BIOTRANSFORMATION OF DIBENZYLBUTANOLIDES BY PEROXIDASE ENZYMES. ROUTES TO THE PODOPHYLLOTOXIN FAMILY.

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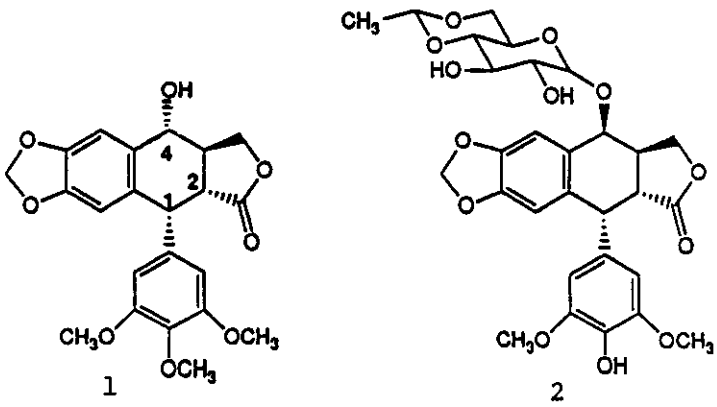
Abstract - In continuing studies on enzyme-catalyzed cyclization of synthetic dibenzylbutanolides to members of the podophyllotoxin family, experiments which involve the biotransformation of such synthetic substrates with horseradish peroxidase and with enzymes produced by the cell culture of Nicotiana sylvestris are presented. Cyclization of synthetic **3** to the desired **5** proceeds efficiently with purified horseradish peroxidase enzyme but not with the acetone powder. A much preferred route to **5**, in gram scale synthesis, involves the biotransformation of **3** with the enzymes produced by the cell culture of Nicotiana sylvestris. This stable cell line, when grown in bioreactors, produces sufficient quantities of enzyme for efficient multi-gram synthesis in short time incubations (5-30 min). The application of this methodology for production of podophyllotoxins utilized as starting materials in etoposide synthesis is under study in our laboratory.

The podophyllotoxin family of natural products and particularly those members which bear the "classical" five-membered lactone system (see **1**) have been extensively studied over the years. Several excellent recent reviews[1,2] provide a summary of the chemistry and syntheses of these compounds. The development of the clinical anti-cancer drug, Etoposide (VP-16) (**2**),[2-5] continues to stimulate further studies directed at improved

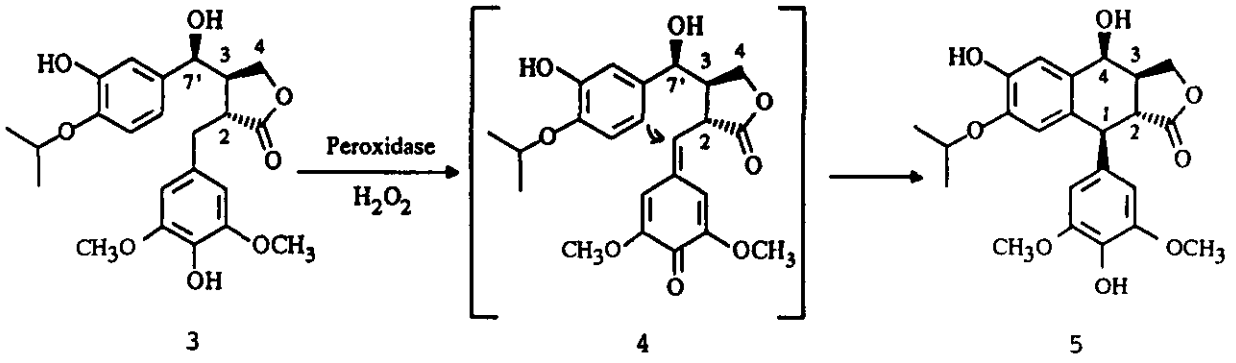
** Dedicated to the memory of the late Professor Yoshio Ban, an outstanding scientist and longtime friend.

synthetic routes to the podophyllotoxins in the hope that such programs would afford more efficient avenues to these important starting materials for Etoposide production. As suggested in several earlier publications[6,7] our approach is directed toward the utilization of a combination of biotechnological and chemical methods so as to achieve these objectives. The present communication describes some recent results pertaining to this area.



In our earlier studies,[6,7] we described the development of a plant cell culture of Podophyllum peltatum, one of the plants from which the present commercial supply of podophyllotoxin (**1**) is derived, and its utilization as an "enzyme source" for the cyclization of synthetic dibenzylbutanolides (see **3**) to the desired cyclic podophyllotoxins. It was clear from those investigations that such enzyme-catalyzed reactions were likely accomplished by peroxidase enzymes (Scheme 1) and it was appropriate to evaluate such enzyme systems as derived from other sources. For comparison, the commercially available horseradish peroxidase (HRP) and a cell culture line of Nicotiana sylvestris,[8,9] an efficient source of peroxidase enzymes, studied extensively in our laboratory were selected for this purpose. The previously synthesized dibenzylbutanolide (**3**)[7] was utilized as the substrate in the following biotransformation experiments.

Two independent sets of experiments were conducted in order to compare the efficiency of cyclization of **3** to **5**. The first set involved these enzyme-catalyzed cyclizations in which a commercially available sample of horseradish peroxidase (HRP) was utilized while the other set of experiments was performed with a crude enzyme preparation obtained from the cell culture of Nicotiana sylvestris previously developed in earlier studies.[8,9] The cells of this culture excrete peroxidases into the broth during growth in an airlift bioreactor. Enzymatic activity was assayed by the published procedure[10] and was shown to vary with the age of cells (3-



Scheme 1. Biotransformation of dibenzylbutanolide (**3**) to cyclic product (**5**) via hypothetical quinone methide intermediate (**4**) with peroxidase enzymes.

11 days). The highest level of peroxidase activity was found in broth from 10 day old cultures and this preparation was selected for the studies presented below. A typical experiment outlining the general procedure employed for the studies summarized in Table 1 is described. It should be emphasized that **5** is an exclusive product and the corresponding C-1 epimer is not obtained.

Typical experimental procedure (Experiment shown as superscript C in Table 1). The dibenzylbutanolide (**3**, 200 mg) was dissolved in ethanol (20 ml) and added to a mixture of water (222 ml) and phosphate buffer (10 ml, 0.2 M, pH 6.3) contained in an Erlenmeyer flask mounted on a rotary shaker (135 rpm) with a controlled water bath. After this mixture had attained a temperature of 14°C (30 min), H_2O_2 (0.5%, 3.14 ml) and the N. sylvestris broth containing a peroxidase activity of 1.05 units/ml (44 ml or 46.2 units of peroxidase and corresponding to 100 units of enzyme per mmol of substrate (**3**)) were added. The final concentration of this mixture was as follows: water (74%); buffer (3.3% or 6.67 mmol); peroxidase (0.154 units/ml); substrate (**3**) (0.667 g/L); ethanol (6.6%); and H_2O_2 (0.0052% or 1.539 mmol).

The stirred mixture was monitored by tlc (chloroform:methanol, 9:1) at 5 min intervals until completion (20-25 min). Ethyl acetate was added, mixture filtered through Celite 545, organic layer concentrated in vacuo to afford the previously established[7] cyclic product (**5**).

The above parameters for the HRP experiments were identical with those presented above except that the reaction temperature was 12°C, a lower amount of ethanol was used (3% versus 6.6%) and the phosphate buffer was more concentrated (40 mmol versus 6.67 mmol).

Table 1. Summary of results obtained in the biotransformation of dibenzylbutanolide (**3**) to podophyllotoxin analogue(s) by peroxidase enzymes.

Peroxidase type	Peroxidase	Substrate 3		Volume			Volume of enzyme solution	H_2O_2	Duration	Recovered substrate 3		Yield of product 5[d]	
	(units/ mmol substrate 3)	(mg)	(g/*l*)	H_2O (m*l*)	EtOH (m*l*)	Buffer (m*l*)	(m*l*)	(mol/mmol substrate 3)	(min)	(mg)	(%)	(mg)	(%)
HRP[a]	1580	549	1.00	394	16.5	27.5	82.5	3.3	35	80	15	350	81.5
HRP	1580	533	1.00	383	16	26.5	80	3.3	35	77	14	340	80
HRP	1580	506	1.00	369	15	25	76	2.2	30	7	1.4	371	81
HRP	1800	500	1.00	368	15	9	91	2.2	30	2	0.4	356	78
HRP	2000	500	1.00	368	15	9	91	2.2	30	5	1.0	354	81
N. sylvestris[b]	50	200	0.67	244	20	10	22	1.0	40	-	-	144	87
N. sylvestris	100[c]	200	0.67	222	20	10	44	1.0	20-25	-	-	140	70
N. sylvestris	150	200	0.67	200	20	10	66	1.0	20-25	-	-	134	67
N. sylvestris	200	200	0.67	178	20	10	88	1.0	20	-	-	116	72
N. sylvestris	250	200	0.67	156	20	10	110	1.0	15-20	-	-	114	69
N. sylvestris	226	200	0.67	164	20	10	99.5	2.0	15-20	-	-	104	70
N. sylvestris	150	1000	0.67	1007	100	50	330	0.8	17	50	5.0	650	68.5
N. sylvestris	150	500[e]	0.67	503	50	25	165	0.8	20	20	4.0	318	66

[a] Horseradish peroxidase was obtained from Sigma (Type VIa) and peroxidase activity was established by a published procedure.[10]
[b] *N. sylvestris* was grown in suspension culture on Gamborg's B5 medium[11] supplemented with 2,4-dichlorophenoxyacetic acid (1.0 mg/*l*). Peroxidase assay in separated broth was performed as in published procedure.[10]
[c] Experiment described in discussion.
[d] Yield based on the consumed dibenzylbutanolide.
[e] 7'α-Hydroxydibenzylbutanolide was used in this experiment.

As noted from Table 1, both purified HRP enzyme and the crude enzyme preparation generated during the growth of the cell culture of N. sylvestris afford interesting results but with some significant variations in the reaction parameters and yields of podophyllotoxin analogue (**5**). One striking variation between HRP and N. sylvestris derived enzymes is the very significant difference between the enzyme units/mmol of substrate required in the two sets of experiments. As the data in Table 1 indicate, good yields of product (**5**) are obtained with purified HRP only when high levels of HRP units are utilized (1500-2000 units) but with N. sylvestris derived "peroxidases" comparable yields of **5** are obtained with a low level of units (50 units, for example). It should be emphasized that an identical assay method[10] was employed in our laboratory to evaluate the "peroxidase" activity in all cases. Although conclusive data to rationalize these differences are not available, one important factor relates to enzymatic "stability". While the commercial HRP employed in the present studies rapidly loses its activity in phosphate buffer (essentially complete loss of peroxidase activity in 30-40 min at room temperature), the enzyme preparation derived from N. sylvestris is remarkably stable exhibiting significant peroxidase activity even after 16 days standing at 4°C (only 11% loss in activity). It is therefore possible that in the experiments involving HRP, significant denaturation of the enzyme is occurring during the duration time involved in the cyclization of **3** to **5** while little or no loss of activity occurs during a similar duration involving the plant cell culture derived enzyme preparation.

The advantages of the plant cell culture derived "peroxidases" over the expensive commercial HRP samples for gram scale preparations are obvious. The problem of enzyme instability has been noted above and the costs involved for large scale experiments with HRP are prohibitive. It should be noted that the less expensive "acetone powder" HRP did not provide encouraging results. In contrast, a conventional 10 liter bioreactor of N. sylvestris cells produces sufficient enzyme for cyclization of **3** to **5** at the level of several hundred grams.

The economically attractive route of a semi-continuous process for the enzyme-catalyzed cyclization reaction described above and evaluated in earlier studies with a cell culture line of Podophyllum peltatum[7] is presently under consideration with N. sylvestris.

ACKNOWLEDGEMENT

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