# STUDIES WITH PLANT CELL CULTURES OF PODOPHYLLUM PELTATUM L. II. BIOTRANSFORMATION OF DIBENZYLBUTANOLIDES TO LIGNANS. DEVELOPMENT OF A "BIOLOGICAL FACTORY" FOR LIGNAN SYNTHESIS

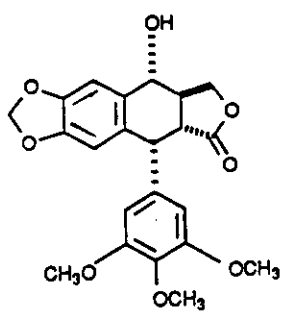
James P. Kutney*, Yung Ping Chen, Shixiang Gao, Gary M. Hewitt, Francisco Kuri-Brena, Radka K. Milanova, and Nikolay M. Stoynov

Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, B.C., Canada V6T 1Z1

Abstract - An efficient and versatile synthetic route to appropriate dibenzylbutanolides suitable for biotransformation to lignans as potential intermediates for the syntheses of the clinical anti-cancer drug etoposide has been developed. Biotransformation of such substrates, for example 10, with cell cultures of Podophyllum peltatum affords a potentially exciting route to this drug. Of particular significance is the development of a semi-continuous fermentation process with these plant cells wherein successive additions of substrate and isolation of end products can be pursued with a given batch of cells and over a period of several months. Biotransformation times for the conversion of 10 to 11 and 12 are short (usually 24-48 h). Although conditions have not yet optimized, this "biological factory" for lignan synthesis provides a

novel approach for the use of plant cells, in combination with chemistry, for continuous and large scale production of such metabolites.

In a previous publication,[1] we described the first successful development of a cell line of Podophyllum peltatum, the plant from which podophyllotoxin (1) is isolated and presently utilized as the starting material in the commercial synthesis of etoposide (2). We indicated that this cell line which produces 1 and several other analogues could be utilized in an alternative approach directed at development of a synthesis of 2 without depending on isolation of 1 from the living plant. In considering such methodology, it must be recognized that, in general, the biosynthesis of complex secondary metabolites by plant cell cultures growing in nutrient media, often takes several weeks and this rather long period is considered one of the drawbacks in the use of this approach in commercial production of plant derived medicinal agents. However, having developed an appropriate cell line, it is possible to consider its utilization in biotransformation experiments in which suitable synthetic sub-strates are converted by the enzymes, inherently present in the cell culture, to the desired end products. Such enzyme-catalyzed processes invariably occur in short time periods, (for example, several hours - 48 h) and gram scale conversions are possible. This approach has received considerable attention in various avenues of our program,[2-5] a substantial portion of which remains unpublished, and we wish to present a novel approach to the use of plant cell cultures in combination with chemistry for the synthesis of lignans as potential routes to 2.

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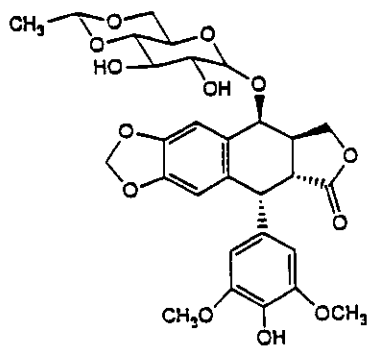


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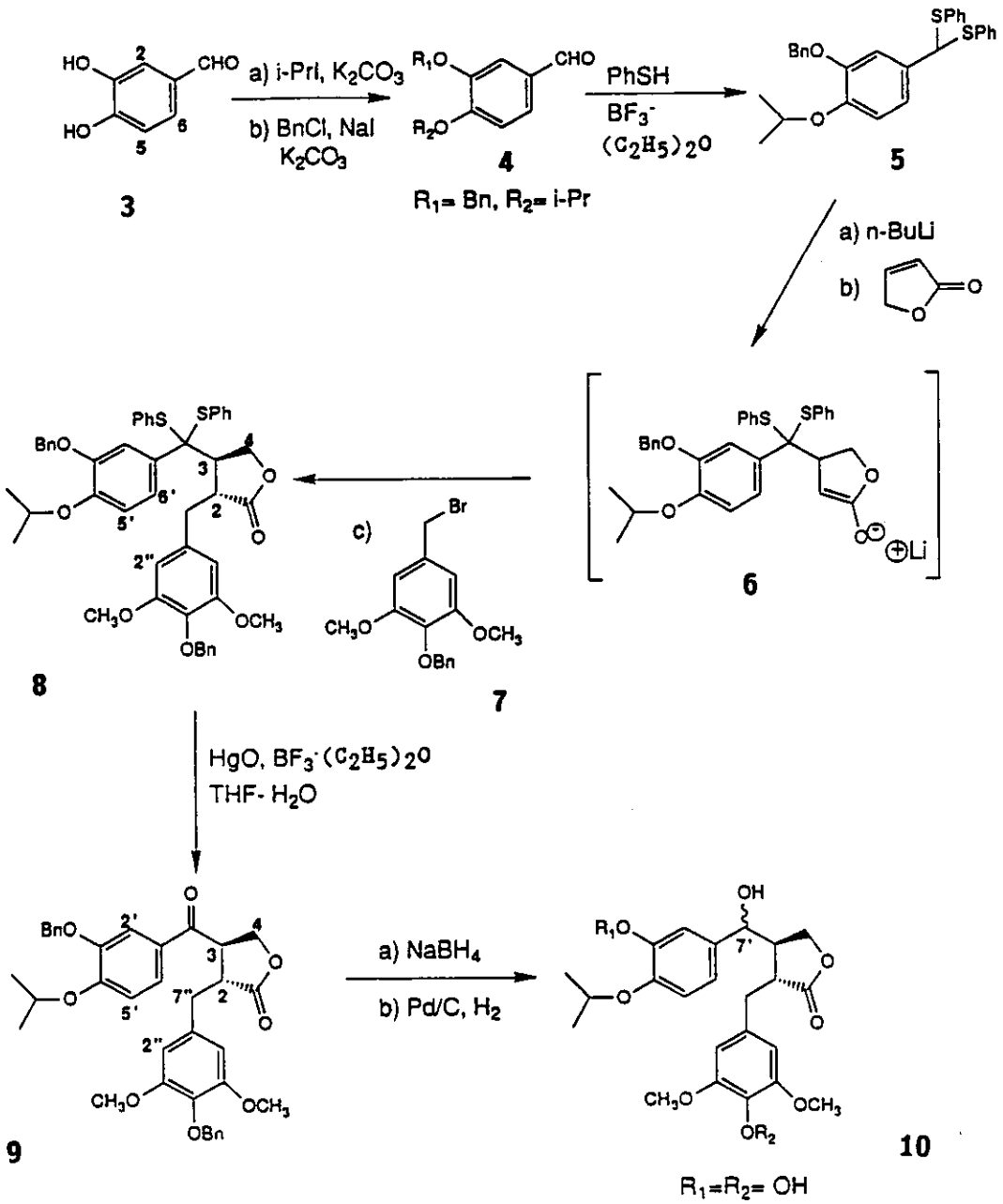
In developing synthetic routes to the substrates to be utilized in the plant cell culture stimulated biotransformations, it was deemed appropriate to consider "templates" which bear close relationship to the proposed biosynthetic pathway and to evaluate reactions which are of general significance within the area. In this regard, from the research of Dewick and coworkers[6] in their study on the biosynthesis of the podophyllotoxins, the importance of dibenzylbutanolides as late stage intermediates in the pathway, was clear and their enzyme-catalyzed oxidative coupling to the cyclic lignan systems became of focus in our study.

A versatile and efficient route to such substrates is outlined in Scheme 1. Reaction of the commercially available catechol (3) with isopropyl iodide (1.3 equiv.) in DMSO using potassium carbonate as base allows selective alkylation at C-4 and the latter is subjected directly to benzylation to afford the protected aldehyde (4) (70% yield). Conversion of 4 to the thioketal derivative (5) required caution (-40° C) to avoid ether hydrolysis. The other component (7) needed for the synthesis of 8, was prepared from commercially available syringaldehyde by benzylation (benzyl chloride, $K_2CO_3$, catalytic amount of NaI, 85% yield), aldehyde reduction ($NaBH_4$ in ethanol, 97% yield) and reaction with $PBr_3$ in ether (76% yield).

The key step in the synthetic plan includes the tandem conjugate addition of the anion of thioketal (5) to γ-crotonolactone and subsequent alkylation of the intermediate enolate (6) with the aryl bromide (7). The conversion, 5 → 6 → 8, can be conducted as a "one-pot" process if reaction conditions are carefully controlled. Optimum results (overall yield of 8, 75%) are obtained when 5 is reacted with n-butyllithium at a stringently controlled low temperature (-70° C, 25 min), stirring continued for 40 min and 7 then added slowly while allowing the reaction temperature to rise gradually to room temperature.

The generality of the above sequence is clear from parallel studies in our laboratory, and not discussed here, in which other functionalized aldehydes (4, $R_1 = R_2 = CH_2$; $R_1 = H$; $R_2 = CH_3$) when subjected to this tandem Michael-alkylation sequence, similarly afford high yields of the corresponding analogues of 8.

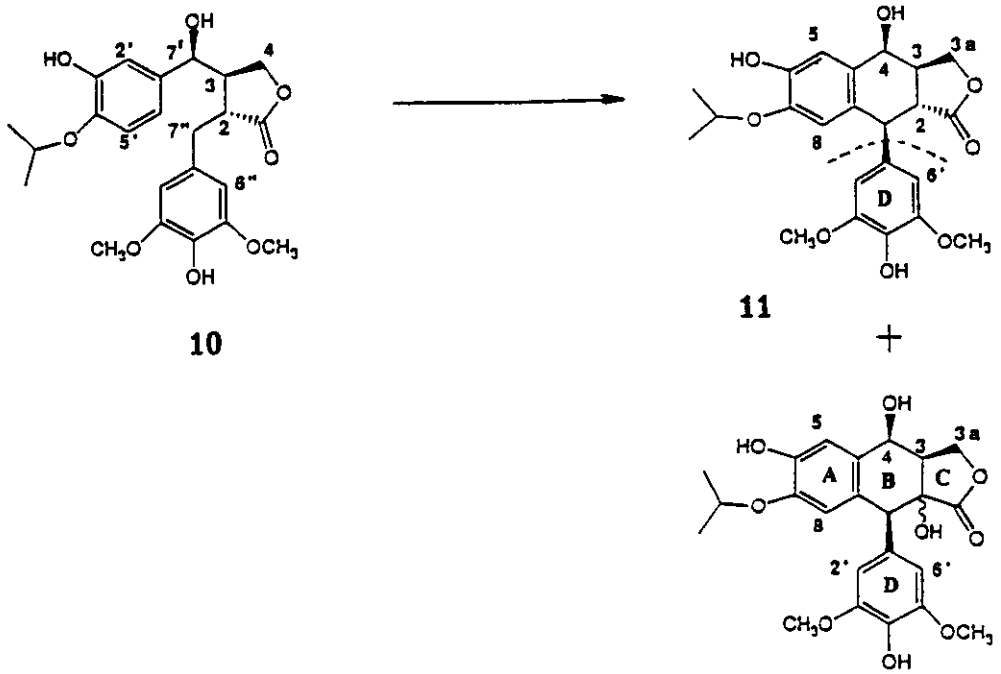
Conversion of 8 to 9 with mercury (II) oxide-boron trifluoride etherate at 0° C, proceeded in excellent yield and subsequent standard reduction of 9 and removal of the benzyl protecting groups provided the desired dibenzylbutanolide (10). The latter compound,


a) i-PrI, K2CO3
b) BnCl, NaI
K2CO3
PhSH
BF3-
(C2H5)2O
a) n-BuLi
b)
c)
HgO, BF3·(C2H5)2O
THF-H2O
a) NaBH4
b) Pd/C, H2
R1= Bn, R2= i-Pr
R1=R2= OH

Scheme 1.

isolated as a colorless foam, possesses the desired stereochemistry at C-7′ (multiplet, $\delta$ 4.52-4.62). The corresponding $\alpha$ isomer obtained as a minor product reveals a distinct doublet ($\delta$ 4.41, J = 7 Hz).

With 10 on hand, enzyme-catalyzed oxidative cyclization to the appropriate cyclic lignan (11) (Scheme 2) was now considered. Incubation of 10 in a Microferm bioreactor was performed under various ages of cells (2-17 days old) and either as (i) one batch addition of 10 or (ii) semi-continuous fermentation of 10. Typical procedures for (i) and (ii) are briefly discussed.




Scheme 2.

In general, inoculum was grown for 15-18 days in shake flasks in Murashige-Skoog medium as discussed in the previous publication,[1] and then transferred to the bioreactor in the earlier described manner[1] where the cells were allowed to grow for 2-17 days prior to substrate addition.

In one study involving one-batch addition of 10 to growing cells of P. peltatum culture, the substrate (3.0 g in 70 ml of ethanol) was added to 2 day old cells in a 3 $\ell$ bioreactor and the biotransformation allowed to proceed for 96 h prior to harvest. Ethyl acetate extraction of broth and cells, followed by chromatographic purification of each extract,

afforded the desired aryl tetralin (11) (34%) and the hydroxylated analogue (12) (6%). Minor amounts (1-3%) of other components, not discussed here, were also obtained and an overall recovery of organic products was only 45%. It was therefore clear that the long biotransformation time was detrimental to recovery, with substantial (>50%) amounts of water-soluble materials resulting.

Spectroscopic data for 11 were consistent with the structure proposed. For example, the $^1H$ nmr spectrum revealed only aromatic proton singlets ($\delta$ 6.46 for protons at C-2'and C-6'; $\delta$ 6.38 and 6.88 for protons at C-5 and C-8 respectively), in contrast to ortho and meta coupling normally observed in the spectrum of 10. The trans relationship of the lactone ring is confirmed from the C-2 proton signal (doublet of doublets at $\delta$ 3.19 with coupling constants of 11.4 and 14 Hz). Mass spectrometry indicated $M^+$ at m/z 430 (compare $M^+$ for m/z at 432 for 10) and a fragmentation pattern typical of cyclic lignans (m/z 154, loss of ring D).

The lignan (12), obtained as a minor component in the above study, possessed the molecular formula $C_{23}H_{26}O_6$ (high resolution mass spectrometry), a strong $M^+$-18 fragment suggesting facile loss of water and a uv spectrum (maxima at 290 and 206 nm) consistent with a typical lignan chromophore. In the $^1H$ nmr spectrum, aromatic proton singlets are observed at $\delta$ 7.08, 6.61 and 6.21 for the protons at C-5, C-8, C-2'(6') respectively. The typical proton signal at C-2 (doublet of doublets at $\delta$ 3.19) present in the spectrum of 11, is now absent.

The majority (approx. 90%) of the above products is present in the broth and therefore the possibillity of a semi-continuous ("draw and fill") fermentation approach was considered. It was felt that if the enzymes present in the cells could remain stable, then a number of additions of substrate (10) could be made and repeated isolations of 11 and 12 from the broth could be possible. Indeed, this novel approach proved highly successful and a brief description is provided here.

In the various experiments utilizing the semi-continuous process, the Microferm bioreactor is fitted with a peristaltic pump and a filter that enables the removal of the broth and resuspension of the cells in situ. Cells are then grown in the bioreactor for different periods, 7 days (growth phase), 17 days (stationary phase), after which time the spent medium containing cell-produced components is removed, new diluted medium (one-tenth

concentration of the original Murashige-Skoog medium[1] and 0.3% sucrose) with just enough nutrients to maintain live cells and containing 10, is added, and the biotransformation allowed to proceed for approximately 24 h. The broth containing the metabolites (11) and (12) is removed, new diluted medium and substrate (10) is added and the process repeated. Our studies, not yet optimized, have already shown that sixteen additions of 10 to the bioreactor could be made with yields of the cyclic products (11) and (12) in the range of 50% and 13% respectively. The cell age in this latter case was over two months and enzymatic activity for the desired cyclization was still present. In fact, interruption after the 16 additions was due to depletion of supply of 10 rather than lack of enzymatic activity. The yields of 11 and 12 do not change significantly with cell age although the younger cells appear to afford slightly higher yields.

Longer biotransformation times favor formation of 12 (40% yield thus far) although again optimal fermentation parameters have not yet been developed.

In conclusion, this "biological factory" allows continuous synthesis of such lignan analogues and we feel confident that higher yields of end products can be achieved with further studies involving control of pH, substrate concentration, etc.

Finally, it should be noted that 11 and 12 are convertible to podophyllotoxin analogues containing the methylenedioxy functionality in ring A since chemical methodology ($BBr_3$, catalyzed removal of the isopropyl side chain followed by reaction of the catechol function in ring A with methylene iodide) has been developed in our laboratory. Dehydration of 12 to a styrene analogue, followed by reduction of the double bond in the latter, allow generation of the natural stereochemistry at C-1. Such studies will be reported in future communications.

ACKNOWLEDGEMENT

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