Natural Products from Plant Cell Cultures

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Abstract

Plants produce complex small molecules–natural products– that exhibit anti-cancer, anti-malarial and anti-microbial activity. These molecules play a key role in human medicine. However, plants typically produce these compounds in low quantities, and harvesting plant natural products is frequently expensive, time-consuming and environmentally damaging. Plant cell culture provides a renewable, easily scalable source of plant material. In this chapter we discuss the successes and pitfalls associated with natural product production in plant cell cultures.

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1. Introduction

Plants are an important source of natural products; these complex, higher eukaryotes make many compounds that are not found in bacteria and fungi. Notably, plants are immobile and interact with their environment largely via the release of complex small molecules. This evolutionary pressure has resulted in the production of an extraordinarily diverse array of natural products by plants. Approximately 100,000 compounds from plants have been reported, with thousands more being discovered each year [1]. Terpene-derived compounds form the largest class of plant derived natural products, with compounds in the alkaloid natural product class following at a close second [2]. Approximately 10% of all drugs considered to be "basic and essential" by the WHO are isolated from plants [3]. A small selection of plant-derived drugs is shown in Figure 1.

Many natural products are isolated from wild plants, and harvesting of these molecules often has a significant, negative impact on the environment. Although some medicinal plants can be farmed– for example, *Hypericum perforatum,* which produces the herbal anti-depressant St Johns Wort– many medicinally important plants cannot be grown under controlled farming

conditions [4]. A particularly compelling example is illustrated in the discovery of the anti-cancer agent Taxol that is isolated from the yew tree, as discussed in Section 3.

Plant cell culture has the potential to greatly expedite the production of natural products in a controlled environment. Controlled fermentation conditions avoid the variations in growing conditions that invariably occur with plants grown in the wild, such as differences in soil composition and attack by pathogens. Importantly, cell culture provides a renewable source of natural products, as plant cell culture can be produced and harvested year round without damage to the environment. Plant cell culture was initiated in the 1930's [5,6] and serious efforts to use plant cell culture as to produce natural products began in the 1970's [7]. These early studies unequivocally established that plant cell cultures produce many natural products; in modern research efforts, the practicality of natural product production in plant cells continues to be assessed [8].

Unfortunately, many pharmaceutically important plant natural products are not produced in cell culture. Biosynthesis of certain compounds requires the cellular differentiation that is not found in a homogenous cell culture. For example, the bis-indole alkaloid anti-cancer agent vinblastine from periwinkle has not been cultured in dedifferentiated cell cultures, because several of the biosynthetic genes are only expressed in epidermal cells of the whole plant [9]. Additionally, expression of natural products in cell cultures is often not stable over years of subculture. Certain plant extracts that contain complex mixtures of many products, such as *Ginkgo biloba*, are used medicinally. However, the relative ratios of the various compounds are often different in cell culture than they are in the plant [10]. From an engineering perspective, culturing of plant cells on a large scale presents technical challenges; the slow growing cells are susceptible to microbial contamination and are sensitive to mechanical stresses as described in

Section 2. Nevertheless, despite these challenges, a number of compounds have been successfully produced on an industrial scale using plant cell culture, as summarized in Table 1.

This chapter aims to give a brief background of the types of plant cell culture, the technology used to culture these cells, and a discussion of some of the genetic tools used to facilitate metabolic engineering in these cells. We then present a series of short sections that describe the biosynthesis of specific natural products in more detail. A comprehensive description of all natural products produced in plant cell culture is beyond the scope of this chapter. Instead, we selected compounds that are clinically relevant in modern medicine, have been subjected to extensive optimization in plant cell culture and which also represent the vast structural variety of compounds that are found in plants. Plant natural products associated with herbal medicine, or that are used as pigments or dyes are not discussed in detail. Furthermore, we do not discuss in detail the production of plant extracts (for example, from *Ginkgo biloba*) that do not contain a defined mixture of specific natural products. Plants have also been used for the production of medically useful proteins and antibodies ("plantibodies") [11], but in this chapter small molecule production is the focus.

2. Techniques of Plant Cell Culture

2.1 Classes of plant cell culture

In the 1934 it was first reported that plant cells could be cultivated on synthetic media in an undifferentiated state and appeared to be capable of unlimited growth [5,6]. In other words, for the first time, plant material could be used to generate tissue culture. Plant tissue culture has had a tremendous impact in the genetic and biochemical study of natural product biosynthesis, and in some cases tissue culture can be used for the large-scale production of plant natural

products. The major types of plant tissue culture are described below (Figure 2). A recent survey has summarized over 90 reports of successful cell culture of medicinal plants [12].

To initiate plant tissue culture, seeds are typically sterilized and germinated under aseptic conditions on solid, agar containing media. The sterile plantlet is then used to generate cell culture. Since sterilization conditions are harsh, it is often easier to sterilize the surface of the seed instead of the soft tissue ultimately used to yield the tissue culture.

Common plant media are Murashige and Skoog, Gamborg, Schenk and Hildebrandt and White [13]. Each of these media recipes contains minerals, vitamins and a carbon source, usually sucrose. Although plant cell cultures are typically initiated on solid media, liquid media is required for large-scale production. It has been commonly observed that changing the amounts of minerals or carbon source in the media often has a profound impact on the levels of metabolite production [14]. The optimal media for natural product production, as with microbial organisms, must be determined empirically, though lowering the amount of phosphate and nitrate usually increases secondary metabolism [15].

An explant– a piece of the sterile plant tissue– from the seedling is then placed on solid culture, and dedifferentiated cells begin to grow. Notably, plants are characterized by totipotency, meaning that each cell can express its genetic potential and can form a complete, fertile plant. Therefore, to maintain cell cultures in a dedifferentiated state, exogenous phytohormones–a mixture of auxins and cytokines– are added to the media to prevent the cells from differentiating. The specific mixture used varies from plant to plant. Changes in the phytohormone ratio and concentration also affect what organ–shoot or root– is regenerated. Over extensive periods of subculturing, cultures may become hormone independent and retain their dedifferentiated state even in the absence of exogenous phytohormones. The mass of cells that

result from the explant are known as callus culture. Callus is typically subcultured every 3-6 weeks and can be maintained for decades. These are heterogeneous cultures and the heterogeneity can be reduced by extensive subculturing, typically with the fastest growing cells being selected for. Callus cultures are typically slow growing and not suitable for large-scale bioreactors.

When callus is suspended in liquid media, the callus breaks into small cell aggregates and cell suspension cultures result. Single cell suspensions are rare, and plant suspension cultures are usually composed of aggregates of cells approximately 40-200 μM in diameter [16]. Cell suspension cultures usually proliferate more rapidly than callus and are one of the most commonly used types of cultures in industry. These cultures are generally considered to be the most suitable cell types for large-scale cultures maintained in a bioreactor.

Hairy root cultures, which are obtained by infecting plants with *Agrobacterium rhizogenes*, are also widely used in basic research and in industrial production studies. When a plant is wounded or cut, signal molecules are produced by the plant at the site of the wound. This allows *Agrobacterium* to infect the plant through the wound, and the bacterial genes Rol A B and C are transferred to the plant and integrated into the plant genome [17]. These genes stimulate root production, resulting in the formation of hairy roots at the site of infection within several weeks. The roots can be removed, adapted to liquid media and grown on large scales. Auxin metabolism is altered in the transformed hairy roots, so exogenous auxins are not required to maintain the root propagation and keep the tissue from regenerating to the whole plant. Hundreds of plants can be transformed into hairy roots as reviewed [18,19] though development of hairy root culture from some species, particularly the *Papaveraceae* and *Rununculaceae*, has been difficult [20]. In general, hairy roots are genetically stable [21] and most have a doubling time of

1-2 days, which is similar to that of cell suspension cultures [22]. Not surprisingly, most compounds formed in hairy root cultures are also found in the root of the differentiated plant. Interestingly however, hairy root cultures of *Artemisia annua* accumulate artemisinin, a natural product that is normally accumulated in the aerial parts of the plant [23,24] (see Section 3).

Root cultures can also be formed by adjusting the hormone balance of the callus culture media. When the appropriate ratio of phytohormones is added to the callus, roots are formed in the callus culture, which can then be excised and cultured. However, it is widely reported that hairy roots derived from *Agrobacterium* transformation are faster growing and generally accumulate higher titers of natural products than non-transformed root cultures [25].

Shoot cultures, which lack roots and undifferentiated callus cells, have also been developed. These cultures are either initiated from the seedling or can be generated from callus by adjusting the phytohormone content of the medium. Shoot cultures can be cultivated on liquid or solid media in the presence of phytohormones. These cultures have been reported to produce certain natural products that cannot be produced in cell suspension culture– for example, artemisinin and vindoline [26]. Typically, shoot cultures accumulate compounds normally observed in the aerial parts of the plant. However, shoot cultures are not well suited for bioreactor production and are unlikely to replace plants for large-scale natural product production.

2.2 Scale up of plant cell culture

For laboratory studies, shake flask cultures are commonly used. However, industrial fermentation of natural products requires highly reproducible conditions in which the mixing, shear forces and gas content do not vary when the culture is grown at different volumes. A

bioreactor has been employed in industrial fermentations to provide a highly controlled culture environment. Plant cells were first cultured in a 10 L bioreactor made of glass or steel in 1959 [27]. The first large-scale production of *Nicotiana tabacum* cells in a 20,000 L stirred reactor was reported in 1977 [28].

Plant cell cultures require low shear mixing, good aeration and absolute sterility. The stirred tank bioreactor predominates in the literature reports of plant cell culture (Figure 3). A mechanical stirrer fitted with a variety of blades is used for mixing, where the best blade type depends upon the individual culture. Plant cells are highly shear sensitive, so slow mixing speeds of 100-300 rpm are used. The air lift fermentor, also commonly used with plant cell cultures, couples aeration to agitation and does not contain a mechanical stirrer (Figure 3).

Compared to microbial cell cultures, plant cell cultures grow slowly with a doubling time of 1-2 days at best. Production levels are in the range of 10-500 mg per L per day, and products are usually stored intracellularly, not exported into the media as they are in most microbial fermentations. These factors lead to an overall less efficient production system so maximizing the cell density of the cultures is essential for making plant cell cultures practical.

Shoot cultures, due to mechanical frailty and light requirements are generally not suited for bioreactor production [16]. Root cultures, however have been successfully cultivated in a variety of bioreactor set ups. Often, roots are treated as immobilized cells; the roots are allowed to adhere to mesh or stainless steel supports in the tank and no mechanical stirring is used. Immobilized cells have also been explored by adsorption of cell suspension cultures on glass, by covalent cross-linking or by entrapment of the cells in agarose [13]. However, since products are not exported into the media, the release of product from the immobilized cells is often problematic.

2.3 Elicitation of natural products in cell culture

A key advance in the development of plant cell cultures was the discovery of elicitors, or small molecules that stimulate natural product production. Elicitors fall into two main categories, biotic and abiotic. Biotic elicitors include fungal derived saccharides and proteins that stimulate a defensive response in plants. Abiotic elicitors include chemicals such as jasmonates, salicylic acid, and hormones (Figure 4). Additionally, various metals such as vanadium, silver and lanthanum have also stimulated natural product production [29]. Elicitation has been used to improve the levels of paclitaxel and shikonin in industrial scale cultures (see Section 3).

2.4 Genetic manipulation in plant cell culture

Genetic manipulation of plant cells has the potential to profoundly impact plant natural product production. For example, genes expressed at low levels can be over expressed at higher levels to make a slow step more efficient. Additionally, genes from other metabolic pathways can be transformed into the wild type pathway to expand the scope of natural products produced. Specific examples of metabolic engineering efforts designed to improved natural product production in plant cell culture are described in Sections 3.3 and 3.4.

Transformation of plants with foreign genes was largely impractical until the mechanism of crown gall formation by *Agrobacterium tumefaciens* was elucidated in the 1970's [30,31]. It was discovered that the *Agrobacterium* transferred DNA material to the plant (T-DNA) and that this DNA was incorporated into the plant genome. Thus, a natural means of plant transformation was identified. The first transgenic tobacco plants expressing recombinant DNA in an integrated T-DNA sequence was reported in the early 1980's [32].

Unfortunately, *Agrobacterium* transformation is limited to certain species of plants, though recent advances have expanded this range [33]. Therefore, alternative methods of transformation are required. Particle bombardment, reported in the late 1980's [34], uses a device to accelerate small tungsten particles coated with DNA at plant cells at a velocity of approximately 400 m/s. This method is generally applicable to plant cells, though subsequent rearrangement of the DNA sequence can be caused by the force used for transformation. Finally, plant cell protoplasts can be formed by partially removing the plant cell wall either enzymatically or mechanically. Foreign DNA can then be introduced into the cell by standard methods such as electroporation. However, protoplasts usually take long culturing periods for cells to recover, and some cell strains do not appear to survive the process [35].

A marker gene is also transferred to allow transformed cells to be easily selected [36,37]. Common marker genes encode enzymes that confer antibiotic resistance. Aminoglycoside adenyltransferase, which confers resistance to spectinomycin, glycopeptide binding protein, which confers resistance to zeocin and hygromycin phosphotransferase, which confers resistance to hygromycin, are all widely used. Additionally, the marker gene mannose-6-phosphate isomerase confers upon transformed cells the ability to use mannose as the sole carbon source.

3. Natural Products Produced from Plant Cell Culture

The production of many natural products in plant culture has been explored. This section describes the advances made in the production of several pharmaceutically important natural products.

3.1 Taxanes

Paclitaxel, also known by its trade name Taxol from Bristol Myers Squibb, is one of the success stories of plant cell culture (Figure 5). This densely functionalized diterpene natural product exhibits potent anti-cancer activity. In 1963, the bark of *Taxus brevifolia* was found to harbor anti-tumor activity in a natural product extract screen at the National Cancer Institute [38]. Paclitaxel, or Taxol, was discovered to be the active agent of this extract in 1971 [39]. Paclitaxel acts by binding to tubulin, promoting the assembly of microtubules and stimulating apoptosis [40]. At the time of its discovery, this represented a completely novel mechanism of cytotoxic activity. Recently an additional target for taxol has been identified as Bcl-2, a human protein involved in apoptosis [41]. Paclitaxel is used against a variety of cancers including breast cancer, ovarian cancer, non-small cell lung cancer and Kaposi's sarcoma [42]. Current sales of paclitaxel and its derivatives are expected to exceed 2 billion US dollars [43].

Paclitaxel is found in the bark of *Taxus brevifolia,* the Pacific yew tree, in small amounts: approximately 0.01% of the dry weight of the bark [44]. The bark is non-renewable– harvesting of the bark results in the death of the tree– and for many years it appeared that no sustainable supply of the drug existed. The structural complexity of the molecule prevented an economic total synthesis for industrial scale production. A number of related diterpene compounds referred to as taxanes are found in a variety of other *Taxus* species. One of these related taxanes baccatin III, a paclitaxel intermediate, is found in the needles of *Taxus baccata*, the European Yew tree, and is currently used to yield commerical supplies of paclitaxel through semi-synthetic strategies [45]. However, although harvesting of the needles it not lethal, it can still have a negative impact on *Taxus* populations. A paclitaxel source from plant cell culture, which is completely renewable, would provide a valuable strategy for paclitaxel production.

Callus cultures of various *Taxus* species were initiated, and the highest producing cultures

were converted to suspension cultures. Disappointingly, *Taxus* cell suspension cultures proved to be exceptionally slow growing [43]. A critical advance was reported in 1996 when it was discovered that addition of methyl jasmonate substantially improved the production of paclitaxel to 0.5% of the dry weight of the plant cell culture [46]. These results were the first suggestion that plant cell cultures might be able to produce taxanes at levels appropriate for commercial production.

A variety of factors have been shown to improve the production of paclitaxel and other related taxanes. Methyl jasmonate, as mentioned above, has been shown to consistently improve levels of taxane production. Heat shock, mechanical stresses such as ultrasound, and peptide regulators (Phytosulfokine A) have also resulted in the improvements of taxane production [47]. Immobilization of cell suspension cultures of *T. cuspidata* on glass fiber mats resulted in production levels of paclitaxel (~0.012%) [48]. Levels of 10-22 mg/L are commonly reported but higher levels of about 100 mg/L have also been reported (Table 2).

Taxol and taxol precursor production in cell cultures has been scaled up, though use of these cultures for industrial production is still somewhat limited. Yields can vary from cell culture to culture, and production of paclitaxel is not favored over other taxanes. Nevertheless, bioreactors up to 75,000 L are being employed by ESC genetic (USA), Phyton (USA), Phyton Biotech (Germany), and Samyang Genex (Korea) for the commercial production of paclitaxel [16].

Use of *Taxus* cell cultures has also made a significant impact in the elucidation of the paclitaxel biosynthetic pathway. In a tour de force, sequencing of a highly productive, elicited cell culture line resulted in the identification of most of the genes in the pathway [49]. An abbreviated biosynthetic scheme is shown in Figure 6, though the exact sequence of biosynthetic

transformations is not clear due to the difficulties in assigning the substrate specificity of many of the enzymes. A recent study that investigated the time resolved expression of these genes in *Taxus* cell culture may shed further light on the timing of the biosynthetic transformations [50]. Additionally, expression of these genes in *E. coli* and *S. cerevisiae* has led to the microbial production of several advanced intermediates of paclitaxel [51].

An endophytic fungal strain isolated from the yew tree that produces small amounts of paclitaxel was reported in 1993 [52]. Since that time, several other taxane producing endophytic fungal strains have been isolated [53]. However, the amounts of taxanes produced by these fungal cultures are quite low (ng-µg per liter) and production levels are often unstable; therefore, these fungal strains are not likely to replace plant-derived cultures in the immediate future.

3.2 Camptothecin

Camptothecin (Figure 7) is a potent anticancer agent, whose mode of action is topoisomerase I inhibition. However due to its severe side effects, camptothecin itself is not used for treatment of cancer though semi-synthetic derivatives of camptothecin have shown more useful clinical properties. First generation camptothecin derivatives irinotecan and topotecan (Figure 7) are used for clinical treatment of lung cancer, and have also shown therapeutic value in the treatment of colon cancer, uterine cervical cancer and ovarian cancer [54]. Progress in the development of new camptothecin analogs to improve pharmacology is reviewed elsewhere [55,56]. The demand for camptothecin and its derivatives is one ton per year and current worldwide sales of clinically used camptothecin derivatives are approximately one billion dollars per year [57]. Since relatively low levels of camptothecin are isolated from natural sources, plant cell culture has been extensively explored as a production model for camptothecin.

Camptothecin, a monoterpene indole alkaloid, was originally isolated from the sap of the stem wood of the Asian tree *Camptotheca acuminata* [58]. All parts of *C. acuminata* contain some camptothecin, with the highest levels found in young leaves at 0.4-0.5% dry weight (DW) [59]. Many derivatives of camptothecin have been isolated from plant extracts and cultures. The most abundant natural derivatives are the water soluble 10-hydroxycamptothecin [60] and 9-methoxycamptothecin [61], both of which display *in vitro* anti-tumor activity comparable to camptothecin [62].

The initial step in biosynthesis of camptothecin– along with all other monoterpene indole alkaloid natural products– is the condensation of tryptamine and secologanin to form the biosynthetic intermediate strictosidine (Figure 7). At this stage, the biosynthesis of camptothecin diverges from other terpene indole alkaloids; typically, strictosidine is immediately deglycosylated, but in the camptothecin pathway strictosidine instead forms the strictosamide intermediate (Figure 7). The biosynthetic conversion of strictosamide to camptothecin is not known on the enzymatic level. However, intermediates observed in camptothecin producing cultures [63, 64] has led to the proposal of a reasonable series of biosynthetic transformations (Figure 7). The lack of genetic and enzymatic knowledge of the biosynthesis of this important class of alkaloids greatly hinders metabolic engineering efforts to improve the production of camptothecin and its derivatives.

Although camptothecin production in plants is sustainable, a readily fermentable source could lower the cost and provide improved access to the compound and its derivatives. Investigation into the production of camptothecin and its derivatives in plant tissue culture was first reported for *Camptotheca acuminata*. Yields of 0.0002 % DW were reported for *C. acuminata* cell suspension cultures [65], a significant drop from the levels found in the whole

plant, which range from 0.02-0.5 %DW depending on the tissue analyzed [59] (Table 3). Media optimization studies revealed that a nitrogen concentration of 70 mM in the media gave the highest biomass of *C. acuminata* cell suspension cultures, while a NH^{4+}/NO^{3-} molar ratio of 5:1 (total of 40 mM nitrogen) gave the maximum camptothecin yield [66]. This is similar to results found in suspension cultures of *Atropa belladonna* for the production of tropane alkaloids [67]. Implementation of a 2 stage bioreactor, where the culture is grown in growth medium containing 70 mM nitrate for the first 18 days, followed by growth in production medium containing 40 mM NH4+/NO3- (5:1), gave the highest reported yield of camptothecin at 12.8 mg/L (Table 3). Callus cultures of *C. acuminata* with production of 0.2 % DW have also been reported [68].

Mature trees of *Nothapodytes foetida* produce 0.075 % DW camptothecin and 0.013% 9-methoxycamptothecin in the shoots, and 0.5 and 0.06 % DW camptothecin and 9-methoxycamptothecin, respectively, in the seeds [69]. The first cell cultures of *N. foetida* produced 100 times less camptothecin than intact plants [69,70]. However, more recently established cell suspension cultures of *N. foetida* have been found to produce camptothecin in levels comparable to *C. acuminata* cultures (approximately 0.1% DW)(Table 3) [71]. This cell line also produced high amounts of 9-methoxycamptothecin (0.08 %DW), which is not a major alkaloid of *C. acuminata.*

Differentiated hairy root cultures of *C. acuminata* have been established by transformation with *Agrobacterium rhizogenes* [72]. These cultures displayed respectable production of camptothecin at 0.1% DW, comparable to levels found in the intact plant [59]. Additionally *C. acuminata* hairy roots yielded 10-hydroxycamptothecin in 0.015% DW compared to 0.002% DW in the intact plant [59]. Hairy root cultures of *Ophiorrhiza pumila, O. liukiuensis* and *O. kuroiwai* have also yielded camptothecin and its derivatives. *O. pumila* hairy

root culture medium was found to contain substantial amounts of camptothecin, which could be increased by the addition of a polystyrene resin Dianion HP-20 to the medium [73]. The production of camptothecin after resin addition was only slightly enhanced; however, the overall secretion into the medium was increased five fold, dramatically simplifying the isolation and purification process. A similar effect was seen after addition of resin to *C. roseus* hairy roots producing the terpene indole alkaloid ajmalicine [74]. Production of this *O. pumila* hairy root line in a 3 L bioreactor did not affect production of camptothecin compared to cells cultured in shake flasks [75]. *O. liukiuensis* and *O. kuroiwai* hairy root lines have been established with different levels of camptothecin and 9-methoxycamptothecin levels compared to *O. pumila* (Table 3) [76].

Despite these advances however, the production of camptothecin in cell cultures is far below the levels needed for commercial production of these compounds. The identification of enzymes involved in the biosynthesis of this important medicinal compound will undoubtedly impact its production in plant cell cultures.

3.3 Tropane alkaloids

The tropane alkaloids hyoscyamine and scopolamine (Figure 8) function as acetylcholine receptor antagonists and are clinically used as parasympatholytics. Scopolamine has a higher commercial market than hyoscyamine, though its yield from plants is much lower. The tropane alkaloids are biosynthesized primarily in plants of the family *Solonaceae*, which includes *Hyoscyamus*, *Duboisia*, *Atropa*, and *Scopolia*; each of these species are capable of biosynthesis of both nicotine and tropane alkaloids. Early studies revealed that tropane alkaloids are formed in the roots and then transported to the aerial parts of the plant [77]. This localization information

suggested that *Agrobacterium rhizogenes* induced hairy root cultures could be used for high yield production of tropane alkaloids. Several hairy root cell lines that produce tropane alkaloids have been established (Table 4) [78].

Metabolic engineering is a powerful strategy often used to improve production of secondary metabolites. However, engineering efforts require knowledge of the enzymes involved in the biosynthetic pathway and the mechanism by which these enzymes are regulated. Tropane alkaloid biosynthesis has been studied at the biochemical level and several enzymes from the biosynthetic pathway have been isolated and cloned [79]. A summary of the biosynthetic steps is shown in Figure 8. Knowledge of the enzymes responsible for catalyzing key biosynthetic steps, in conjunction with *Agrobacterium* mediated transformation of plants, provided an excellent platform to initiate metabolic engineering efforts with this plant alkaloid. The levels of tropane alkaloid production in a variety of hairy root cultures were altered by overexpression of methyltransferase putrescine-N-methyltransferase (PMT) and hyoscyamine 6B-hydroxylase (H6H) (Figure 8).

Hyoscyamine 6B-hydroxylase (H6H) catalyzes the hydroxylation of hyoscyamine to 6B-hydroxyhyoscyamine, as well as the epoxidation to scopolamine (Figure 8) [80,81]. H6H was first cloned from *Hyoscyamus niger* [82]. Transformation and overexpression of the H6H clone from *H. niger* into *Atropa belladonna,* under control of the cauliflower mosaic virus 35S (CMV 35S) promoter, resulted in plants with increased scopolamine production [83]. This demonstration of improved alkaloid production by heterologous expression of a pathway enzyme in intact plants led researchers to seriously explore genetic strategies as a method for increasing production and altering the alkaloid profiles of tropane alkaloid producing plant hairy root cultures.

Transformation of plants with *Agrobacterium rhizogenes* is a common technique used to heterologously express genes in plant hairy root culture as described in Section 2. Using this transformation system, the cDNA encoding *H. niger* H6H was expressed in hairy root cultures of various *Solonaceae* plants (Table 4). The overexpression of H6H under control of the CMV 35S promoter in transgenic hairy root cultures of *Atropa baetica* [84], *Hyoscyamus niger* [85], *Hyoscyamus muticus* [86], Scopolia *parviflora* [87], and a *Duboisia* hybrid [88] cultures systematically resulted in an increase in the production of scopolamine when compared to control cultures not expressing H6H (Table 4). Therefore, transgenic expression of H6H appears to be a general method for increasing scopolamine production in hairy root cultures of tropane alkaloid producing plants.

The conversion of putrescine to the N-methylputrescine by putrescine N-methyltransferase (PMT) is the first committed step in the biosynthesis of both tropane alkaloids and nicotine (Figure 8). Heterologous expression of *Nicotiana tabacum* PMT in *Scopolia parviflora* resulted in an 8-fold increase in scopolamine production and a 4.2 fold increase in hyoscyamine production [89]. A similar effect was observed with the expression of *Nicotiana tabacum* PMT in *H. muticus* and *D. metel* [90]. However the expression of *Nicotiana tabacum* PMT in other tropane alkaloid producing hairy root cultures including *Hyoscyamus niger, Atropa belladonna, Duboisia* hybrid had no effect on alkaloid production [85, 91, 92].

H6H and PMT were co-expressed in *Hyoscyamus niger* on a plasmid containing two separate expression cassettes both under the control of the CMV 35S promoter. Co-expression of H6H and PMT resulted in the highest production of scopolamine in hairy root culture reported to date of 411.2 mg/L. This was a 10-fold increase over control cultures and a 2-3 fold increase over cultures only expressing H6H (Table 4) [85].

The tropane alkaloid biosynthetic pathway has been used as a model system for the generation of high alkaloid producing cell cultures through genetic engineering. The extent to which these cultures can be modified is dependent on the identification of new genes encoding regulatory and biosynthetic enzymes involved in tropane alkaloid secondary metabolism.

3.4 Benzylisoquinoline Alkaloids

The benzylisoquinoline alkaloids (BIA) include medicinally important compounds such as the analgesics morphine and codeine, and the antibiotics berberine, and sanguinarine (Figure 9). Although used for over a century, morphine and codeine are still two of the most important analgesics in medicine worldwide. Additionally, the morphinan alkaloid thebaine is converted semi-synthetically to yield the analgesics oxycodone and buprenorphine (Figure 9). Due to their complexity and multiple chiral centers, commercial synthesis of morphine, codeine and other BIA is not economically feasible, and opium poppy plants remain the main commercial source of the alkaloids [93].

Development of plant cell cultures for the BIA producing medicinal plants *Eschscholzia californica*, *Papaver somniferum* and *Coptis japonica* aided in the isolation and cloning of many of the enzymes involved in the biosynthesis of BIA [for reviews see 94, 95]. In brief, the biosynthesis of BIA begins with the condensation of dopamine and 4-hydroxyphenylacetaldehyde, both derived from tyrosine, by norcoclaurine synthase to form (S)-norcoclaurine (Figure 9). (S)-Norcoclaurine is converted to (S)-reticuline by a number of enzymes, all of which have been isolated and cloned. (S)-reticuline is the last common intermediate for the berberine, benzophenanthridine, and morphinan alkaloids. In morphine biosynthesis, (S)-reticuline is converted to (R)-reticuline through a 1, 2-dehydroreticuline

intermediate. Many of the downstream enzymes of this pathway have been isolated and cloned; for example, codeinone reductase and salutidarinol-7-O-acetyltransferase have very recently been cloned [96,97].

In the biosynthesis of berberine, an antibacterial benzylisoquinoline alkaloid, (S)-reticuline is converted to (S)-scoulerine (Figure 9). All the enzymes involved in the biosynthesis have been isolated and cloned except the final enzyme. Overproduction of berberine in *C. japonica* cell suspension cultures was achieved by selection of a high producing cell line [98], with reported productivity of berberine reaching 7 g/L [99]. This was one of the first demonstrations of production of a benzylisoquinoline alkaloid in cell culture at levels necessary for economic production. Another BIA, sanguinarine, a benzophenanthridine type alkaloid, is used as an antibiotic and antiplaque agent in toothpastes and mouthwashes. It is commercially isolated from the root of *Sanguinaria Canadensis* L. The production of sanguinarine in cell suspension cultures has been optimized by elicitation [100], medium optimization [101], resin addition [102], and metabolic engineering [103] to a maximum reported yield of 2% DW in cell suspension cultures of *Papaver Somniferum* after elicitation.

The morphinan alkaloids, the most desirable benzylisoquinoline alkaloids from an economic perspective, are not produced in high amounts in cell suspension cultures. Several strategies for increasing the production of morphinan alkaloids in plants and plant cell cultures have been employed including media optimization, differentiated cell cultures, RNA mediated suppression of off pathway enzymes, and over-expression of rate limiting pathway enzymes.

The low production of morphinan alkaloids in cell suspension cultures is thought to be due to the need for differentiation for production of these secondary metabolites. Removal of hormones from the media is often used to induce embryogenesis and promote cellular

differentiation. In one study, a two stage process where cell suspension culture were grown in media containing hormones and then transferred to hormone free media for production of secondary metabolites, resulted in increased production of morphinan alkaloids [104]. Often the removal of hormones results in the formation of shoots roots and meristemoids. In this study the culture maintained its dispersed character after removal of hormones, and was maintained in hormone free medium for 9 months. Removal of exogenous hormones from the medium of cell suspension cultures of *Papaver somniferum* resulted in the accumulation of 0.3 % DW and 0.25 % DW codeine and morphine respectively, a 3-fold increase over control cultures supplied with hormones [104]. However, these levels are still significantly lower than the 1.23-2.45% commonly found in field grown whole plant extracts and the 20 % DW found in the latex of *Papaver somniferum*. Since differentiated cell cultures often lead to increased production of certain secondary metabolites, hairy root cultures of *Papaver somniferum* and *Eschscholzia californica* were established in the hopes that benzylisoquinoline alkaloid production would be increased. Production of morphine, codeine and sanguinarine was achieved in several hairy root lines of *P. somniferum* [105]. Transformed root cultures obtained by infection with *Agrobacterium* contained a 1.4 fold higher alkaloid content compared to non-transformed roots and yielded a 3-fold increase in production of codeine (0.18% DW) when compared to non-transformed roots. Morphine was produced in similar concentration to non-transformed roots at 0.26 % DW. The hairy roots also produced sanguinarine (0.02 % DW), which is not produced in non-transformed roots. Additionally, the hairy root culture media contained 0.26 and 0.014 % DW morphine and sanguinarine respectively.

RNA mediated suppression of pathway enzymes is a potential tool for the metabolic engineering of secondary metabolism. Blocking the action of enzymes at pathway branch points

could lead to an increase in the levels of a central intermediate that could in turn be converted to a greater amount of the desired end product. Suppression of enzymes may also give insight into regulatory effects of certain pathway enzymes. Both berberine and morphinan alkaloids arise from a central intermediate (S)-reticuline (Figure 9). One strategy to increase production of the morphinan alkaloids is to prevent (S)-reticuline from being converted to the berberine or benzophenanthridine type alkaloids (Figure 9). Presumably, more (S)-reticuline will then be available for conversion to alternative end products though additional metabolic engineering.

Berberine bridge enzyme (BBE) catalyzes the conversion of S-reticuline to S-scoulerine, the intermediate that leads to the berberine and benzophenanthridine alkaloids, berberine and sanguinarine respectively. Antisense RNA-mediated suppression of BBE in cell suspension cultures of California poppy, which produces benzophenanthridine alkaloids but not morphinan or berberine alkaloids, resulted in a 10 fold decrease in total alkaloid production [106]. The suppression of BBE did not result in the accumulation of the (S)-reticuline biosynthetic intermediate. Antisense suppression of BBE in hairy root cultures of California poppy gave results similar to those observed in cell suspension cultures. Over expression of *Papaver somniferum* BBE, under the control of the cauliflower mosaic virus 35S promoter, in hairy root cultures resulted in a 5 fold increase in total alkaloid content [107].

In a separate study, a RNAi knockdown of BBE in California poppy cell suspension cultures resulted in the accumulation of (S)-reticuline [108], which was not accumulated in any previous RNA antisense mediated suppression of cell suspension and hairy root cultures. The reason for the differences in these results is unknown. BBE RNAi knockdown cultures accumulated a maximum of 0.031 % fresh weight (S)-reticuline versus 0.00045 % fresh weight found in control cultures. Additionally, 3 healthy growing BBE knockdown cell lines secreted

significant amounts of (S)-reticuline into the medium (6 mg/20 mL after 2 weeks). (S)-reticuline has been shown to be a substrate for the manufacturing of various compounds that possess anti-malarial and anticancer activity, so this cell line may have some commercial interest [93]. Additional products were 7-O-methylreticuline ((S)-laudanine) and 1, 2-dehydroreticuline, both minor components in wild type cultures and formed in greater amounts in BBE suppressed cultures. This study showed the accumulation of the central branch point intermediate, (S)-reticuline, and reduction of the naturally produced benzophenanthridine class of benzylisoquinoline alkaloids in California poppy. Antisense BBE was transformed into opium poppy plant and showed a similar increase in (S)-reticuline along with other alkaloids; including laudanine, laudanosine, 1, 2-dehydroreticuline, salutaridine and (S)-scoulerine and no change in the relative concentration of the morphinan alkaloids [109].

In attempts to accumulate the precursor to the analgesic oxycodone, thebaine, and decrease production of morphine (a precursor to the recreational drug heroine), RNAi mediated silencing of codeinone reductase, in opium poppy plant, was performed [93]. Codeine reductase is the penultimate step in morphine biosynthesis. Opium poppy was transformed with a chimeric cDNA hairpin RNA construct designed to silence codeinone reductase. However, silencing of codeinone reductase resulted in the accumulation of (S)-reticuline, but not the substrate codeinone or other compounds on the pathway from (S)-reticuline to codeine [93]. Allen and coworkers postulated that this accumulation could be due to several factors. First, accumulation of codeinone and morphinone could result in negative feedback on one of the enzymes, such as the reductase responsible for the reduction of (S)-reticuline to 1, 2-dehydroreticuline. Also, the biosynthetic intermediates and final product may regulate the transcription of pathway enzymes, though analysis of the transcript levels of a number of the morphine biosynthetic enzymes

showed no change in suppressed plants. Finally, codeinone reductase could be a part of a multienzyme complex, which can not function when one of the enzymes is removed. This study highlights that the complex metabolic networks found in plants are not easily or predictably redirected.

Secondary metabolite profiles can also be altered by the over expression of enzymes that are rate limiting in the synthesis of the desired product. Since the silencing of codeinone reductase leads to accumulation of (S)-reticuline, this enzyme may play a role in regulating the morphinan alkaloid biosynthetic pathway and overexpression of the reductase may lead to increased production of morphinan alkaloids. The over expression of codeinone reductase in opium poppy plants did in fact result in an increase in morphine and other morphinan alkaloids, such as morphine, codeine, and thebaine compared to the control plants [110]. This demonstrates that increase of morphinan alkaloid levels can be achieved by increased expression of a single pathway enzyme.

In another study , a cytochrome P450 (CYP80B3) responsible for the oxidation of (S)-N-methylcoclaurine to (S)-3'-hydroxy-N-methylcocluarine (Figure 9), was over expressed in opium poppy plants, and morphinan alkaloid production in the latex was increased to 4.5 times the level in wild type plants [111]. Additionally, expression of antisense CYP80B3 resulted in a decrease in morphinan alkaloids to 16% of the wild type level. The increase (or decrease) in alkaloid levels was found to correlate with overexpression (or suppression) of a number of other biosynthetic enzyme transcript levels in plants overexpressing (or suppressing) CYP80B3. These results support the regulatory role CYP80B3 plays in the production of benzylisoquinoline alkaloids.

Moreover, this work highlights the complexity of the regulation of biosynthesis of

secondary metabolites, where enzymes that perform the catalytic roles also have a regulatory function using mechanisms that are not yet understood. Understanding of these mechanisms will impact the future of metabolic engineering of secondary metabolism in both plants and plant cell cultures. The effective expression and suppression of pathway enzymes in hairy root cultures, cell suspension cultures and whole plants indicates a positive future for the optimization of production of benzylisoquinoline alkaloids compounds in plant cell culture for drug manufacturing.

3.5 Artemisinin

Approximately 100 million people are infected with malaria each year, with 1 million dying from the disease [112]. The third world is particularly affected and there is a great need for inexpensive, easily administered, effective drugs. Malaria is caused by the parasite *Plasmodium*, and a number of strains resistant to older antimalarials have recently emerged [113]. Notably, used in combination with older antimalarials, artemisinin and its derivatives appear to be effective against drug resistant *Plasmodium falciparum* strains found in Southeast Asia and Africa (Figure 10) [114]. The biological mechanism of action of this drug is not entirely clear. Although a variety of biological mechanisms have been proposed, the sarco/endoplasmic reticulum Ca2+ ATPase orthologue PfATP6 appears to be the likely target [115].

The extracts of *Artemisia annua* (annual or sweet wormwood), a plant that is native to China but is now endemic in many countries, were found in the 1970's to have antimalarial activity. The active agent, artemisinin (also known as qinghaosu [118] was isolated in 1972 from the aerial portions of the plant at concentrations of 0.01-0.86% [116]. The compound was

structurally elucidated in 1979 and shown to be a sesquiterpene natural product containing an endoperoxide moiety (Figure 10).

The low production levels in the plant make commercialization of artemisinin difficult. Total synthesis does not provide a commercially viable solution. Therefore, plant cell culture is being examined as a viable strategy for artemisinin production. Although production levels to date still remain low, it is hoped that optimization and genetic efforts can improve the prospects of plant cell culture as a source for artemisinin.

Efforts at establishing artemisinin producing *A. annua* cultures began in the early 1980's [117]. Although a range of artemisinin levels have been found in callus, shoot and root cultures, no artemisinin has been found in cell suspension cultures, suggesting that some degree of differentiation is required for production [114]. Transformation of *A. annua* with *Agrobacterium rhizogenes* yielded hairy root cultures that produced artemisinin at modest levels. Currently, many research efforts are directed toward optimizing artemisinin production in hairy root cultures [118]. Bioreactors designed for the production of artemisinin from hairy root cultures have been developed, though much more optimization needs to be done before commercial levels are obtained.

The biosynthesis of artemisinin involves cyclization of farnesyl pyrophosphate to the germacrane skeleton ((amorpha-4,11-diene) by a terpene cyclase. The exact order of the steps following cyclization has not been elucidated. The gene encoding the terpene cyclase has been cloned, as well as a number of genes involved in the biosynthesis of the farnesyl pyrophosphate precursor [119, 120, 121, 122]. Since the complete biosynthetic pathway has not been elucidated, artemisinin cannot be expressed in a microbial host. However, attempts have been made to over express the genes in the mevalonic acid biosynthetic pathway as well as the terpene cyclase gene

(amorpha-4,11-diene synthase) in *E. coli* to yield 24 μg/mL of the artemisinin precursor amorphadiene [123]. Notably, an efficient transformation system for *A. annua* is available, suggesting that as additional genes in the biosynthetic pathway are elucidated, genetic manipulation in plant cell cultures may improve production levels [124].

3.6 Shikonin

Shikonin represents one of the earliest of the plant cell culture success stories (Figure 11). Shikonin is a naphthoquinone pigment accumulated in a variety of boraginaceous species [125]. Shikonin displays anti-microbial, anti-inflammatory and anti-tumor activity. Mechanistic studies indicate that shikonin acts as a topoisomerase I inhibitor and that it also induces apoptosis in certain leukemia cell lines [126]. Shikonin appears to inhibit angiogenesis in *in vitro* studies. Shikonin is also used as a lipstick pigment in certain countries [127]. It was first demonstrated that *Lithospermum erythrorhizon* callus cultures yielded shikonin acyl esters in the 1970's [128]. High producing cell lines, producing up to 1.2 mg/g (fresh weight), could be selected for visually, since shikonin is red. Production in cell suspension cultures was subjected to extensive optimization both in terms of generation of cell biomass and product production. It was found that sucrose, oligogalacturonide and methyl jasmonate upregulated the production of shikonin [127]. A 750 L bioreactor culture yielded 1.4-2.3 g/L of shikonin in 23 days [129]. Commercial production of shikonin was reported by Mitsui Petrochemical Industry in 1983 [127].

A hairy root culture of *L. erythrorhizon* was established by infection with *Agrobacterium rhizogenes*. The hairy root culture produced shikonin in the root culture medium and also secreted it into the medium [130]. Addition of absorbents increased the concentration of shikonin produced to levels of approximately 9 mg/day. The plant cell cultures were also used to

investigate the mechanism of biosynthesis and regulation [131]. Feeding studies in the cell cultures were used to demonstrate that shikonin is derived from *p*-hydroxy-benzoic acid and geranyl diphosphate [132]. A proposed biosynthetic pathway is shown in Figure 11. However, most of the enzymes that catalyze the biosynthetic transformations remain to be cloned.

3.7 Podophyllotoxin

Podophyllotoxin, a liganin, was discovered to possess antitumor activity in the early 1980's (Figure 12) [133]. This compound binds to the protein tubulin and prevents association of tubulin into microtubules [134]. Although podophyllotoxin proved to be too toxic for clinical use, a variety of semi-synthetic podophyllotoxin derivatives were screened for anti-tumor activity and the clinical drugs etoposide, etopophos and teniposide, both topoisomerase II inhibitors, were developed from these studies and are produced by Bristol-Myers Squibb (Figure 12) [135,136].

Podophyllotoxin is produced in highest levels (4.3 % DW) in *Podophyllum hexandrum* [137]. Many *Podophyllum* species are endangered, so establishment of a renewable plant cell culture source of this compound would be highly desirable. Although a cell culture that produces appropriate amounts of *Podophyllum* have not been established yet, efforts are being made to develop a cell culture line capable of sustaining podophyllotoxin production. However, cell cultures of *P. hexandrum* produced greatly reduced levels of the compound [138]. A variety of plant species have been screened for podophyllotoxin production and it appears that *Linum album*, *L. flavum* and *L. nodiflorum* all produce podophyllotoxin and grow well as cell suspension cultures. Cell suspension cultures of *L. album* produce 0.2-0.5 % DW podophyllotoxin, which, although an improvement over earlier cell lines, still does not approach

the levels observed in the whole plant [139]. Root cultures of *L. flavum* have been shown to produce up to 5 % DW podophyllotoxin, suggesting that root cultures may provide the most viable production strategy for this compound in culture [139].

4. Future Directions

Plant derived natural products play a critical role in modern medicine. However, harvesting these products from the plant is expensive, requires large amounts of plant material and is often damaging to the environment. Due to the structural complexity of natural products, total synthesis does not present an economical option for industrial production for many of these metabolites. Plant cell cultures have been established for a wide variety of species using nominal amounts of plant material, a particularly useful strategy for the production of metabolites from endangered or rare plants. Although plant cell culture represents an important alternative for natural product production, only a small fraction of plant natural products can be expressed at industrially useful levels in culture. However, as biosynthetic genes are cloned, the opportunities to metabolically engineer plant cells become more feasible, and the commercial prospects of plant cell cultures may improve.

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Figure 1. Representative pharmaceuticals found in plants.

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Figure 3. Schematic of stirred and airlift bioreactors.

Figure 4. Chemical structures of jasmonate elicitors.

Figure 5. Structures of taxanes.

Figure 6. Paclitaxel biosynthetic pathway. GGPS (geranyl geranyl pyrophosphate synthase); TASY (taxadiene synthase); T5αH (taxadiene 5α hydroxylase); TDAT (taxadiene5α-ol O-acetyltransferase); T10βH (taxane 10β-hydroxylase); TBT (taxane 2a-O-benzoyltransferase); DBAT (10-deacetylbaccatin III-10-O-acetyltransferase); PPT (phenylpropanoyl transferase); NDBzT (3'-N-debenzoyl-2-deoxytaxol-N-benzoyltransferase)

Figure 7. Biosynthetic scheme for camptothecin. Structure of camptothecin and derivatives.

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Figure 9. Tetrahydroisoquinoline biosynthesis. NCS (norcoclaurine synthase); 6-OMT (O-methyl transferase); NMT (N-methyltransferase); CPY80B3 (cytochrome P450); 4OMT (O-methyl transferase) BBE (berberine bridge enzyme); STS (salutaridinol synthase); SAT (salutidarinol-7-O-acetyltransferase), COR (codeine reductase)

Figure 10. Artemensinin.

Figure 11. Shikonin.

Figure 12. Podophyllotoxin biosynthesis and chemical structures of derivatives.

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Cell culture	Product	Function	Manufacturer
Duboisia	Scopolamine	Anticholinergicum	Sumitomo Chemical Industries (Japan)
Podophyllum	Podophyllotoxin	Anti-tumor	Nippon Oil (Japan)
Coptis japonica Thalictrum minus	Berberines	Anti-tumor	Mitsui Petrochemical Industries (Japan)
Taxus	Paclitaxel	Anti-tumor	ESCAgenetics (USA), Phyton Catalytic (USA/Germany) Nippon Oil (Japan)
Coleus blumei	Rosmarinic acid	Anti-inflammatory	Nattermann (Germany)
Panax ginseng	Ginseng biomass	Dietary supplement	Nitto Denko (Japan)
Echinacea purpurea Echinacea augustifolia	Echinaceae	Immunostimulant	Diversa (Germany)
Lithospermum erythrorhizon	Shikonin	Pigment Antibiotic	Mitsui Petrochemical Industries (Japan)
Geramineae	Geraniol	Essential oil	Mitsui Petrochemical Industries (Japan)
Catharanthus roseus	Arbtin	Pigment	Mitsui Petrochemical Industries (Japan)
Carthamus tinctorius	Carthamin	Pigment	Kibun (Japan)
Vanilla planifolia	Vanillin	Flavor	ESCAgenetics (USA)
Beta vulgaris	Betacyanins	Color	Nippon Shinyaku (Japan)
Euphorbia milli Aralia cordata	Anthocyanins	Dye, color	Nippon Paint (Japan)

Table 1. Plant cell cultures used in industry. (Adapted from reference 16.)

Cell Type	Paclitaxel (mg/L)	Culture Type	Reference
Taxus canadensis	117	Shake flask, MeJa	74
Taxus media	110	Shake flask, MeJa	26
Taxus media	21	Stirred reactor, MeJa, precursor	82
Taxus cuspidata	22	Wilson type	22
Taxus chinensis	138	Shake flask, silver nitrate	49

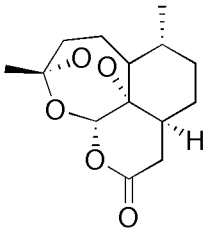
Table 2. Plant cell suspension cultures producing paclitaxel. (Adapted from Baldi A, Bisaria VS, Srivasrava AK (2007) Biotechnological approaches for the production of some promising plant based chemotherapies. In: O Kayser, W Quax (eds): *Medicinal Plant Biotechnology* Wiley VCH, Weinheim, 128)

Species	Culture	Conditions	CPT	HCPT	MCPT	Ref.
			% DW (mg/L)			
C acuminate	Leaves		0.4-0.5	0.003		59
	Roots		0.04	0.002		59
	Bark		0.18-0.2	0.009		59
	CSC	Control	0.009			66
		2 stage bioreactor Optimal nitrogen	0.036 (12.8)			
	Callus		0.232	0.008		68
	Hairy roots		0.1	0.015		72
N foetida	Shoot		0.075		0.013	69
	Seed		0.5		0.06	69
	CSC		0.11(35)		0.08 (26)	71
O pumila	Leaves		0.03			73
	Young roots		0.1			73
	Hairy root		0.1 (8.9)			73
		3L bioreactor	(8.7)			75
O luikiunesis	Hairy root		0.083 (4.6)		(0.5)	76
O. Kuroiwai	Hairy root		0.022 (9.4)		(0.3)	76

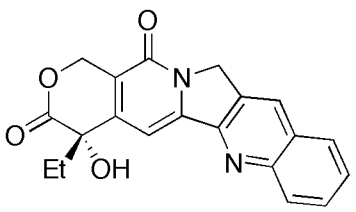
Table 3. Select examples of production of camptothecin and its analogs in various plants and plant cell culture. CSC is cell suspension culture, CPT is camptothecin, HCPT is 10-hydroxycamptothecin, MCPT is 9-methoxycamptothecin.

Species	Condition	Scopolamine	Hyoscyamine	Ref
		%DW (mg/L)		
Duboisia hybrid	Tranformed with *Hn*H6H	2.48 (74.5)	0.14 (4.5)	88
	Control	1.16(24.7)	0.39 (8.3)	
Atropa baetica	Transformed with *Hn*H6H	0.385	0.02	84
	Control	0.29	0.50	
Scopolia Parviflora	Transformed with *Nt*PMT	0.52	0.35	89
	Transformed with *Hn*H6H	0.81		87
	Control	0.08	0.10	
Hyoscyamus niger	Transformed with *Hn*H6H and *Nt*PMT	(411.2)		85
	Control	(43.7)		
	Transformed with *Hn*H6H	(184.4)		
Hyoscyamus muticus	Transformed with *Hn*H6H	0.11 (14.4)	0.98 (125.4)	86
	Control	(0.12)	(134.9)	

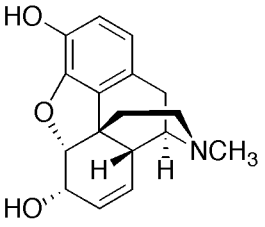
Table 4. Select examples of scopolamine and hyoscyamine production in wild type and transformed hairy root cultures. *Hn*H6H is *Hyoscyamus niger* H6H, NtPMT is *Nicotiana tabacum* PMT.



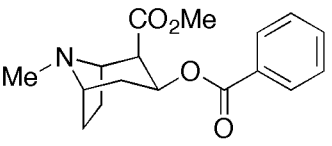
artemensin



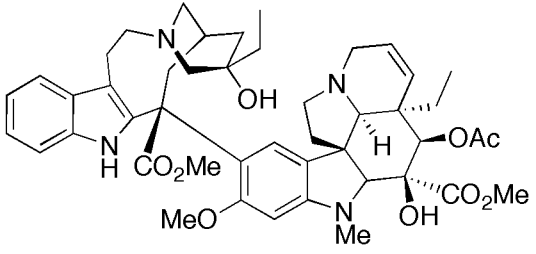
camptothecin



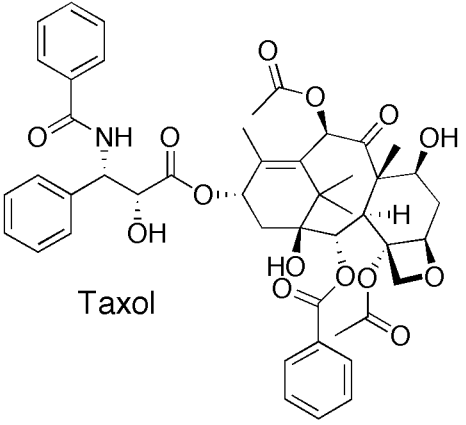
morphine



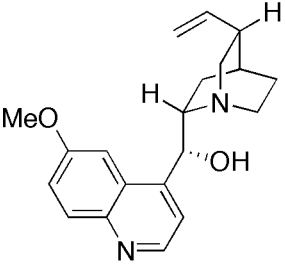
cocaine



vinblastine

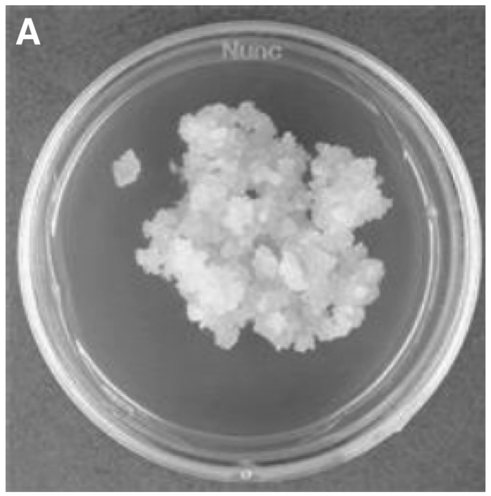


Taxol

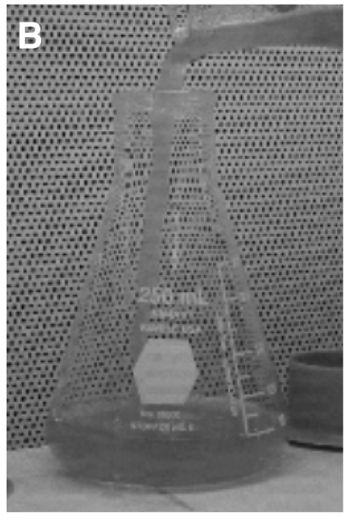


quinine

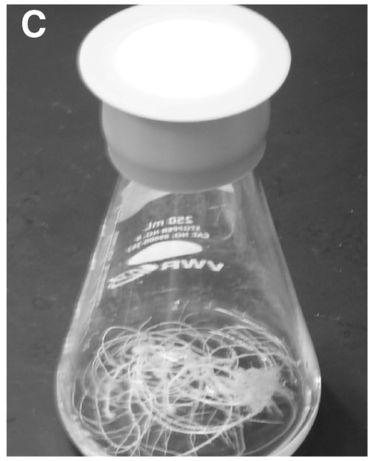
Figure 1



callus culture

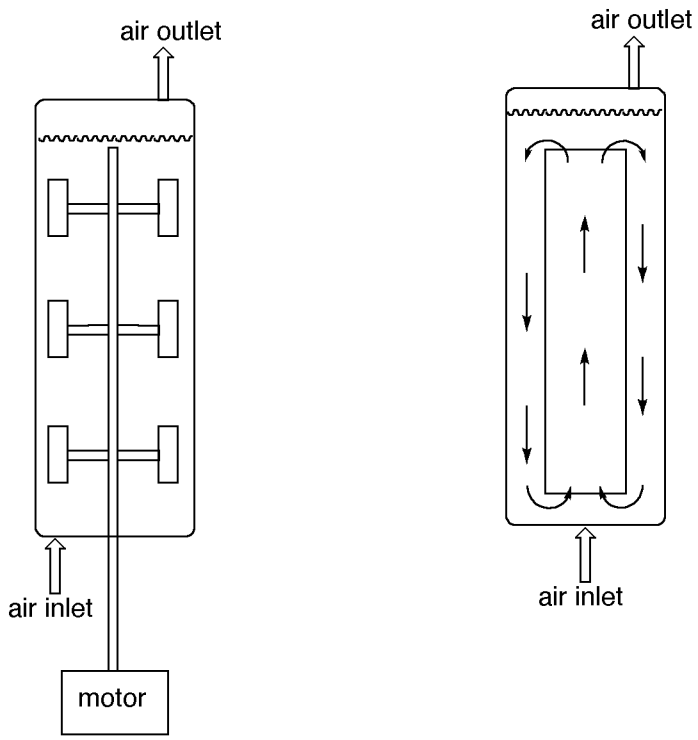


cell suspension culture



hairy root culture

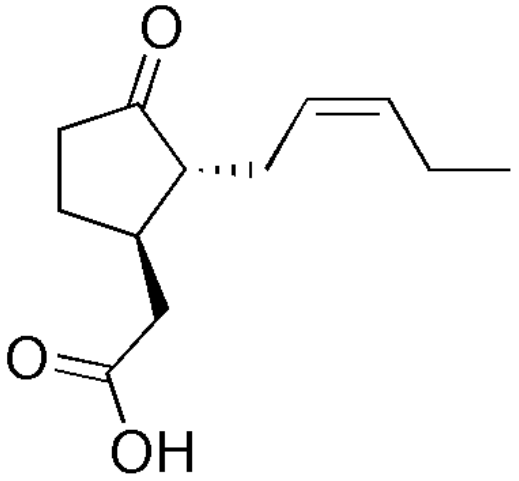
Figure 2



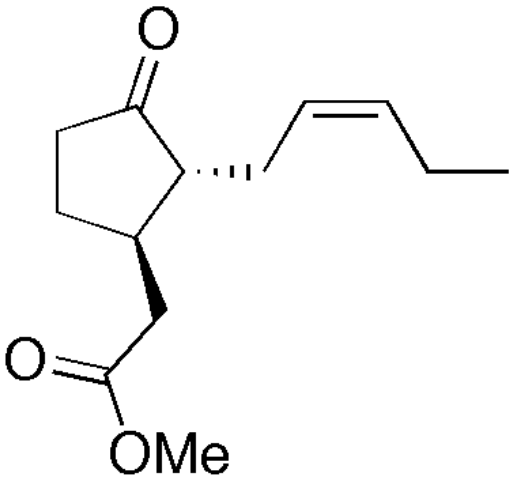
stirred reactor

airlift reactor

Figure 3

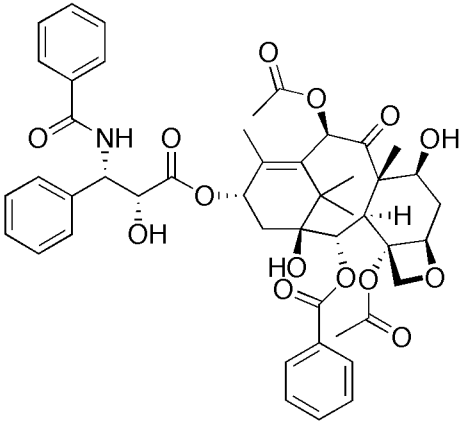


jasmonic acid

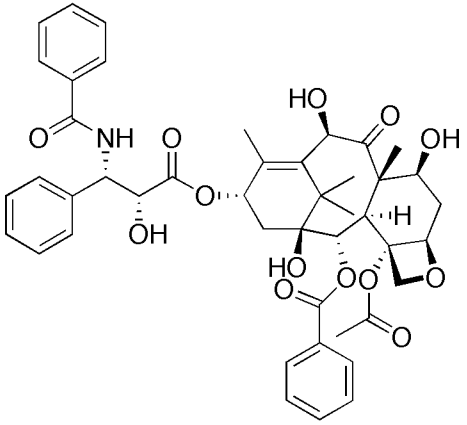


methyl jasmonate

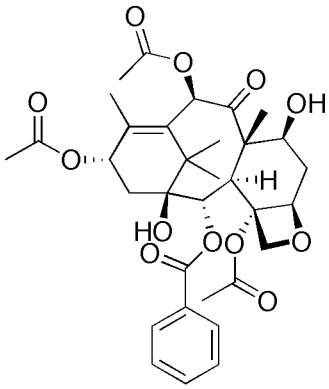
Figure 4



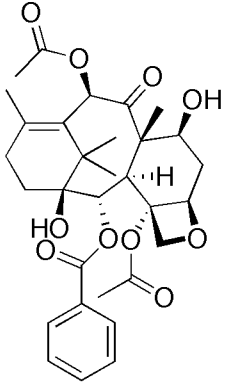
paclitaxel (Taxol)



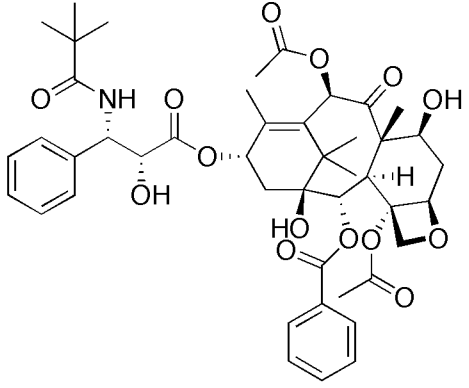
10-deacetylpaclitaxel



taxuyunnanine C



13-dehydroxybaccatin III



docetaxel (Taxotere)

Figure 5

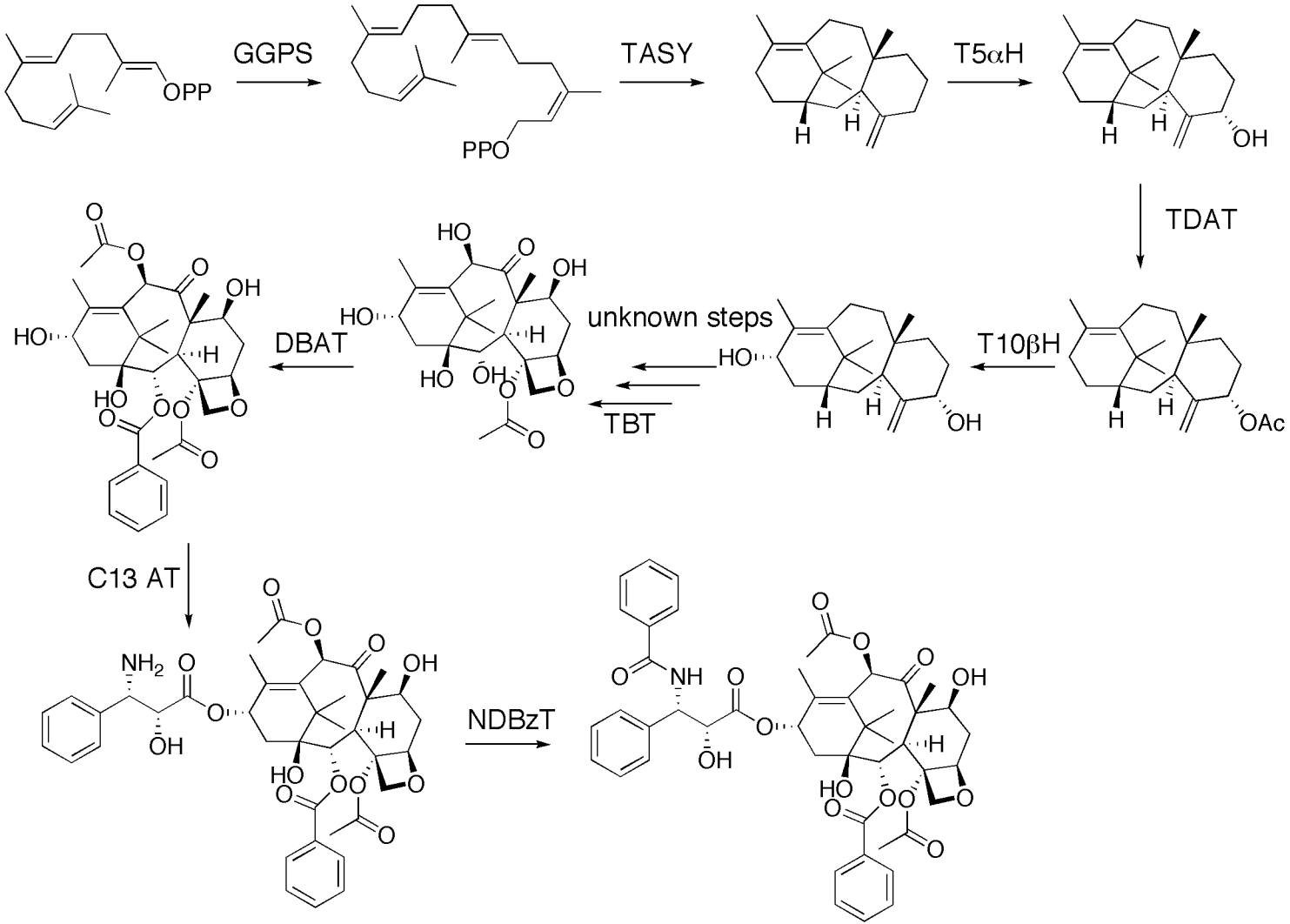
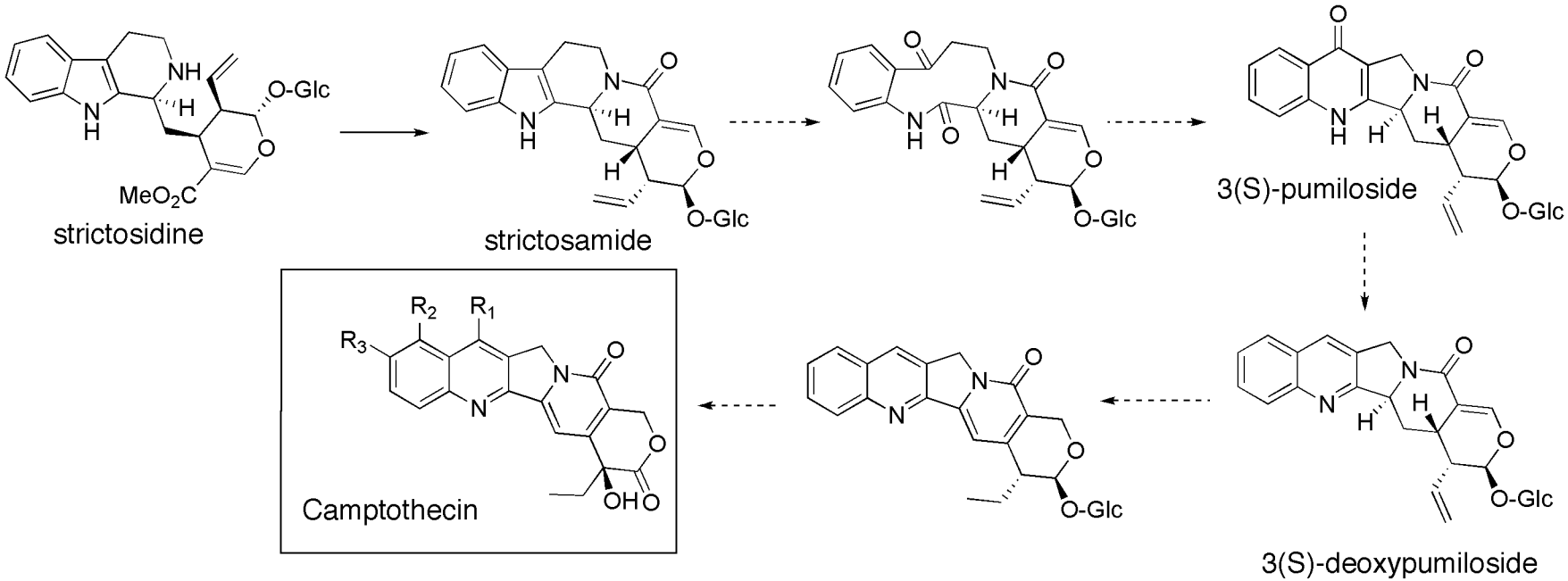

OPP
GGPS
PPO
TASY
T5αH
OH
TDAT
OAc
T10βH
unknown steps
TBT
DBAT
C13 AT
NH2
NDBzT
NH

Figure 6



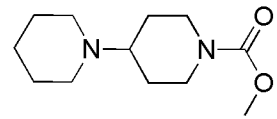
	R1	R2	R3
Camptothecin	H	H	H
9-methoxycamptothecin MCPT	H	OMe	H
10-hydroxycamptothecin HCPT	H	H	OH
Topotecan	H	$CH_2N(CH_3)_2$	OH
Irinotecan	C_2H_5	H	

Figure 7

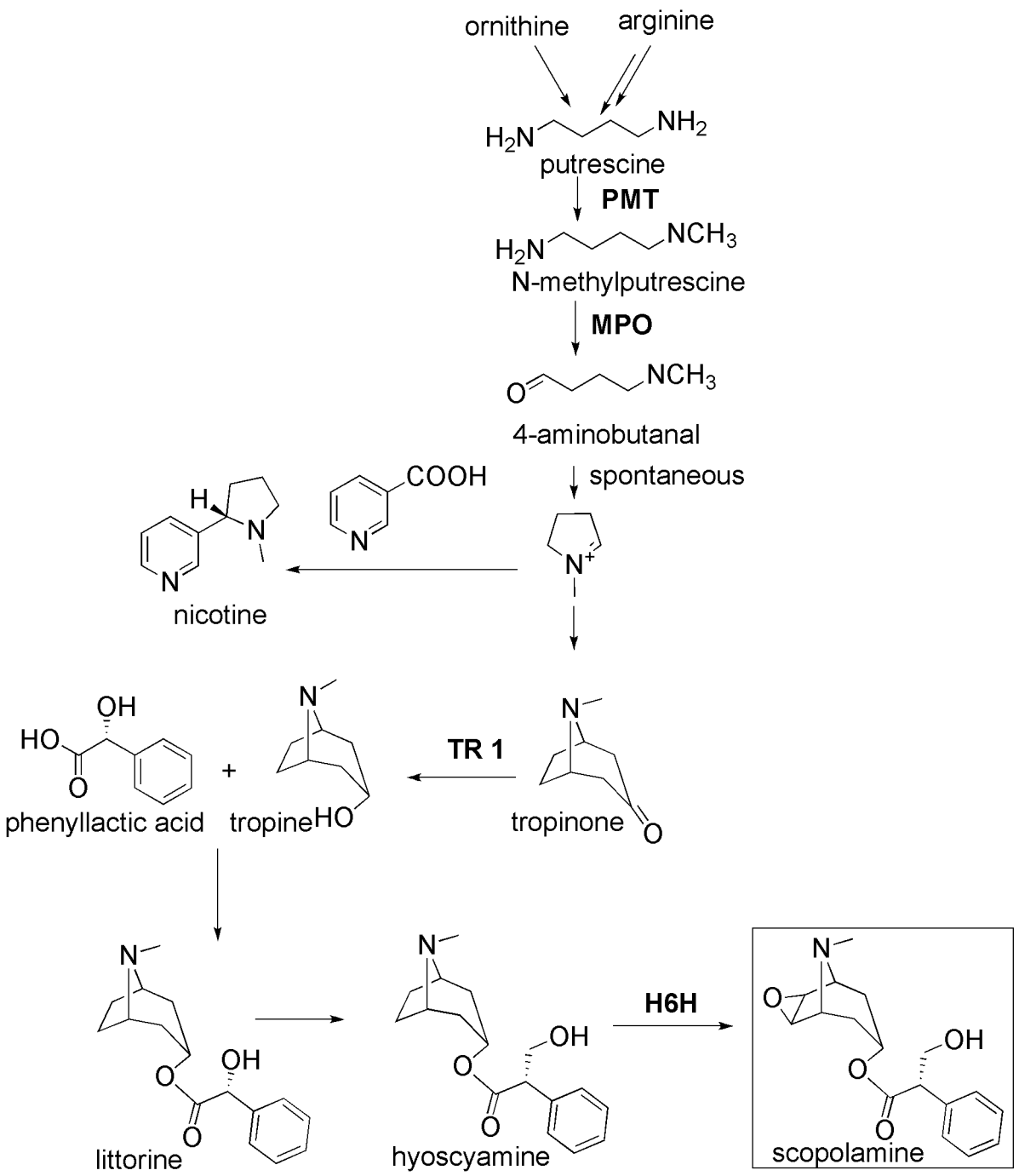
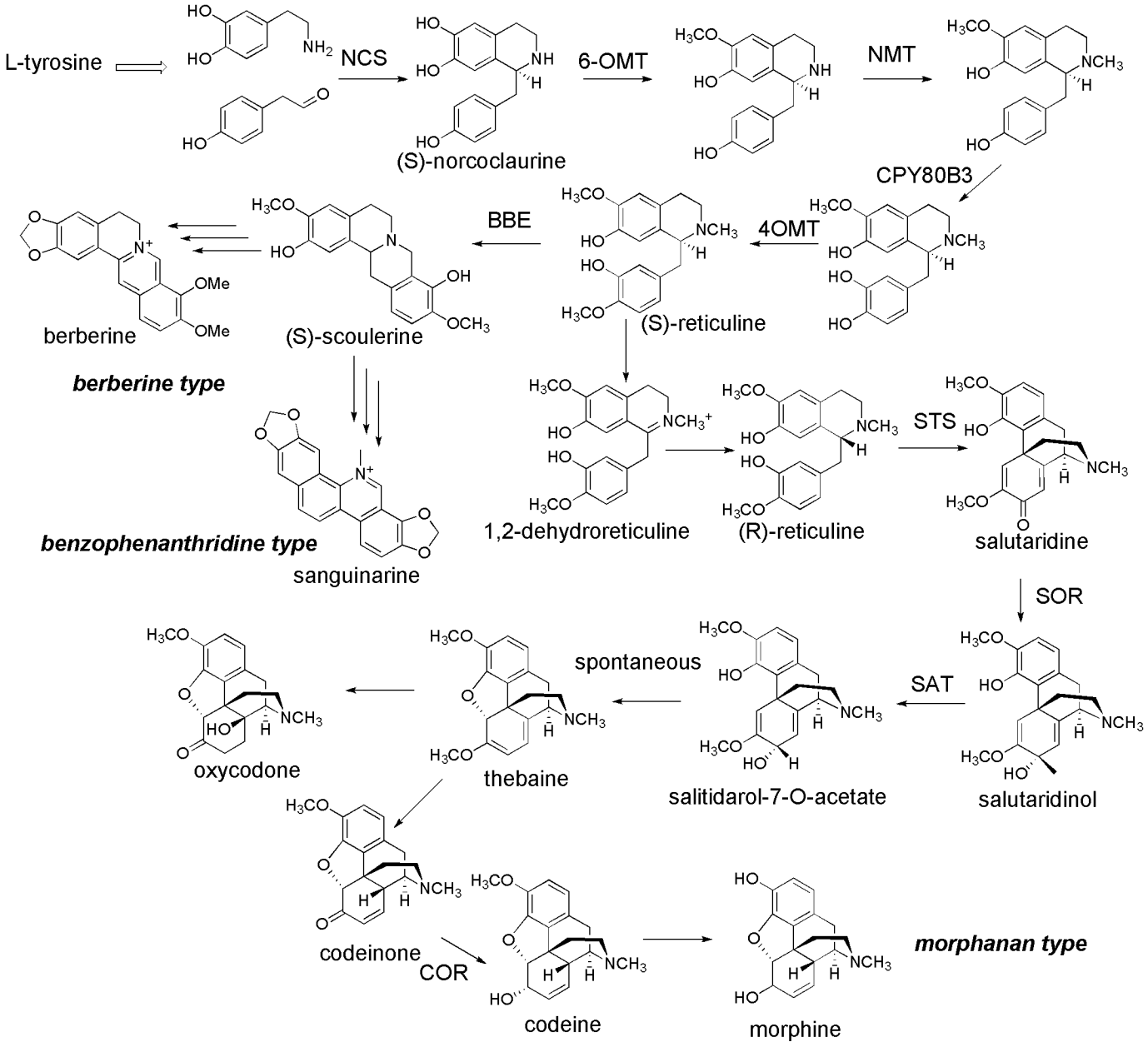

ornithine
arginine
putrescine
PMT
N-methylputrescine
MPO
4-aminobutanal
spontaneous
nicotine
phenyllactic acid
tropine
TR 1
tropinone
littorine
hyoscyamine
H6H
scopolamine

Figure 8


L-tyrosine
NCS
(S)-norcoclaurine
6-OMT
NMT
CPY80B3
4OMT
(S)-reticuline
BBE
(S)-scoulerine
berberine
berberine type
benzophenanthridine type
sanguinarine
1,2-dehydroreticuline
(R)-reticuline
STS
salutaridine
SOR
salutaridinol
SAT
salitidarol-7-O-acetate
spontaneous
thebaine
oxycodone
codeinone
COR
codeine
morphine
morphanan type

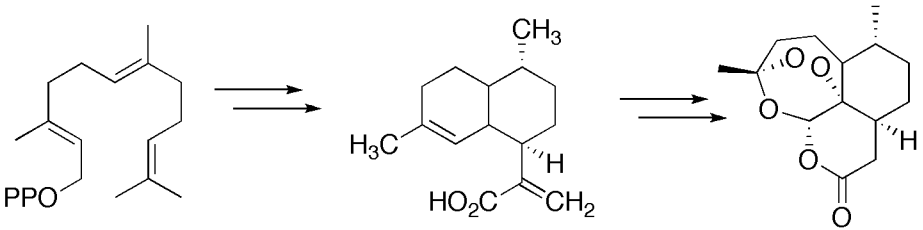

PPO
CH3
H3C
H
HO2C
CH2
O
O
O
O
O
H
farnesyl
pyrophosphate
artemisinin acid
artemisinin

Figure 10

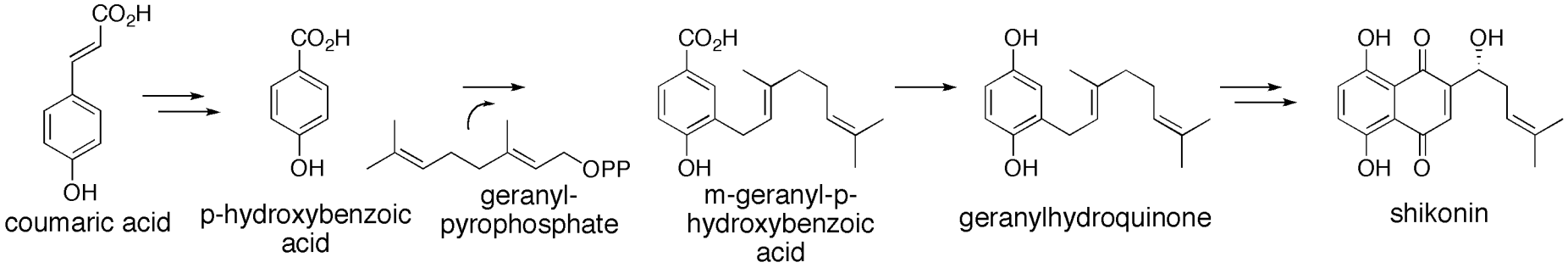

CO2H
OH
coumaric acid
p-hydroxybenzoic acid
OPP
geranyl-pyrophosphate
m-geranyl-p-hydroxybenzoic acid
geranylhydroquinone
O
shikonin

Figure 11

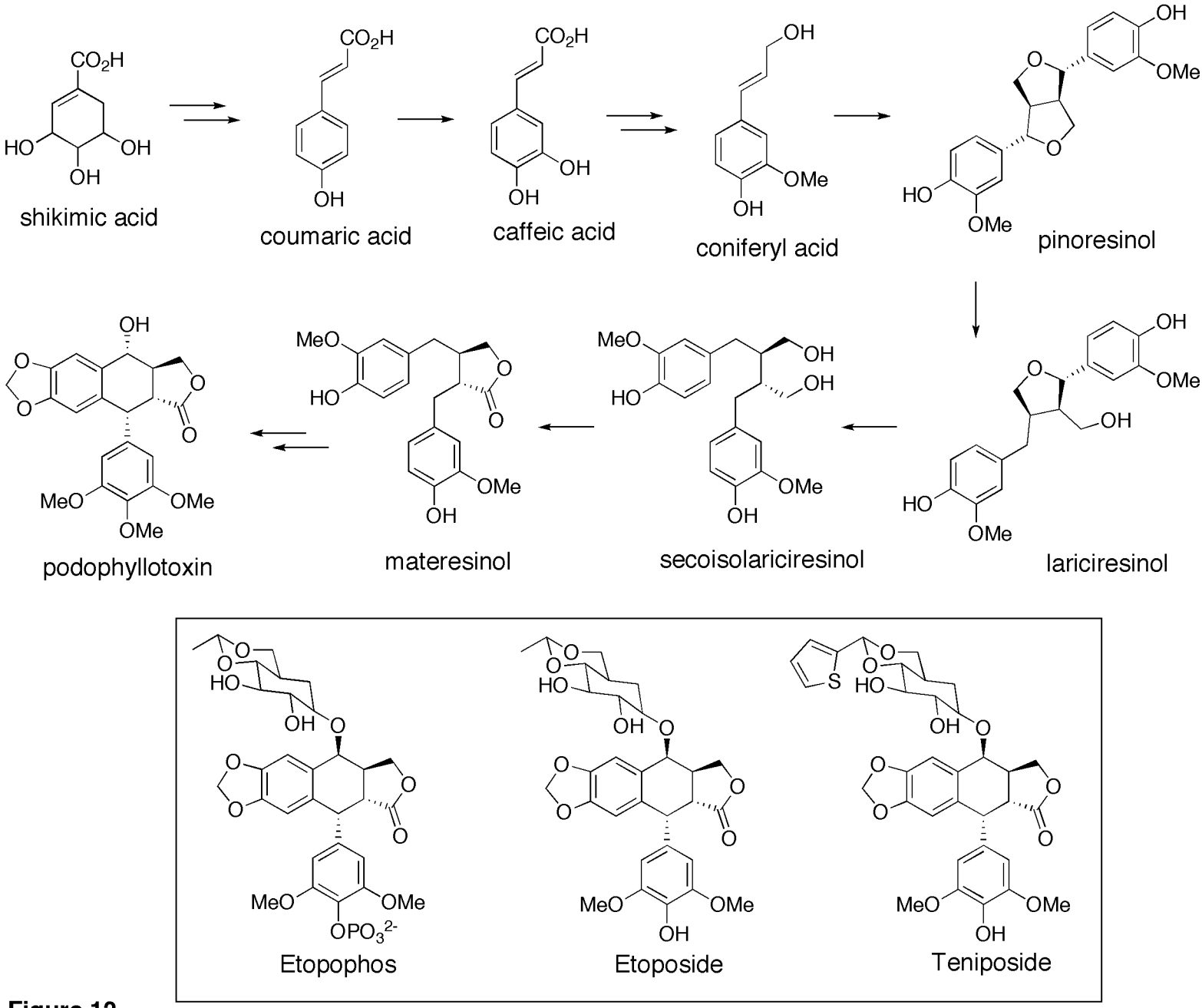

shikimic acid
coumaric acid
caffeic acid
coniferyl acid
pinoresinol
podophyllotoxin
materesinol
secoisolariciresinol
lariciresinol
Etopophos
Etoposide
Teniposide

Figure 12