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Plant Cell Culture Combined with Chemistry: A Powerful Route to Complex Natural Products

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Introduction

Plant-derived natural products continue to stimulate extensive investigations among a wide family of chemists interested in their structure, synthesis, and biosynthesis. In particular, those natural systems which portray important applications in the pharmaceutical, agrochemical, and fragrance industries, for example, attract attention from synthetic chemists who attempt to develop efficient synthetic routes to the target compound, thereby eliminating the dependence on the living plant as the source. However, often the structural complexity, inherent in such natural products, demands multistep syntheses, which although of distinct academic interest are rarely of practical importance for large-scale industrial production. One possible solution to the latter requirement may come from the development of plant cell cultures derived from tissue of the living plant used as the source of the natural product, and in combination with chemistry, routes may be developed which ultimately provide an efficient source of such compounds. Fermentation technology, utilizing fungal and bacterial cultures, has made dramatic advances particularly in the pharmaceutical industry, but similar technology with plant cells has not yet reached this level of application. It was our desire to initiate a program with the latter objective in mind. The present Account illustrates, with specific examples from the author's laboratory, how a combination of plant cell culture methodology with chemistry does allow the development of efficient routes to complex natural products. Apart from the "academic" interest, such a strategy, at least in selected instances, can afford entries into large-scale (commercial) production of industrially important compounds.

The methodology of plant cell culture development is not new and is a rapidly expanding area of research. It is beyond the scope of this brief Account to detail the large number of elegant contributions from laboratories all over the world, so the reader is directed to extensive reviews[1-7] on this subject. It should be noted, however,

that a substantial percentage of these studies are not specifically directed to the production of natural products (secondary metabolites) but rather to the use of plant cell cultures to evaluate the more "biological" aspects of such cultures (cell growth regulation, cell structure, cytodifferentiation, somatic embryogenesis, morphogenesis, physiology, etc.). Other investigators have utilized these cultures in elegant biosynthetic studies, as sources of enzymes, etc. Although more recent investigations have been more focused in the direction of metabolite production, the overall approach is generally rather different from the strategy developed in our laboratory. Our approach, which emphasizes a continual interplay of synthetic chemistry with plant cell culture methodology, can provide different results, and the examples cited herein illustrate the various objectives achieved.

Avenues of Research

Our program can be conveniently divided into four sections, and the discussion following will present studies within these areas: (1) Biosynthetic Information Forms a Basis for Efficient Synthesis of Complex Natural Products; (2) Plant Cell Cultures as "Reagents" in Organic Syntheses; (3) Use of Plant Cell Cultures To Produce Higher Levels of Pharmaceutically Interesting Compounds; (4) Use of Plant Cell Cultures To Separate Pharmacological Activities in Complex Herbal Medicine Extracts.

1. Biosynthetic Information Forms a Basis for Efficient Synthesis of Complex Natural Products. A large number of investigators have employed plant cell cultures with considerable advantage over living plants, for unraveling biosynthetic pathways of a variety of natural products. A stable cell line, once developed, can afford a reliable source of enzymes, the latter obtained under established conditions, to provide the opportunity for controlled experiments designed to obtain biosynthetic intermediates in a selected pathway. One of the particular interests in our program was to utilize information derived from such biosynthetic

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(2) Fujiwara, A., Ed. *Plant Tissue Culture 1982*; Maruzen Co., Ltd.: Tokyo, 1982.
(3) Neumann, K. H.; Ed. *Primary and Secondary Metabolism of Plant Cell Cultures*; Springer-Verlag: Berlin, 1985.
(4) Misawa, M. *Adv. Biochem. Eng. Biotech.* 1985, *31*, 59.
(5) Charlwood, B. V.; Rhodes, M. J. C. *Secondary Products from Plant Tissue Culture*; Clarendon Press: Oxford, 1990.
(6) Komamine, A., Ed. *Plant Cell Culture in Japan*; CMC Co., Ltd.: Tokyo, 1991.
(7) Payne, G. F.; Bringi, V.; Price, C. L.; Shuler, M. L. *Plant Cell and Tissue Culture in Liquid Systems*; Hanser Press: Munich, 1992.

C. roseus Tissue Culture

  ↓ homogenized in 0.1 M potassium
      phosphate buffer, pH 6.3

 ↓ centrifuged at 30,000 x g for
      20 min

Crude Enzyme

  ↓ ammonium sulfate precipitation
      (70% saturation) dialysis

  ↓ DEAE-cellulose chromatography

  ↓ Sephadex G-200 chromatography

Partially Purified Enzyme

Figure 1. Isolation of enzymes from cell culture of *C. roseus*.

experiments to develop highly efficient syntheses of natural products. In the initial stages, we directed our attention to the clinically important anticancer drugs vinblastine (VLB, **7**, R = Me) and vincristine (VCR, **7**, R = CHO)—a problem that has attracted the interest of synthetic chemists for many years.

1.1. Studies with the *Catharanthus roseus* Cell Line. Detailed accounts of our extensive studies in this area, involving development of cell lines, indole alkaloid production, and isolation and immobilization of enzymes derived from the *C. roseus* cell line, are published. These experiments, which also include biotransformation of "precursors" to unravel the probable structures of biosynthetic intermediates involved in the biosynthesis of VLB, form the subject of approximately 20 publications and of several recent summaries.[8,9] For this reason, only a brief description of the most salient features to illustrate the approach of Section 1 will be presented here.

The isolated enzymes obtained from the *C. roseus* cell line (Figure 1) were utilized in biosynthetic experiments with the alkaloids catharanthine (**1**) and vindoline (**3**) as "precursors" to initially establish the unstable dihydropyridinium intermediate (**4**) (Figure 2) as the first formed biosynthetic intermediate. With an enzyme preparation immobilized, via the technique of affinity gel chromatography, on 2′,5′-ADP–Sepharose,[9,10] a 90% "coupling" of **1** and **3** to **4** could be achieved in 15–30-min incubations. The enzyme(s) involved in this coupling process were shown to be of the "peroxidase" family. Longer incubation times (30–45 min) led to the disappearance of **4** (HPLC monitoring) and the appearance of enamine **5**. A separate experiment involving reduction of **4** to **5** by a commercial preparation of NADH established that such a reductive process involves NADH-like enzymes present in the enzyme preparation obtained via the isolation procedure shown in Figure 1. Further incubation studies with the crude enzyme preparation revealed disappearance of **5** to afford a new intermediate, proposed as **6**, and the latter finally converts to VLB (**7**). In summary, the sequence from **1** + **3** → **4** → **5** → **6** → **7** involves oxidation, reduction, oxidation, and reduction in successive fashion. These data suggest that the biosynthesis of these
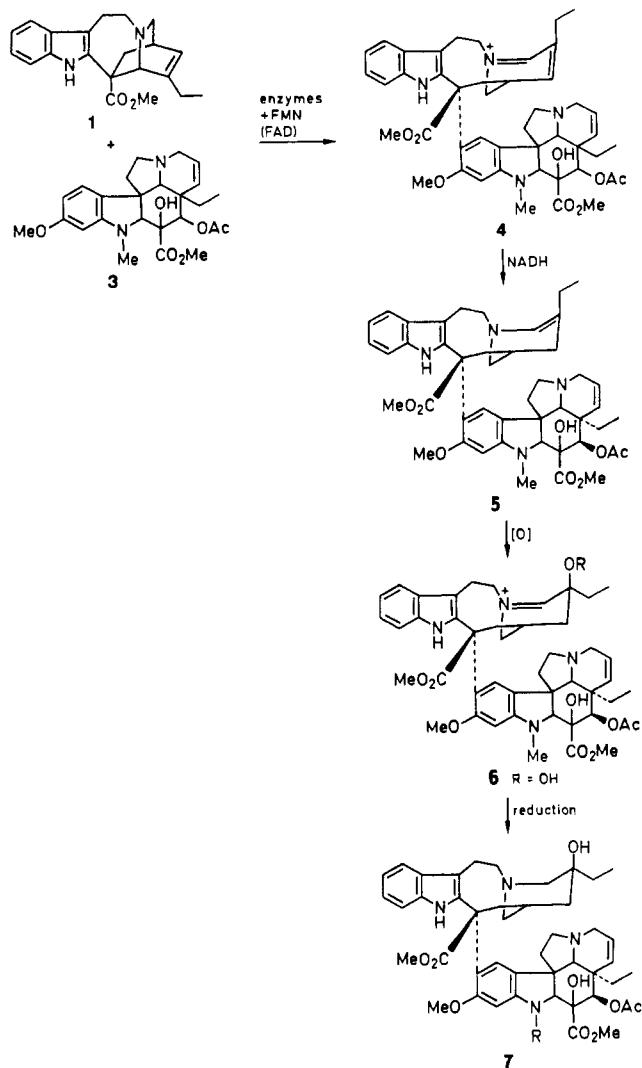
Figure 2. Overall summary of the biosynthetic pathway of vinblastine (**7**) from catharanthine (**1**) and vindoline (**3**).

bisindole alkaloids within the plant is likely a result of careful enzymatic control involving oxidative and reductive processes.

1.2. Biomimetic Studies—Biosynthetic Information Sets the Stage for an Efficient Synthesis of Vinblastine. The overall enzymatic conversions summarized in Figure 2 could now form the basis of a chemical synthetic route to VLB. Earlier studies in our laboratory[11] and elsewhere[12] had established that the *chemical* coupling of catharanthine (**1**) and vindoline (**3**) to **4** could be achieved via catharanthine N-oxide (**2**, Figure 3), the latter being produced by MCPBA reaction with **1**. Coupling of **2** with **3** is successful with trifluoroacetic anhydride as a catalyst to stimulate a Polonovski-type reaction to afford **4**. In effect, MCPBA becomes the chemical "mimic" for the peroxidase enzyme involved in the enzyme-catalyzed coupling of **1** and **3** as shown in Figure 2. We have established that the N-oxide (**2**) is *not* accepted as a precursor in the enzymatic process to **4**, so a different "activation" of **1** by the enzyme is involved (see discussion in refs 8 and 9 for details). From the

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(9) Kutney, J. P. *Nat. Prod. Rep.* **1990**, *7*, 85 and references therein.
(10) Kutney, J. P.; Boulet, C. A.; Choi, L. S. L.; Gustowski, W.; McHugh, M.; Nakano, J.; Nikaido, T.; Tsukamoto, H.; Hewitt, G. M.; Suen, R. *Heterocycles* **1988**, *27*, 621.

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Route to Complex Natural Products

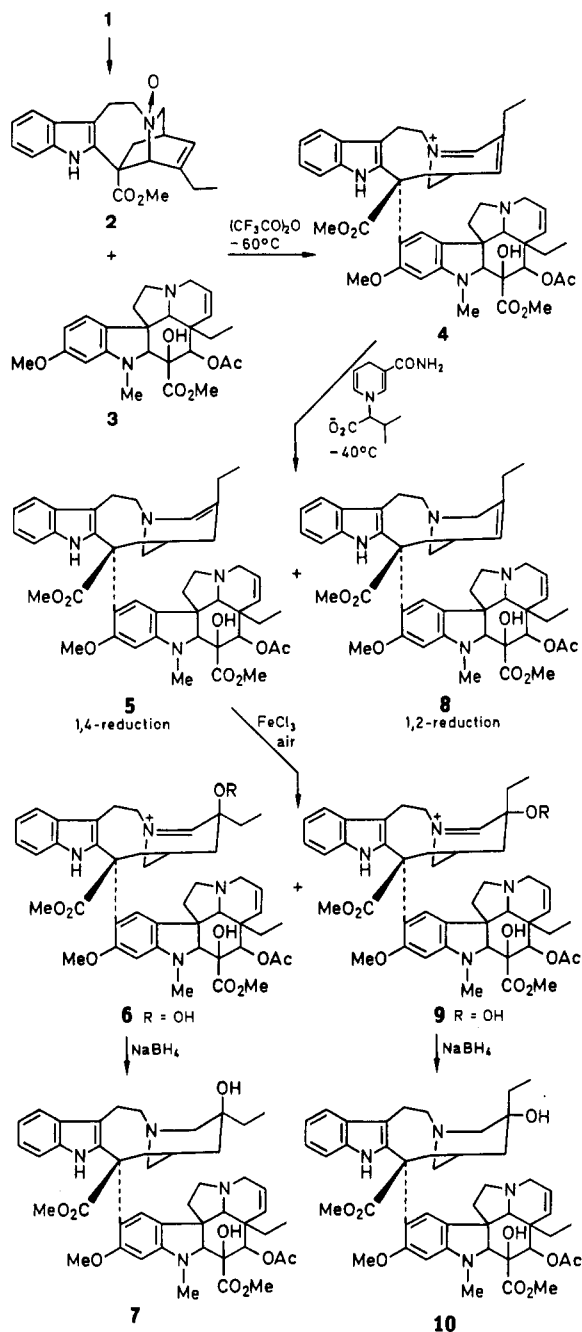*Acc. Chem. Res., Vol. 26, No. 10, 1993* **561**

Figure 3. A highly efficient "one-pot" process for the synthesis of vinblastin (7) and leurosidine (10) from catharanthine (1) and vindoline (3).

standpoint of developing an efficient chemical route to the clinical drugs VLB (7, R = Me) and VCR (7, R = CHO), a solution for the initial chemical coupling of 1 and 3 via the Polonovski reaction was now available, and only refinements in reaction conditions to optimize the yield of 4 were required. The major focus in further studies directed to VLB was an efficient regiospecific 1,4-reduction of 4 to enamine 5—a process which had not been achieved in our earlier studies or in other laboratories. The important information derived from the biosynthetic experiments was the realization that, instead of utilizing the more conventional reducing agents (NaBH₄, etc.), which afforded only 1,2-reduction of 4 to anhydrovinblastine (8),[11,12] *chemical* models mimicking the NADH enzyme-catalyzed process of 4 to 5 may become the reagents of choice. Indeed, an

extensive study[13,8] with various dihydronicotinamides as reducing agents revealed an effective solution to this problem, as summarized in Figure 3. After study with various oxidants (O_2, H_2O_2, $FeCl_3$), the chemical parallel to the enzymatic oxidation of 5 to 6 was best achieved with $FeCl_3$, and the final reduction, 6 → 7, was highly successful with $NaBH_4$. The final objective, the direct conversion of 1 and 3 to vinblastine (7, R = Me) and, in turn, vincristine (7, R = CHO) *without isolation of intermediates*, that is, a "one-pot" process, could be realized. The overall process, involving five separate chemical reactions and providing a 40% overall yield of vinblastine, demands that each reaction must proceed with yields in excess of 80%. When the two additional bisindole products, anhydrovinblastine (8, 18%) and leurosidine (10, 17%), formed in this process are taken into account, it is clear that the majority of the reactions proceed in almost quantitative yield.

In conclusion, the studies with plant cell cultures of *C. roseus* have provided biosynthetic information which has formed the basis on which the development of an efficient synthetic route to a complex natural product has been achieved.

2. Plant Cell Cultures as "Reagents" in Organic Synthesis. Frequent criticism levelled against the use of plant cell culture methods for production of secondary metabolites relates to the normally long periods (generally 2–3 weeks) associated with the synthesis of such natural products *if* production is desired directly within the nutrient media utilized for cell growth and/or propagation. An approach which alleviates this problem concerns the use of developed culture lines as "reagents" wherein the enzymes present within the culture, or isolated from the culture, can serve to biotransform "precursors" to suitable end products. Such enzyme-catalyzed processes should generally complete in short time periods (minutes to hours) and should provide respectable yields of desired end products. An extensive program in our laboratory with well-developed cell lines derived from the plants *C. roseus*, *Podophyllum peltatum*, *Tripterygium wilfordii*, and *Nicotiana sylvestris* has revealed that the enzyme systems present in such cell cultures *do* indeed possess considerable versatility. Such enzymes are capable of biotransforming "foreign" precursors, that is, substrates not normally produced in the plants from which the cultures are derived, to novel end products. A large majority of the results obtained remain unpublished, but it is appropriate to exemplify the overall strategy and the results obtained with two cell culture lines, *C. roseus* and *P. peltatum*.

2.1. Studies with the *C. roseus* Cell Line. Section 1 above has provided a summary of experiments concerning our *C. roseus* cell line (coded as AC3) and its utilization in biosynthetic studies within the indole alkaloid area. It is of interest to present some studies in which this cell line is utilized in experiments where enzymes derived therefrom (Figure 1) can be effectively employed as "reagents" in the biotransformation of "foreign" precursors to end products of interest in developing an efficient route to the clinical anticancer drug etoposide (13, Figure 4).

The podophyllotoxin family (11, Figure 4) has been well studied over the years. Excellent reviews of the

(13) Kutney, J. P.; Choi, L. S. L.; Nakano, J.; Tsukamoto, H.; McHugh, M.; Boulet, C. A. *Heterocycles* **1988**, *27*, 1845.
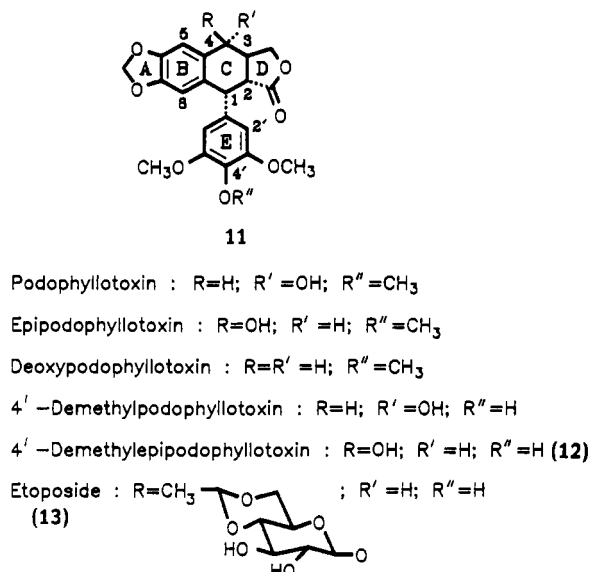
Figure 4. The podophyllotoxin family of compounds.

investigations pertaining to their chemistry and synthesis are available,[14,15] and the studies concerning their pharmacological properties, particularly the clinical importance of the anticancer drug etoposide, have been also reviewed.[14,16,17] The present route to this drug requires the isolation of podophyllotoxin, via extraction of *P. peltatum* plants, and subsequent utilization of this natural product as a starting material to afford etoposide. In brief, podophyllotoxin is converted, via a two-step chemical process, to 4'-demethylepipodophyllotoxin (12, Figure 4), and the latter, via a five-step process to attach the carbohydrate unit, finally completes the synthesis of this important drug.

In our program concerning the combined utilization of plant cell cultures and synthetic chemistry, we focused our attention on deriving an efficient route to 12. Such a strategy would eliminate the dependence on plant extraction and, hopefully, some chemical conversions, to afford a more direct synthesis of 12. If the subsequent attachment of the carbohydrate unit to 12, so as to obtain the clinical drug, could be similarly improved through biotechnological methods, to be studied later, a highly attractive sequence to etoposide would be on hand. The discussion following summarizes our recent results with our well-developed *C. roseus* cell line.[18]

In order to ascertain which synthetic "precursors" may be suitable for biotransformation to 12 with the "reagent" enzymes present in the *C. roseus* cell line, and subsequently in other studies discussed below, an understanding of pertinent features of a proposed biosynthetic pathway leading to the podophyllotoxins is desirable. Dewick and co-workers[19] have published

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(15) Ward, R. S. *Synlett* 1992, 8, 719 and references therein.
(16) Issell, B. F.; Muggia, F. M.; Carter, S. K. *Etoposide (VP-16)—Current Status and New Developments*; Academic Press: Orlando, FL, 1984.
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(18) Kutney, J. P.; Hewitt, G. M.; Jarvis, T. C.; Palaty, J.; Rettig, S. J. *Can. J. Chem.* 1992, 70, 2115.
(19) Broomhead, A. J.; Rahman, A. M. M.; Dewick, P. M.; Jackson, D. E.; Lucas, J. A. *Phytochemistry* 1991, 30, 1489 and references therein.
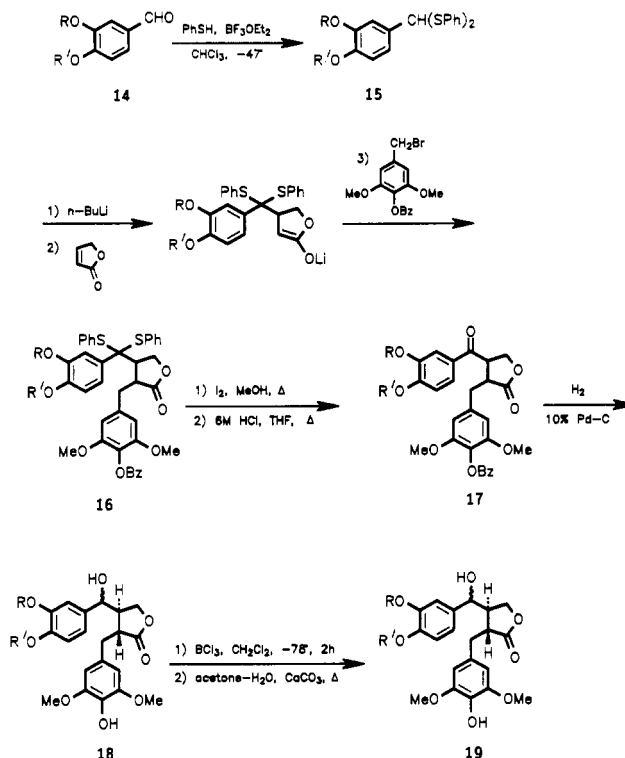

Figure 5. Synthesis of a 4'-demethylepipodophyllotoxin precursor.

detailed studies in this area and have suggested that dibenzylbutanolides could act as substrates in enzyme-catalyzed oxidative cyclization reactions to the corresponding podophyllotoxins. Such enzymatic processes of carbon–carbon bond formation with phenolic systems are expected to involve radical intermediates, and within living systems, the latter are usually generated by peroxidase enzymes.[20,21] It is to be noted that we had already demonstrated the presence of peroxidase-type enzymes in our studies with the *C. roseus* cell line, as presented in Section 1, so it was of interest to ascertain whether such peroxidases could serve as "reagents" in biotransforming appropriate dibenzylbutanolides to the podophyllotoxins.

A requirement for this study involved the development of a versatile synthetic route to the requisite dibenzylbutanolides from commercially available starting materials. Figure 5 summarizes the completion of the essential objectives.

The route shown employs readily available aldehydes 14 [R = R' = H; R = H, R' = alkyl; R, R' = (methylenedioxy)], and excellent overall yields of the corresponding precursors of general structure 19 [R = R' = H; R' = CH₃ or isopropyl, R = H; R, R' = (methylenedioxy)] were obtained.

If synthetic intermediate 16 was treated with Raney nickel, desulfurization is achieved, and the precursors, for example, 20 (R = R' = H, R'' = alkyl), corresponding to the deoxypodophyllotoxin series, become available. Since Japanese workers[22] had demonstrated the conversion of deoxypodophyllotoxin to podophyllotoxin by the use of *Penicillium* strains, any successful

(20) Kohler, H.; Jenzer, H. *Free Radical Biol. Med.* 1989, 6, 323.
(21) Hewson, W. D.; Hager, L. P. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, Vol. 7, 1977; p 295.
(22) Kondo, A.; Ogura, M.; Midorikawa, Y.; Kozawa, M.; Tsujibo, H.; Baba, K.; Inamori, Y. *Agric. Biol. Chem.* 1989, 53 (3), 777.
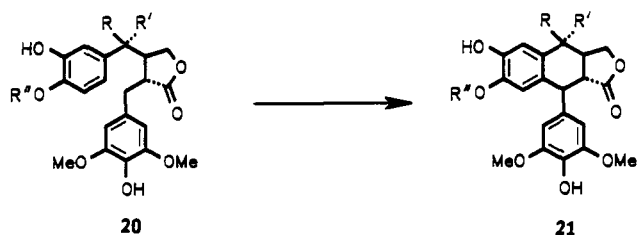
Route to Complex Natural Products

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Figure 6. Biotransformation of dibenzylbutanolide **20** with cell free extracts of *C. roseus* cell cultures and whole cells of *P. peltatum*.

cyclization of **20**, by our plant cell culture-derived enzymes, could also prove of interest in relation to etoposide production.

Initial studies with the deoxy series and the crude enzyme preparation obtained from *C. roseus* cell culture (Figure 1) are now briefly described.

A large number of experiments with substrate **20** (R = R' = H, R'' = CH₃) (Figure 6) and the crude enzyme preparation (Figure 1) obtained from our *C. roseus* cell line coded as AC3 were conducted in order to achieve optimum conditions for the cyclization of **20** to podophyllotoxin analogues, for example, **21** (R = R' = H, R'' = CH₃). The reaction parameters (pH, H₂O₂ as cofactor, units of peroxidase enzyme per millimole of substrate, reaction time) were evaluated. Details are published elsewhere,[18] and only a summary of the most salient features is provided here.

One of the important parameters was the pH of buffer used to prepare the crude enzyme and its effect on the enzymatic activity. From a number of experiments, it was concluded that a pH of 6.3 appeared to provide the highest activity in terms of bioconversion of **20** to **21**.

The ideal ratio of peroxidase to substrate was determined using an amount of 2.0 molar equiv of hydrogen peroxide as cofactor and conducting the reaction at pH 6.3. These studies established that **21** was very nearly consumed within 15 min when an amount of crude enzyme corresponding to 400 units of peroxidase/mmol of substrate was used. Increasing the peroxidase:substrate ratio or the reaction time (180 min) failed to improve the yield of **21**; instead, the sole effect was to promote the reaction of aryltetralin **21** to a dimer obtained by radical coupling at C(5) (see **11**, Figure 4, for numbering system and details published elsewhere).[18]

It was concluded that a peroxidase:substrate ratio of approximately 250 units/mmol of butanolide **20** would give optimum yields of the aryltetralin **21**. Higher values promoted dimerization while lower values resulted in incomplete conversion of substrate.

The optimum hydrogen peroxide:substrate ratio was determined using 250 units of peroxidase/mmol of **20** in buffer at pH 6.3. In the absence of hydrogen peroxide, no reaction occurred after 15 min. A similar result was noted when the reaction was allowed to proceed for 180 min. The yield of **21** then increased with hydrogen peroxide concentration but remained approximately constant at about 80% when more than 2.0 molar equival (3.1 mM) were present. It was concluded that 2.0 molar equiv of hydrogen peroxide would give optimum yields of **21**. Lower values resulted in incomplete biotransformation of **20** while higher ratios promoted the dimerization of **21**. There also appeared

to be a distinct decrease in enzyme activity when more than 3 equiv of hydrogen peroxide were used as cofactors.

The optimum biotransformation pH for the cyclization of **20** to **21** could now be reliably determined as the ideal peroxidase:substrate and peroxide:substrate ratios had been established. From the studies conducted, a pH value of 6.3 was selected. In fact, the yield of **21** showed only minor variations over the pH range of 6.0–6.6 but decreased markedly at more basic values.

In summary, the best conditions for the biotransformation of **20** to **21** are as follows: 250 units of *C. roseus*-derived peroxidase/mmol of substrate **20**, conducted for 180 min at a pH of 6.3, whereupon 80% yields of **21** were consistently obtained.

Finally, it could be determined that the crude enzyme preparation, when stored at 4 °C, was quite stable with respect to the biotransformation of **21**.

It was now clear that the enzymes of *C. roseus* cell cultures, originally developed for the production of indole alkaloids, could tolerate "foreign" substrates of the dibenzylbutanolide family and afford high yields of the desired podophyllotoxin analogues.

It is of interest to note that, for the sake of comparison, the biotransformation of **20** to **21** was investigated with commercial horseradish peroxidase. The reaction mixture was complex and the best yields of **21** were 15–19%, thereby revealing that the *C. roseus*-derived enzymes are *far superior* for the present study.

Extension of the above studies to suitable dibenzylbutanolides, which, upon cyclization, would afford podophyllotoxin analogues possessing the required C(4) hydroxyl function, was now undertaken. For this purpose, compound **20** (R = OH, R' = H, R'' = isopropyl) (Figure 6), available via the chemical route outlined in Figure 5 and starting with aldehyde **14** (R = H, R' = isopropyl), was considered. The expected cyclized product **21** (R = OH, R' = H, R'' = isopropyl) (Figure 6) can then be converted, through established chemical methods, to the C(6)–C(7) dihydroxy system and, in turn, to the methylenedioxy functionality required for the crucial starting material **12** (Figure 4) for etoposide synthesis.

Indeed, when this substrate was subjected to the above-noted biotransformation conditions with the crude enzyme preparation obtained from *C. roseus*, a 84% yield of **21** (R = OH, R' = H, R'' = isopropyl) was obtained.

2.2. Studies with *P. peltatum* Cell Line. 2.2.1. Studies with Whole-Cell Fermentations—Batch Process. While the above studies were underway, we were fortunate in establishing the first successful cell culture of *P. peltatum*, which, *in cell suspension*, afforded podophyllotoxin, deoxypodophyllotoxin, and 4'-demethylpodophyllotoxin (Figure 4).[23] It should be noted that Kadkade[24] reported earlier the development of callus cultures of *P. peltatum* which produce podophyllotoxin, while Dewick and co-workers[25] similarly established callus cultures of *Podophyllum hexandrum* for this purpose. Neither group has reported

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(24) Kadkade, P. G. *Plant Sci. Lett.* **1982**, *25*, 107.
(25) Hegenga, G. A., Lucas, J. A.; Dewick, P. M. *Plant Cell Rep.* **1990**, *9*, 382.

a successful cell suspension. Dutch workers[26] have reported a cell suspension of *P. hexandrum* which provides podophyllotoxin. It was clear that enzyme systems inherent in such cell cultures may be ideally suited for the biotransformation of the above-noted dibenzylbutanolides since as noted earlier, closely related intermediates have been implicated in the biosynthesis of the podophyllotoxins.

From a practical standpoint, it would be preferable to evaluate, and hopefully to obtain, successful cyclization reactions, for example, 20 → 21 with *whole-cell* fermentations rather than isolating crude enzymes as noted in the above section. We turned our attention to this aspect with the now available cell line of *P. peltatum*. A large number of experiments have been conducted, but the following discussion will focus on a few selected areas to illustrate the successful approach.

One avenue of research which was pursued with dibenzylbutanolide 20 (R = OH, R′ = H, R″ = isopropyl) involved addition of this substrate to "growing" cells of *P. peltatum* cell cultures. In this approach the substrate, dissolved in a small amount of ethanol, was added to cell cultures of varying ages (5–21 days old) growing in nutrient medium in shake flasks or bioreactors. After an appropriate time of incubation (generally 24–120 h) the fermentation broth and cells were extracted (ethyl acetate) and extracts analyzed for products formed. In general, the major cyclized product 21 (R = OH, R′ = H, R″ = isopropyl) was obtained in yields of 50–55% although conditions for this conversion were *not* optimized in view of success in the semicontinuous process discussed below.

2.2.2. Studies with Whole-Cell Fermentations—Semicontinuous Process Provides a "Biological Factory". The above studies indicated that the desired cyclization product 21 was extracted almost entirely from the fermentation broth with only insignificant quantities remaining within the cells. It therefore became of interest to determine whether a semicontinuous fermentation process could be established. In this approach, the fermentation broth, after a certain incubation period with 20 (R = OH, R′ = H, R″ = isopropyl), would be withdrawn under aseptic conditions, new medium and a new batch of 20 added, and the process repeated over a number of cycles. Obviously, if successful, one batch of cell culture could be utilized for successive productions of 21 (R = OH, R′ = H, R″ = isopropyl) and this "biological factory" could afford a highly attractive approach toward multigram quantities of end products. Indeed, a successful approach has been developed in our laboratory,[27] and only a brief description of a large number of experiments is provided here.

Incubation of 20 (R = OH, R′ = H, R″ = isopropyl) in a Microferm bioreactor maintained according to the published procedure,[23] with cells of varying ages (2–17 days old), was performed for 24–50-h periods. In a typical experiment, involving 24-h incubation periods between cycles, the fermentation broth containing the metabolites is withdrawn, new medium (generally at

one-tenth to half the original MS medium) and a new batch of 20 are added, and the process is continued for a further 24 h, whereupon the extraction, etc., is repeated. Although studies are still underway, we have already demonstrated that *one* batch of cells maintains the necessary enzymatic activity to biotransform 20 to 21 for *several months*, during which time *16* batches of 20 had been added and biotransformed to 21. Indeed, we have not yet evaluated how long such activity persists since the supply of 20 was depleted, after numerous additions, and the study had to be terminated before enzymatic activity within the bioreactor had been exhausted. In effect, the *P. peltatum* cell line is a "biological factory" in terms of its ability to achieve oxidative coupling of dibenzylbutanolides to podophyllotoxin analogues. The present yields of 21 are in the range of 50% when 20 is added in 24–48-h cycles, although higher yields are anticipated when conditions are optimized.

In summary, the semicontinuous process, once optimized, will afford a highly attractive route to various analogues of the podophyllotoxin series.

Finally, it should be noted that an efficient process in removing the isopropyl group in 21 and regeneration of the methylenedioxy function as required in 12 (Figure 4) has already been completed in our laboratory.

3. Use of Plant Cell Cultures To Produce Higher Levels of Pharmaceutically Interesting Compounds. Plant cell culture methodology, in combination with chemistry, can provide significantly higher levels of the plant-derived natural products *and* often affords the opportunity to derive novel compounds for more in-depth pharmacological screening within a given "family" of compounds. Since enzyme stimulation within the cell cultures is generally achieved through "artificial" nutrient media changes, it is not surprising that plant cell cultures often *do* produce, in addition to the normal natural products present in the plant, novel metabolites which are not found in plant extracts. The following discussions, involving an extensive program in our laboratory with plant cell cultures of the Chinese herbal plant *T. wilfordii*, illustrate our results pertaining to these areas.

Tripterygium wilfordii Hook, commonly called Lei Gong Teng (Thunder God vine) or Mang Cao (rank grass) in China, is a perennial twining vine which has been used in herbal medicine in that country for several centuries. A refined extract from the root xylem of this plant, available in Chinese markets as tablets, has been used for the treatment of rheumatoid arthritis, various skin disorders, and more recently as a potential male contraceptive agent.[28] Furthermore, the cytotoxic effects of two of the minor plant constituents, tripdiolide (22) and triptolide (23), isolated by Kupchan[29] have also been noted. These data stimulated studies in our laboratory to develop cell cultures of *T. wilfordii* in an effort to obtain higher yields of these natural products. In particular, we hoped to ascertain which chemical structural types present in the tablets used in China were responsible for the various pharmacological properties noted above.

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Route to Complex Natural Products

Acc. Chem. Res., Vol. 26, No. 10, 1993 **565**

22 R = OH
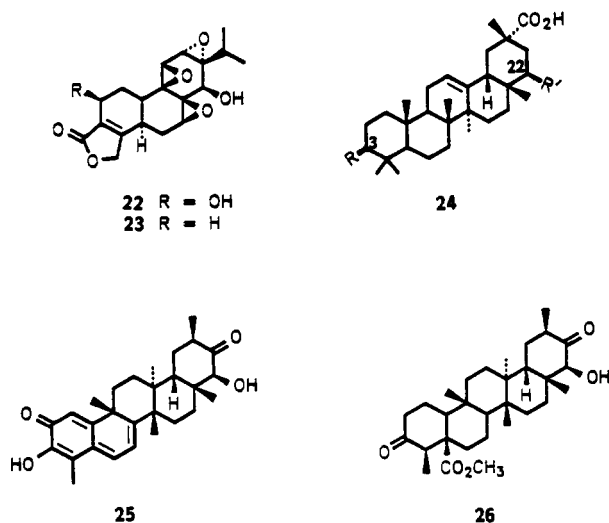**23** R = H

24

25

26

Figure 7. A summary of structural types of secondary metabolites isolated from *T. wilfordii* cell cultures.

Earlier studies with our developed cell line of *T. wilfordii* have been published,[30,31] so only a summary of the most salient features of these studies will be presented here. Major emphasis is provided on the most recent[32] and, as yet, unpublished results.

The cell line of *T. wilfordii*, coded as TRP4a, has been stable since its original development in the late 1970s and continues to produce the same spectrum of metabolites. Scale-up of TRP4a in bioreactors, for example, 60-L Chemapac and other airlift bioreactors, has allowed several hundred-liter quantities of broth and significant amounts of cells, from which 21 compounds within the diterpene and triterpene families have been isolated and fully characterized. Figure 7 provides a summary of the structural types that have been observed. As expected, in addition to providing some metabolites identical to those produced by *T. wilfordii* plants, novel triterpenes of general structure **24** (R = OH or carbonyl, R' = OH or carbonyl) and **26** and diterpenes[32] have been produced by the TRP4a cell line. As will be noted below, various approaches to increase metabolite production can be utilized, and several of these are discussed presently.

3.1. Increased Production of Tripdiolide (Td) and Triptolide (Tl) by Nutrient Media Changes. One approach which can be utilized for increasing yields of secondary metabolites in plant cell cultures concerns a study directed at evaluating the relative importance of growth media composition. Such a study was undertaken with the above-noted TRP4a cell line, and the results are presented in a detailed publication.[31] In brief, the objective was directed toward increasing the yield of the diterpene triepoxides, Td (**22**) and Tl (**23**), in view of their potentially important pharmacological properties.

An extensive study with three basal media (PRL-4, MS, and SH; see ref 31 for details on these growth media) was completed to determine that the MS

medium was preferred. Further studies then revealed that specific concentrations of $CaCl_2$, NH_4NO_3, and sucrose were important in affording higher yields of the above-noted triepoxides. Although optimum conditions for direct syntheses of these metabolites would require further study, the above experiments did allow the increase in Td production from an initial yield of less than 2.0 mg/L to a level of 4.0 mg/L *or* a yield of about *36 times greater* than that reported by Kupchan[29] from the living plant.

3.2. Increased Production of Triterpenes by Fungal Elicitation. In a more recent[33] study with the TRP4a cell line, we have evaluated the approach of "elicitor stimulation" to determine increases, if any, in metabolite production.

This approach is based on earlier studies by numerous laboratories involving living plants and phytoalexin production. It is well-known that plants produce phytoalexins, that is, secondary metabolites with antimicrobial activity, generally in response to an imposed stress (infection by pathogenic organisms).[34,35] It seemed reasonable to assume that plant cells growing in appropriate media may similarly stimulate "phytoalexin" production if exposed to such organisms, and if such metabolites were identical and/or structurally related to the target compounds, an effective approach to elevating metabolite yields in such fermentation processes would become available.

In order to determine whether elicitation of the desired metabolites (Figure 7) was indeed occurring upon addition of fungal preparations to growing cells of the TRP4a cell line, a large number of small-scale experiments were conducted. In these studies, fungal cultures of strains of *Botrytis* sp., *Sclerotinia sclerotiorum*, and *Trichoderma viride* were grown to maturity, treated in a homogenizer until the mycelia were fragmented, autoclaved, and then added to growing cultures of TRP4a, at various stages of the growth phase. The cultures were then allowed to continue growing for varying time periods (18–72 h). Extraction with ethyl acetate, followed by monitoring of tripdiolide and triptolide production by quantitative fluorimetric detection and triterpene production by gas–liquid chromatography, revealed that *Botrytis* and *Sclerotinia* were indeed effective in elevating triterpene levels in the TRP4a cell line when compared to control cultures to which no fungal preparations had been added. It was of interest that *neither* of the diterpene triepoxides **22** or **23** were elicited by addition of any fungal preparations.

Subsequent studies with TRP4a in airlift bioreactors containing 1.0% elicitor (*Botrytis*), followed by 72-h incubation periods after fungus addition, revealed very significant increases in yields of selected triterpenes, specifically, the four oleanane triterpenes **24** (R = O, R' = βOH), **24** (R = O, R' = αOH), **24** (R = R' = βOH), and **24** (R = βOH, R' = αOH), labeled as A, B, C, and D, respectively, in Figure 8. Figure 8 provides the most pertinent results from five separate experiments.

Addition of the fungal preparation to the growing cells of TRP4a was made at different ages of the cell

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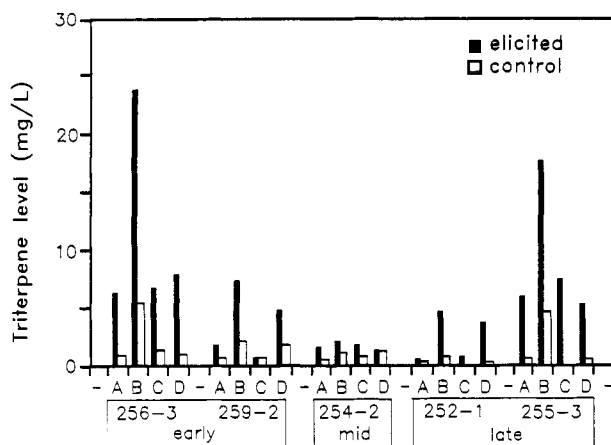
Figure 8. Increase in triterpene production in TRP4a cell line by fungal elicitation with *Botrytis*.

culture, and it is clear that "elicitation" of triterpene production varies depending on time of addition. The experiments coded as 256-3 and 259-2 in Figure 8 indicate addition of *Botrytis* (1% concentration) after a 10-day initial growth of TRP4a cells in the nutrient medium [refractive index (R.I.) = 1.3360] followed by a 72-h incubation period prior to harvest and extraction of metabolites. Experiment 254-2 represents addition of fungal preparation in the middle of the growth period (14 days of growth, R.I. = 1.3355) while 252-1 and 255-3 represent addition of *Botrytis* in late stages of growth (16 days, R.I. = 1.3332; 19 days, R.I. = 1.3334, respectively). It should be noted that the refractive index, generally a measurement which reflects consumption of nutrients by the growing cells, is used in our studies to provide a measure of cell development and growth.

In summary, the time course studies shown in Figure 8 demonstrate that maximum triterpene yield is obtained from cultures elicited at the beginning of the rapid growth period (experiment 256-3) and that an approximate 5-fold increase in production of triterpene **24** (R = O, R′ = αOH, labeled as B), for example, is observed relative to the control culture. These, and other data not provided here, reveal that elicitation with 1% *Botrytis* produced a steady increase in production levels of the triterpenes of general structure **24** for at least 6 days of growth after addition of the elicitor and usually until the end of culture growth (18–22 days).

4. Use of Plant Cell Cultures To Separate Pharmacological Activities in Complex Herbal Medicine Extracts. Well-developed plant cell lines can be utilized to provide a constant series of metabolites, often in yields substantially higher than the living plant. Furthermore, they allow more facile separation of components from a less complex mixture than normally encountered in a plant extract. Consequently, it becomes advantageous to use this methodology to try and understand which components of a plant extract are responsible for the specific pharmacological effects claimed. This approach was undertaken with our TRP4a cell line in order to establish which components of the herbal mixture derived from *T. wilfordii* were responsible for the various pharmacological activities claimed by the Chinese. The metabolites isolated[32] (Figure 7) have been submitted for evaluation. The diterpene triepoxides **22** and **23** are highly active as immunosuppressive agents as determined by inhibition of lymphocyte proliferation with mice spleen cells. This activity is believed to be related to treatment of rheumatoid arthritis. In addition, **22** and **23** exhibit significant antileukemic activity against mice L-1210 and P-388 leukemias *in vivo* and antispermatogenic activity in male rats (see details in ref 28). On the other hand, the triterpene family, exhibited by the structures **24** and **25**, have shown antiinflammatory activities for possible treatment of dermatological disorders.

Concluding Remarks

Utilizing specific examples from the author's laboratory, a summary of the possible avenues of research in which plant cell culture methods combined with chemistry can afford interesting routes to biologically active compounds is provided.

The results presented illustrate the potential of such an interdisciplinary program and, in particular, reveal that various "shortcomings" in the utilization of plant cell cultures as sometimes expressed by various members of the scientific community (for example, cell line instability, long-term and low-level production of metabolites, etc.) are invalid. With proper selection of methodology, gram-scale production of end products, often in short-term experiments similar to those with microbial systems, can be achieved. The use of semi-continuous fermentation methods, for example, provides an approach of distinct interest in large-scale efficient production of commercially important products.