyllotoxin (3), podophylllotoxone (4) and 4'-demethylpodophyllotoxin (5), via fermentation techniques is described.

The podophyllotoxin (1) family of naturally occurring lignan lactones has been the subject of extensive studies over the years.[1] The more recent development of the clinical anti-cancer drug etoposide[2,3] for which 1 becomes an important starting material in commercial production, has stimulated further studies directed toward achieving efficient routes to 1 and/or its closely related analogues. Our program on plant cell culture development for the production of secondary metabolites has focussed, in one of its phases, on the development of an appropriate cell line of Podophyllum peltatum L., a plant from which 1 and a series of related lignans are

isolated, in order to afford such compounds by fermentation technology. The present communication describes our success in this direction.

Leaf, stem and root explants of P. peltatum L. were initiated on one of Kadkade's[5] variations of Murashige-Skoog[4] (MS) medium that contained 2,4-D (0.1 mg/l), kinetin (0.2 mg/l), casamino acids (Difco: 500 mg/l) and agar (Difco Bacto: 8 g/l). As van Uden et al.[7] found with P. hexandrum Royal, we found extreme difficulty in initial propagation of callus due to severe intertissue fungal contamination, especially in root material, and to lethal tissue browning. Although many stem and three root-derived calli survived, extreme sensitivity to the effects of tissue browning persisted and required scrupulous removal of any dark tissue at each transfer. Currently, calli are maintained on half-strength MS agar supplemented with napthalene-acetic acid (NAA: 1.0 mg/l), kinetin (0.2 mg/l) and casamino acids (100 mg/l).

Shake flask studies for growth improvement of suspension cultures led to media with low auxin content in which root-derived cell line R3 showed signs of organogenesis. Repeated manual selection of root structures from filtered culture provided inocula that eventually gave healthy, differentiated suspensions. Subsequent removal of hormones, optimization of sucrose content (15 g/l) and rebalancing of the medium to one half standard salts concentration gave a healthy, predominantly embryonic, habituated cell line that has been perpetuated in hormone-free, half-strength MS broth (initial pH 5.8) since September 1988. Subculture requires filtration to obtain cell aggregates free of spent medium and inoculation of fresh medium at a rate of 15 ml aggregates per 250 ml broth. Biomass doubles once (from 4 to 8.5 g/l) and stationary phase is reached in 14-16 days. Shake flask cultures were agitated at 135 rpm with a 4.8 cm throw.

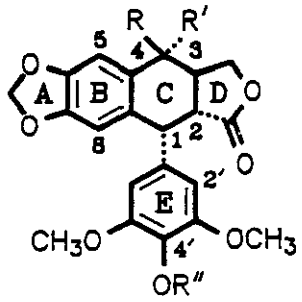
Bioreactor cultivations were done in continuously-stirred glass reactors fitted with turbine impellers (New Brunswick Scientific, Microferm model, 7.5 l total capacity). Agitation was set at 440 rpm and aeration at 0.1 vvm. Stationary phase cultures of 21-22 days were harvested for extraction and analysis. (Note: All cultures were grown at 26° C in darkness.)

The same extraction procedure was generally employed for the purpose of isolating the various metabolites produced in either shake flasks or bioreactors. After separation of broth and cells by filtration through Miracloth (Calbiochem), the broth was extracted in a liquid-liquid continuous extraction apparatus (dichloromethane, 100 h) and the resulting crude

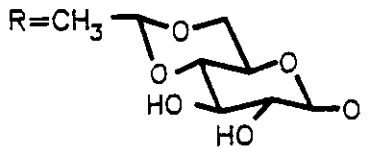
extract was subjected to column chromatography (silica gel, dichloromethane:acetone 4:1). Two compounds, β-sitosterol, obtained in the initial chromatography fractions and podophyllotoxin (1) in subsequent fractions were isolated and characterized by the appropriate (nmr and ms spectra) spectroscopic comparison with authentic samples.

The cells were homogenized (Ultra-Turrax T-25 disperser), extracted (Soxhlet, dichloromethane, 116 h) and the resulting extract chromatographed (silica gel, dichloromethane: acetone 4:1) to afford, in order of elution, deoxypodophyllotoxin (3), podophyllotoxone (4) and 4'-demethylpodophyllotoxin (5). These compounds (3-5) were compared (nmr and ms) with authentic samples kindly provided by Prof. H. Yamaguchi, Osaka University of Pharmaceutical Sciences, Osaka, Japan.

In a 5.5 liter bioreactor culture, the following yields were obtained: podophyllotoxin (1, 22 mg in broth and 127 mg in cells for a total of 149 mg), 4'-demethylpodophyllotoxin (5, 36 mg), and a mixture (17 mg) of deoxypodophyllotoxin (3) and podophyllotoxone (4). The dry biomass of mature suspension cultures has been determined to be 7.5 - 8.5 g/l. On this basis, the percent yield of 1 in this bioreactor study is estimated at 0.32 to 0.36%.



1, R = H; R′ = OH; R″ = CH_3
2, R = OH; R′ = H; R″ = H
3, R = R′ = H; R″ = CH_3
4, R = R′ = O; R″ = CH_3
5, R = R″ = H; R′ = OH

etoposide: 

R′ = H; R″ = H

Although Kadkade[5] first reported podophyllotoxin presence in P. peltatum callus in 1982, only recently have new studies of the production of podophyllotoxins by cell culture techniques been published. Dewick[6] reported the isolation of 1 and 5 from a callus culture of P. hexandrum. Dutch workers[7] reported isolation of 1 (0.1% based on dry weight) from a cell suspension of P. hexandrum. Cell suspension cultures of Linum flavum[8,9] have been established and shown to produce 5-methoxypodophyllotoxin.

In L. flavum, the highest yields of 5-methoxypodophyllotoxin (0.7-1.3% dry weight basis) were obtained from root cultures[8], and yields in undifferentiated suspension cultures could be improved (to a maximum of 0.16-0.20%) by transfer to auxin-free media[9]. In light of our findings that undifferentiated suspension cultures of P. peltatum were unstable and produced only trace amounts of podophyllotoxins and that a habituated embryonic suspension culture of P. peltatum produces significant amounts, it may be a general property of more highly differentiated cultures of these species to produce higher yields of podophyllotoxins.

The present study affords the first successful development of a stable cell line of P. peltatum which can be successfully grown in larger scale bioreactors and which affords several podophyllotoxin analogues. The isolation of 5 is of particular interest in terms of its relationship to etoposide, the latter also lacking a methoxyl function at C-4'.

In conclusion, it should be noted that the fermentation parameters for optimum yields of 1 and 3-5 have not yet been determined so that substantially higher yields of these compounds are anticipated after further studies in our laboratories.

Studies with synthetic "precursors" and their biotransformation by cell cultures of P. peltatum to novel podophyllotoxin analogues will be published later.

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