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Biotechnology and synthetic chemistry—routes to clinically important compounds*

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Abstract: The combination of plant cell culture methodology with synthetic chemistry affords an attractive route to the synthesis of complex natural products and related compounds of industrial importance. Plant cell cultures which often provide 'unique' enzyme systems can be employed effectively as 'reagents' in the biotransformation of synthetic substrates to desired end products. Such conversions are often superior to those utilizing chemical reagents with the result that the overall synthetic route is more efficient. Alternatively, the use of plant cell culture derived enzymes in the evaluation of biosynthetic pathways can often afford important information for synthetic design. The overall strategy illustrating such an approach is presented herein with specific examples.

INTRODUCTION

The plant kingdom has provided a wide variety of natural products with diverse chemical structures and a vast array of biological activities, many of which have found applications in the health sciences. For years, synthetic chemists have been afforded the challenge of developing syntheses of such components but often due to structural complexity the resulting multistep syntheses rarely find application in large scale production as required in the pharmaceutical drug industry. As a result, starting materials for such drug production, or indeed the final clinical drug, are frequently obtained from tedious and often costly extraction from the living plant. This latter solution is often fraught with the well known problems: (a) active agent present in minute amounts in the plant extract; (b) separation of target compound may be difficult and, in turn, expensive; (c) varying concentrations of the target compound depending on seasons during which plant collection is performed; (d) desired plant species growing in geographically or politically inaccessible regions, etc.

Appropriate solutions to at least some of the above difficulties are possible by the use of plant cell culture methodology particularly when such studies are coupled with chemical methods. The advantages of plant cell cultures over living plants, in terms of secondary metabolite production, are clear: (a) growth conditions are laboratory controlled therefore reproducible yields of end product are achieved; (b) growth parameters such as pH, changes in nutrient media, temperature, etc. can be optimized to achieve metabolite production in yields significantly higher than in the living plant; (c) separation of target compounds is much easier due to lower complexity of extract; (d) plant cell cultures are an excellent source of enzymes, much superior to living plants where isolation often leads to enzyme denaturation. With enzyme availability, the opportunity to perform biosynthetic and/or biotransformation experiments related to metabolite production, is clear.

The present discussion will summarize studies performed in the author's laboratories, in several select areas, in order to portray to the potential reader the various avenues of research that such an interdisciplinary program can achieve.

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AVENUES OF RESEARCH

For the purpose of this lecture, our program can be conveniently divided into several sections and the discussion following will present studies within these areas.

Section 1. Plant cell cultures as 'reagents' in organic syntheses.

Section 2. Use of plant cell cultures to produce higher levels of pharmaceutically interesting compounds.

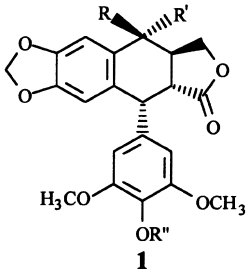
Section 3. Use of plant cell cultures to separate pharmacological activities in complex herbal medicine extracts.

Section 1. 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 cell 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 – hours) and should provide respectable yields of desired end products. An extensive program in our laboratory with well developed cell lines derived from the plant *Catharanthus roseus* [1–6], *Podophyllum peltatum* [1,3,7], *Tripterygium wilfordii* [8,9] and *Nicotiana sylvestris* [10–12] 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. It is appropriate to illustrate this approach with some examples taken from our overall program.

1.1. Studies with the Podophyllum peltatum cell line

The podophyllotoxin family (**1**, Fig. 1) has been well studied over the years. Excellent reviews of the investigations pertaining to their chemistry and synthesis are available [13,14] and the studies concerning their pharmacological properties, particularly the clinical importance of the anti-cancer drug etoposide [3] have been also reviewed [13–16]. The present route to this drug requires the isolation of podophyllotoxin, via extraction of *Podophyllum 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 (**2**, Fig. 1) and the latter, via a five step process to attach the carbohydrate unit, finally completes the commercial synthesis of this important drug.



1

Podophyllotoxin: R = H; R' = OH; R" = CH_3

Epipodophyllotoxin: R = OH; R' = H; R" = CH_3

Deoxypodophyllotoxin: R = R' = H; R" = CH_3

4'-Demethylpodophyllotoxin: R = H; R' = OH; R" = H

4'-Demethylepipodophyllotoxin: R = OH; R' = H; R" = H (**2**)

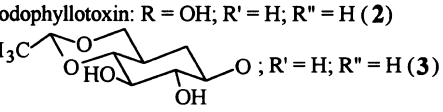
Etoposide: R =  ; R' = H; R" = H (**3**)

Fig. 1 The podophyllotoxin family of compounds.

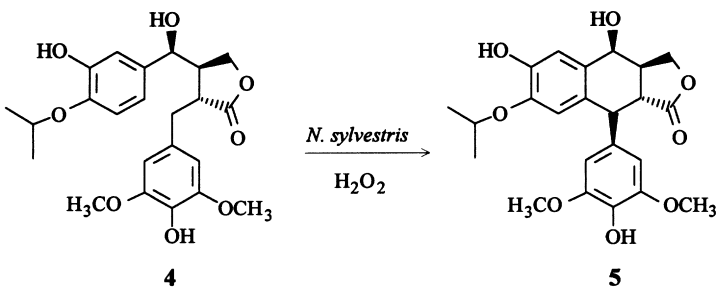
In our program concerning the combined utilization of plant cell cultures and synthetic chemistry, we focussed our attention on deriving an efficient route to **2**. Such a strategy would eliminate the dependence on plant extraction and hopefully, some chemical conversions, to afford a more direct synthesis of **2**. If the subsequent attachment of the carbohydrate unit to **2**, 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. In order to ascertain which synthetic 'precursors' may be suitable for biotransformation to **2** with the 'reagent' enzymes present in the *P. peltatum* cell line, an understanding of

the pertinent features of a proposed biosynthetic pathway leading to the podophyllotoxins is desirable. Dewick and co-workers [17] have published detailed studies in this area and have suggested that dibenzylbutanolides could act as substrates in enzyme-catalyzed oxidative cyclization reactions to the corresponding podophyllotoxins. Since our *P. peltatum* cell line had *already* provided several podophyllotoxins as metabolites [18], it was reasonable to assume that such substrates may be appropriate for the enzyme-catalyzed cyclizations with this cell line.

A requirement for this study involved the development of a versatile synthetic route to the requisite dibenzylbutanolides, for example, from commercially available starting materials. Indeed, such a route from the readily available aromatic aldehydes was completed [7] and studies with these substrates were undertaken.

1.1.1 Studies with whole cell fermentations—Semi-continuous process provides a 'biological factory'. With a successful cell culture of *P. peltatum* which afforded podophyllotoxin, deoxypodophyllotoxin and 4′-demethylepipodophyllotoxin (Fig. 1) on hand [18], it was appropriate to evaluate the enzyme-catalyzed cyclization of the above-noted synthetic substrates. For the sake of brevity, one particular study involving the butanolide **4** and a semicontinuous process will be presented.

In this approach, the fermentation broth, after a certain incubation period with **4**, would be withdrawn under aseptic conditions, new medium, and a new batch of **4** 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 **5** (Fig. 2, Table 1), 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 [7]. In brief, *one* batch of cells maintains the necessary enzymatic activity to biotransform **4–5** for *several months* during which time *16* batches of **4** had been added (yield of **5**, 50%, not 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 **5** and regeneration of the methylenedioxy function as required in **2** (Fig. 1) has already been completed in our laboratory.



4 **5**

Fig. 2 Biotransformation of a dibenzylbutanolide with the cell culture of *Nicotiana sylvestris* (see Table 1).

Table 1 Biotransformation conditions

Substrate	0.67 g/L	Possibility of large scale
Enzyme source	broth from *N. sylvestris* cell culture (10-day-old)	biotransformation:
Peroxidase	50 units/mmol substrate (pyrogallol method)	Broth from 80 L *Nicotiana* cell culture contains sufficient
Cofactor (H_2O_2)	1.0 equivalent	peroxidase enzyme to convert
Phosphate buffer, pH 6.3	6.7 mM	1000 g of the above
Temperature	14 °C	substrate to 870 g of ring
Biotransformation time	40 min	closed product
Yield of product	87%	

1.2 Studies with the Nicotiana sylvestris cell line

While the above studies were underway, we developed a stable cell line of *N. sylvestris* [10,11] and were able to demonstrate, via the appropriate enzyme assay, that this cell line produces *high levels* of 'peroxidases' which are excreted into the broth. It was therefore of interest to determine whether such peroxidases are capable of biotransforming these dibenzylbutanolide precursors to the desired cyclic products. Figure 2 summarizes the conditions which provided an 87% yield of **5** in a short time period (40 min). Although conditions have not been optimized, it is clear that this cell line provides a highly attractive route for *gram scale* production of podophyllotoxins. A summary of the most pertinent results has been published [12].

While the above studies were underway, a parallel series of experiments in the chemical laboratory were undertaken. Figure 3 summarizes the overall sequence from the readily available 1,3-dithiane derivative of piperonal to the target molecule, 4-demethylepipodophyllotoxin [2] and its C-1 epimer [6].

Section 2. Use of plant cell cultures to produce higher levels of pharmaceutically interesting compounds

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 [19]. Furthermore, the cytotoxic effects of two of the minor plant constituents, tripdiolide and triptolide, have also been noted [20]. 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. Some of our studies have been published ([9] and cited therein) so only a brief summary of the most salient features are presented. The cell line of *T. wilfordii*, coded as TRP4a, produces a variety of diterpenes and triterpenes, including tripdiolide and triptolide, obtained in the cell line at levels 40 times that in the living plant. In order to assess the pharmacological importance of the triepoxide functionality, it was of interest to obtain novel analogues of the basic structural template to determine whether the presence of epoxide functions was essential for the activities noted. The approach selected was to synthesize appropriate 'substrates' and subsequently to incubate these in whole cell fermentations and/or in the presence of TRP4a cell line derived enzymes, in order to achieve biotransformation to novel end products (Scheme 1).

Synthetic routes to various diterpene analogues from the readily available dehydroabietic acid were developed [21] and biotransformation experiments were conducted. Some of the results obtained in the biotransformation of one of these substrates, isotriptophenolide (**7**), are summarized in Fig. 4. Other minor products obtained in these studies are presented in [21].

The biotransformation of **7**, dissolved in ethanol, was investigated with 21-day-old TRP4a cells with incubation times varying from 96 h to 7 days, and the two isomeric epoxides **8** and **9** were obtained in a combined yield of 54%. It should be noted that reaction conditions were not optimized. Pharmacological evaluation of a number of these compounds is published [22].

In order to develop a family of novel diterpene epoxides so as to afford a structure-activity relationship within this series of pharmacologically interesting compounds, a combination of biotechnology and synthetic chemistry program was undertaken. Figure 4 exemplifies the advantages of this strategy. *T. wilfordii* cell cultures are first utilized to afford the novel epoxides **8** and **9**, the latter being *inaccessible* via chemical methods in spite of numerous unsuccessful chemical experiments to obtain **8** and **9** from **7**. These epoxides are then subjected to epoxidation in the chemical laboratory to afford **10** and **11** for pharmacological screening.

Since it was suspected that the TRP4a cell line was converting **7** to **8** and **9** via oxidative enzymes, likely to be of the peroxidase family, it was of interest to compare the enzyme-catalyzed conversion of **7** with chemical conversions utilizing oxidizing (dehydrogenating) reagents which proceed via free radical

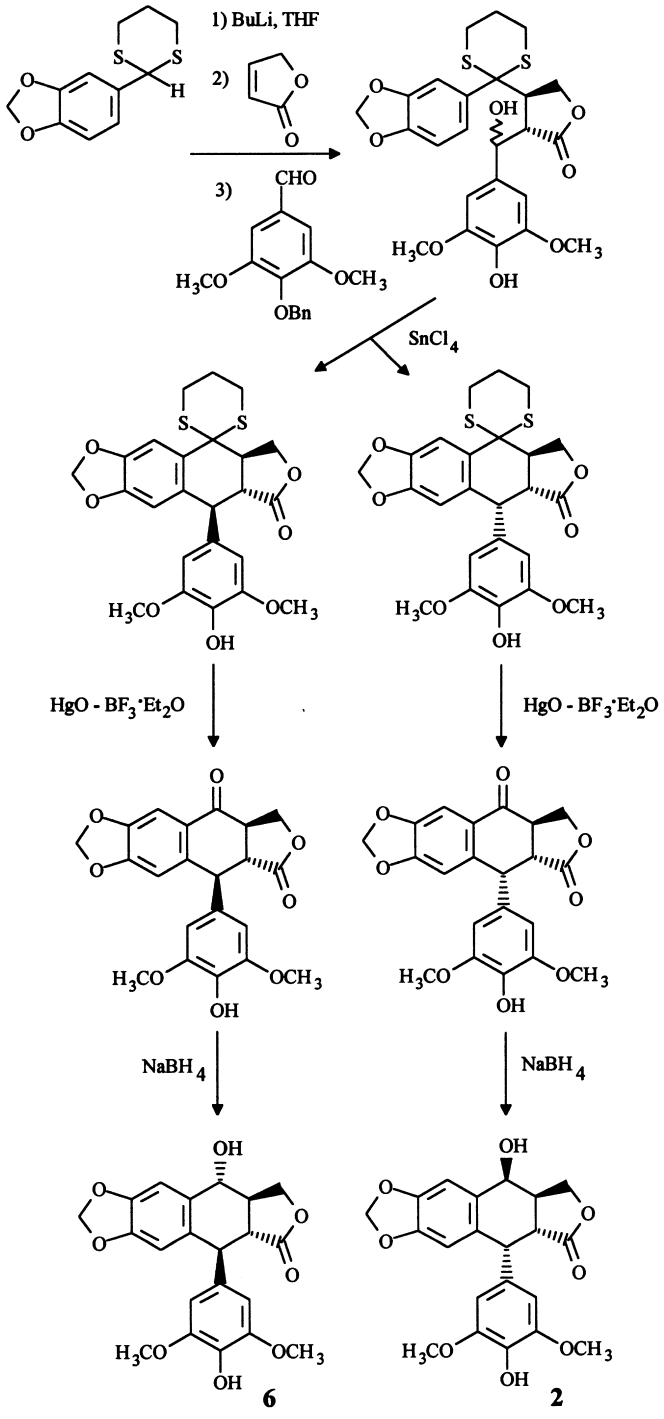
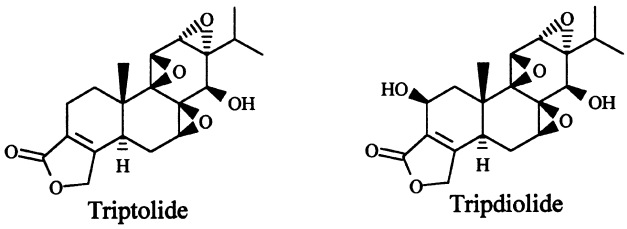


Fig. 3 Synthesis of podophyllotoxin analogues from piperonal 1,3-dithiane.

processes. For this purpose, **7** was treated with DDQ at room temperature in a methanol solution. The products obtained were **13** (62%) and **14** (15%) (Fig. 5). It is believed that these products arise via the quinone methide intermediate **12**. Demethylation of **13** (BCl_3, –75 °C) afforded the alcohol **15** (62% yield). When **15** was reacted with PIDA (CH_3CN, 0 °C), a low yield (22%) of the epoxydienone **9** was obtained. On this basis, it was clear that the biotransformation of **7–9** was preferable to the chemical route.



Scheme 1

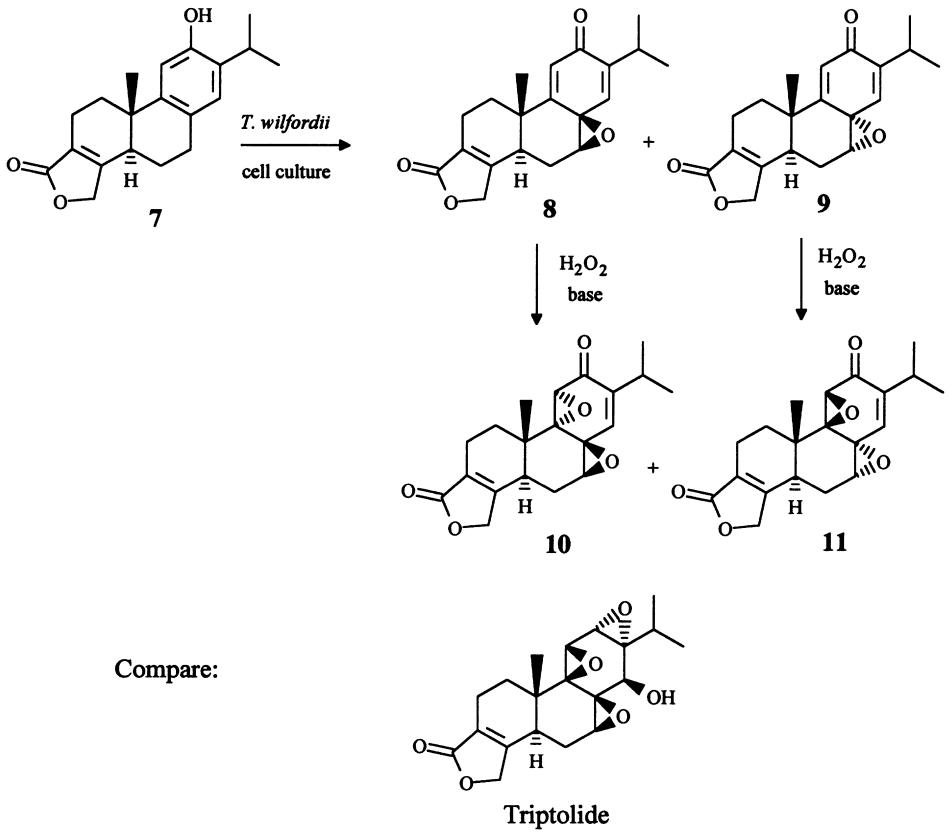


Fig. 4 Combination of biotechnology with synthetic chemistry affords a versatile route to novel diterpene epoxides.

Section 3. Use of plant cell cultures to separate pharmacological activities in complex herbal medicine extracts

As noted above, *T. wilfordii* plant extracts have revealed interesting pharmacological properties in the treatment of various diseases in man. The obvious advantages of plant cell culture vs. living plants in terms of providing higher yields, a less complex and more easily handled mixture of metabolites, afford an attractive route to separate the pure components and assess their pharmacological properties. As noted earlier, studies with our TRP4a cell line allowed the separation of pure compounds and subsequent evaluation will shed some light on the properties of *T. wilfordii* derived herbal mixtures. A brief summary is presented here.

(i) Immunosuppressive activity. In developing drugs of potential interest in the treatment of rheumatoid arthritis, immunosuppression is considered as a significant parameter. For this reason, various metabolites from the TRP4a cell line were submitted for such assays, for example, lymphocyte proliferation, and in

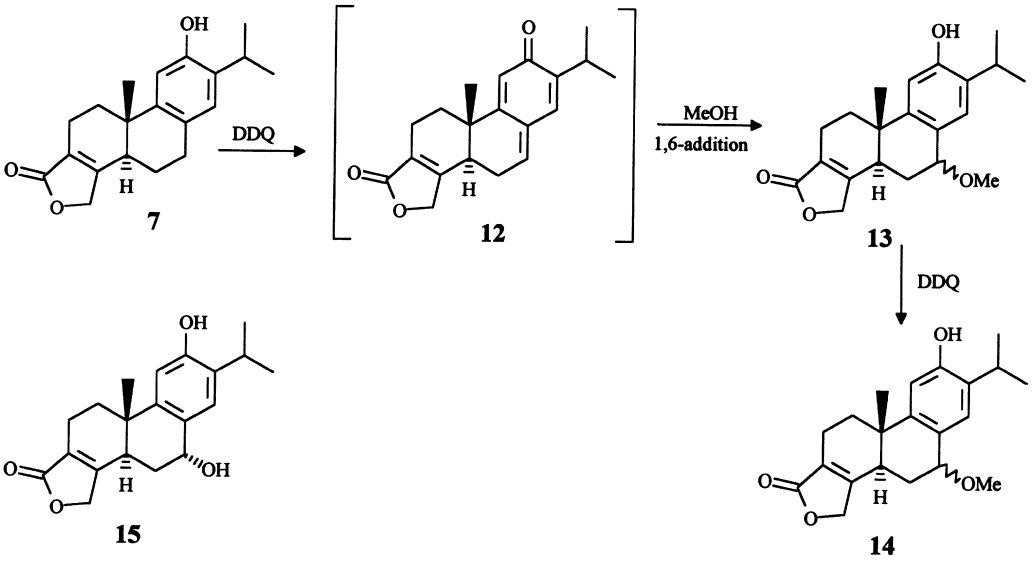


Fig. 5 Chemical oxidation of isotriptophenolide (**7**).

comparison with cyclosporine. The diterpene triepoxides, tripdiolide and triptolide, were highly active (95–99% immunosuppression at 100 pg/mL) and further studies are clearly required to establish structure-activity relationships.

(ii) Cytotoxic activity. In *in vitro* tests conducted recently against a spectrum of human cancer cell lines [21], the above-noted triepoxides have revealed significant growth inhibition at nanogram levels and their potential in cancer chemotherapy is of interest.

(iii) Male contraceptive activity. Clinical data, available from extensive studies in China [19] have shown that tripdiolide and triptolide possess significant anti-spermatogenic activity in human males. Triptolide chlorohydrin, tripchlorolide, is presently in clinical trials.

CONCLUSION

The above discussion has revealed that the interplay between plant cell culture methodology and synthetic chemistry provides a unique opportunity for entertaining areas of interest related to a broad spectrum of scientists. The results obtained afford information of interest to synthetic and medicinal chemists, pharmacologists and enzymologists and finally scientists involved in the commercial production and development of drugs. Of particular significance, this interdisciplinary approach has afforded efficient routes for the *commercial* production of several clinical anti-cancer drugs, vinblastine, vincristine, and Etoposide. To be specific: (a) studies with the cell cultures (enzymes) of *Catharanthus roseus* afforded important biosynthetic information concerning the plant biosynthesis of vinblastine. This information was then utilized to develop a commercially attractive 'one-pot' process to vinblastine and vincristine. This process is presently undergoing development for commercial production of these drugs (see [1–3]) (b) studies with the cell cultures of *Podophyllum peltatum* and *Nicotiana sylvestris*, in combination with synthetic chemistry, has afforded commercially attractive routes to the commercial precursor, 4-demethylepipodophyllotoxin (**2**), for large scale production of Etoposide. The dependence on *P. peltatum* plants as a source of podophyllotoxin (**1**) and, in turn **2**, is now eliminated (see Figs 2 and 3).

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