Chapter 17
Higher Plant Sources of Cancer Chemotherapeutic Agents and the Potential Role of Biotechnological Approaches for Their Supply

Peter J. Blanco Carcache, Ermias Mekuria Addo, and A. Douglas Kinghorn

Abstract There are five major structural groups of cancer chemotherapeutic agents derived from higher plants approved clinically in the USA. These are used either in their unmodified naturally occurring form, as semi-synthetic derivatives, or as a conjugate as part of a larger drug molecule. In this chapter, those compounds used currently as approved oncolytic drugs will be described, following by a brief mention of selected plant-derived compounds in current clinical trials as potential cancer chemotherapeutic agents. Next, details on the methods proposed for the production for the oncology drug market of three examples of different structural groups of plant-derived anticancer agents will be given [*viz.*, bisindole (Vinca) alkaloids, podophyllotoxin lignan analogs, and taxane diterpenoid derivatives]. The plant natural products surveyed represent sustainable sources of specialized pure chemicals that are valuable in treating cancer.

Keywords Higher plants · Cancer chemotherapeutic agents · Clinical trials · Biotechnological methods of production · Secondary metabolites

17.1 Introduction

Cancer continues to be the second-leading cause of death, and more than 1.8 million people will be diagnosed with this disease in the USA alone in 2020, with the mortality rate for this same year projected to be above 9 million worldwide (Siegel et al. 2020; WHO 2018). This disease condition has many initial symptoms and complications that lead to a negative impact on an afflicted person's quality of life. Such symptoms include pain, bleeding, extreme fatigue, neurological problems, and dangerous weight gain or loss (National Cancer Institute 2019). The leading cancer types in new estimated cases in the USA for 2020 include those of the prostate or breast, lung and bronchus, colon and rectum, urinary corpus or bladder, melanoma

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H. M. Ekiert et al. (eds.), *Medicinal Plants*, Sustainable Development and Biodiversity 28, https://doi.org/10.1007/978-3-030-74779-4_17

of the skin, kidney and renal pelvis, non-Hodgkin's lymphoma, oral cavity, pharynx or thyroid, and pancreas, as well as leukemias and lymphomas (Siegel et al. 2020). However, the availability of cancer treatments is still somewhat limited, and many are not easily affordable. Therefore, additional treatments for cancer from sustainable resources need to be found and utilized.

Nature is a rich source of potential therapeutic agents obtained from various different life forms (Butler et al. 2014; Newman and Cragg 2020a). In particular, plant-based traditional medicine continues to play an essential role in the primary health care of approximately 80% of the world's population (Agarwal et al. 2020; Cragg and Newman 2005). Furthermore, nature continues to produce viable effective anticancer agents that are sustainable, such as those in clinical use that come from diverse origins, including plants, terrestrial microbial, and marine organism sources (Agarwal et al. 2020; Basmadjian et al. 2014; Butler et al. 2014; Cragg and Newman 2005; Cragg and Pezzuto 2016; Khazir et al. 2014; Li and Vederas 2009). Moreover, natural products have been established as being chemically diverse with optimal chirality to interact with different drug targets (Crüsemann et al. 2016; Pietra 2002).

Plant constituents have served as an important group of naturally occurring anticancer drugs. A particularly notable example is that of taxol (paclitaxel), a potent antineoplastic taxane diterpenoid isolated from *Taxus brevifolia* Nutt. of very wide clinical use that first reached the clinic in the USA to treat ovarian cancer in 1992 (Donehower 1996; Wani et al. 1971). There are also members of other compound classes of higher plant origin that have afforded anticancer compounds, namely the bisindole alkaloids, the podophyllotoxin lignans, the camptothecin derivatives, and the cephalotaxine analog, omecetaxine mepesuccinate (Agarwal et al. 2020; Butler et al. 2014; Henkin et al. 2018; Newman and Cragg 2020a). Over the past few decades, natural products have been used in various forms to treat various cancer types more efficiently. In more recent years, such natural products have evolved to incorporate some of the modern approaches of drug delivery used to develop safer, more efficaceous anticancer drugs. One type of approach is the use of antibody drug conjugates (ADCs), which are targeted therapeutics composed of three components (a cytotoxic drug, a linker, and an antibody) and are designed for selective delivery of very potent anticancer drugs (Agarwal et al. 2020). In December 2019, the first example of a higher plant-derived ADC was approved by the US FDA, namely famtrastuzumab deruxtecan-nxki (also known as DS-8201a, Enhertu®; Daiichi Sankyo), for the treatment of unresectable or metastatic HER2-positive breast cancer (US Food and Drug Administration 2019). DS-8201a is an ADC derived from the conjugation of the camptothecin derivative DXd with the monoclonal antibody trastuzumab via an enzyme cleavable peptide linker (Nakada et al. 2019).

Thus, higher plants have afforded numerous purified compounds of promise in treating cancer, which can be utilized in not only their unmodified naturally occurring forms, but also as semi-synthetic derivatives, or as conjugates in larger molecules. In this chapter, the major pure compounds of plant origin used currently as approved oncolytic drugs will be surveyed briefly, following by a selection of such compounds in clinical trials as potential cancer chemotherapeutic agents. After this, information on different approaches proposed of the production for the oncology drug market of

three selected groups of plant-derived anticancer agents (viz., bisindole alkaloids, podophyllotoxin analogs, and taxane derivatives) will be provided. Overall, plant natural products may be seen to have afforded sustainable sources of valuable rare compounds that now have major use in treating cancer.

17.2 Approved Plant-Derived Anticancer Drugs

In the paragraphs below, the plant-derived anticancer agents obtained from renewable resources that have been developed as sustainable anticancer agents for the market in Western medicine will be described (Fig. 17.1). These clinically used agents may be classified into several major groups.

The first group of plant-derived anticancer drugs introduced to the US market nearly 60 years ago were the bisindole alkaloids. This class of bisindole alkaloid drugs includes two compounds isolated from *Catharanthus roseus* G. Don (Apocynaceae), namely vinblastine (**1**) and vincristine (**2**). Additionally, three synthetic derivatives vindesine (**3**), vinflunine (**4**), and vinorelbine (**5**) have been developed. The two natural product drugs **1** and **2** have antimitotic effects and antimicrotubule properties. The US FDA approved these for the treatment of different cancer types, including certain forms of breast and lung cancer, leukemia, and lymphoma (Panda et al. 1996; Rowinsky and Donehower 1991; Tafur et al. 1975). Recent optimization of the formulation of vincristine has improved both its pharmacokinetic and pharmacodynamic profiles, to produce the FDA-approved vincristine sulfate liposome injection (Silverman and Deitcher 2013). The synthetic bisindole alkaloid derivatives also are marketed as anticancer drugs for clinical use. Vindesine (**3**) has been approved for childhood acute lymphocytic leukemia. Vinflunine (**4**) is approved in Europe as monotherapy for the treatment of metastatic bladder cancer and vinorelbine (**5**) also is approved for use against non-small cell lung cancer (Ianniello 1996; Roussi et al. 2012).

The second group of plant-derived anticancer drugs that was introduced commercially includes compounds of the podophyllotoxin lignan class. The epipodophyllotoxin-type lignan derivatives, etoposide (**6**), etoposide phosphate (**7**), and teniposide (**8**), were chemically optimized from the natural compound, podophyllotoxin, isolated from *Podophyllum peltatum* L. (Berberidaceae) (Chen et al. 2013; Clark and Slevin 1987). The main mechanism of action of these chemical derivatives of podophyllotoxin is by inhibiting topoisomerase II (Hartmann and Lipp 2006). Etoposide (**6**) and its close chemical analog etoposide phosphate (**7**), a more water-soluble version, have been used against non-small cell lung, small cell lung, and testicular cancers. Teniposide (**8**) has been utilized in various combination chemotherapy regimens, against neuroblastoma, acute lymphoblastic leukemia, and small cell lung cancer (Lee and Xiao 2012).

The third group of plant-derived anticancer drugs that were introduced to the market are representatives of the taxane diterpenoid class. This group comprises the taxane derivative paclitaxel (**9**) and its semi-synthetic derivatives docetaxel (**10**)

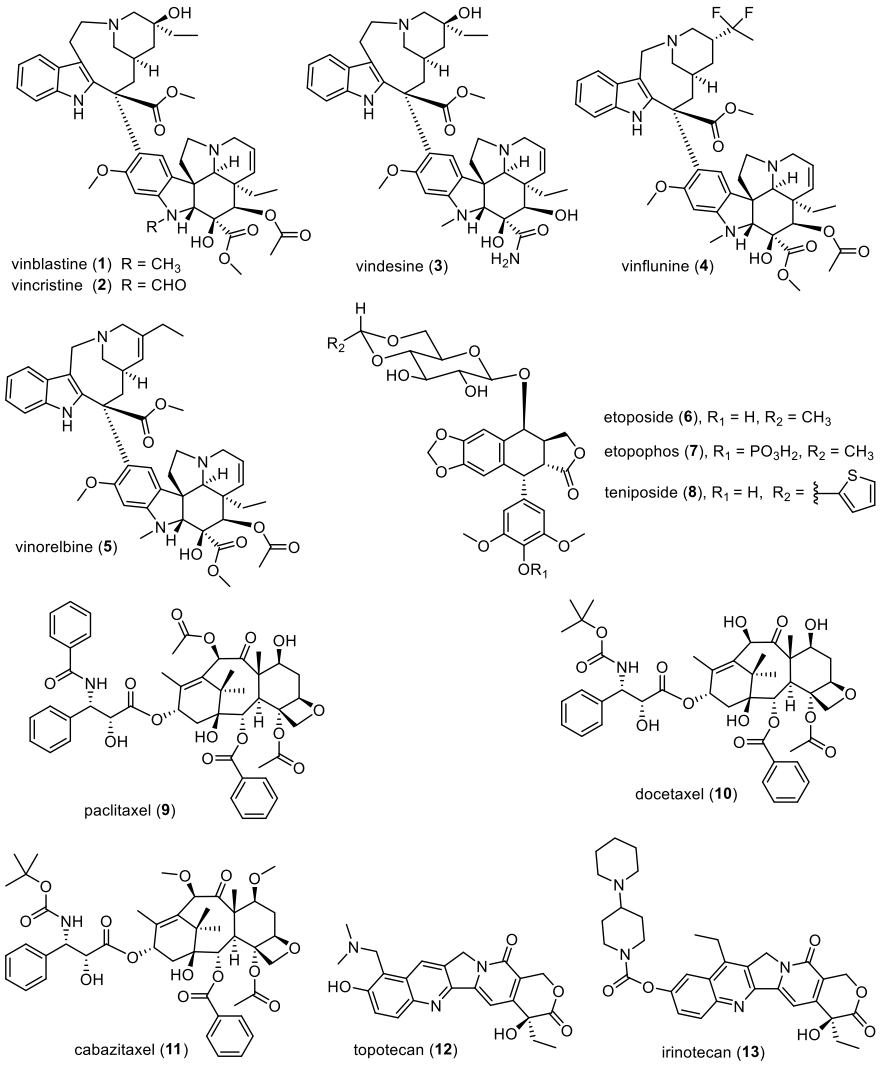

Fig. 17.1 Structures of plant-derived natural products used as anticancer agents

and cabazitaxel (**11**). In 1971, the parent compound, paclitaxel, was isolated and structurally characterized under the trivial name "taxol" from *Taxus brevifolia* Nutt. (Taxaceae) (Wani et al. 1971). Approved initially by the FDA in 1992, paclitaxel was used for the treatment of refractory ovarian cancer (Donehower 1996).

The limited water solubility of paclitaxel led to its chemical optimization as the semi-synthetic derivative, docetaxel (**10**) (Rowinsky and Donehower 1991). The primary mechanisms of action described for these taxane drugs are based on stabilizing the assembly of microtubules as well as inhibiting depolymerization of tubulin

during cell division. At present, both paclitaxel and docetaxel are used for different types of cancer, including breast, ovarian, and non-small cell lung cancer, while a newer analog, cabazitaxel (**11**), is prescribed for the treatment of hormone-refractory prostate cancer (Kingston 2012; Liu et al. 2016; Weaver 2014). Abraxane® is a nanoformulation of paclitaxel, and it has been approved for the treatment of advanced forms of breast cancer, non-small cell lung cancer, and pancreatic cancer (Hare et al. 2017).

A fourth group of plant-derived anticancer drugs to be introduced comprises several camptothecin alkaloid derivatives. In 1966, a new quinolone alkaloid from *Camptotheca acuminata* Decne. (Nyssaceae) was isolated and named camptothecin (Wall et al. 1966). Among camptothecin derivatives of interest for cancer treatment are topotecan (**12**) and irinotecan (**13**). Camptothecin proved to have suboptimal solubility and toxicity; hence, the chemical analogs **12** and **13** were developed successfully to improve its efficiency, by enhancing its bioavailability, and thus gained FDA approval about 25 years ago (Ciardiello et al. 1999; Hörmann et al. 2012). Members of the camptothecin compound class act as topoisomerase I inhibitors, with topotecan utilized for metastatic ovarian cancer (Ciardiello et al. 1999; Hörmann et al. 2012). Irinotecan, on the other hand, is used for metastatic colorectal cancer (Takeba et al. 2007; Villalona-Calero and Kolesar 2002). The very recently US FDA-approved ADC, fam-trastumazab deruxitecan-nxki (Enhertu®, Fig. 17.2) (**14**), is also a camptothecin derivative (Nakada et al. 2019).

The initial member of a fifth group of plant-derived anticancer drugs is categorized under the *Cephalotaxus* alkaloid class. Homoharringtonine was first isolated from an alkaloid fraction of *Cephalotaxus harringtonia* Kitam. (Taxodiaceae) (Powell et al. 1970). This compound, as the derivative, omacetaxine mepesuccinate (**15**), has been shown to exert antitumor and antiangiogenic activity, apoptotic induction, and protein synthesis inhibition. Omacetaxine mepesuccinate (**15**) is the first member of a new class of FDA-approved anticancer agents that acts as a protein translation inhibitor. Omacetaxine mepesuccinate (**15**) has been approved for the treatment of chronic

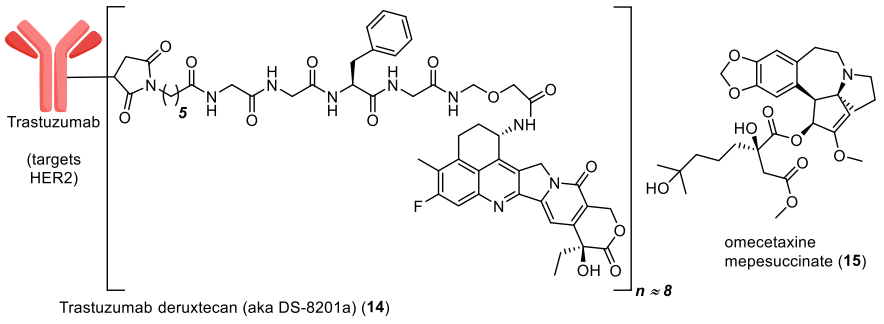

Fig. 17.2 Structures of fam-trastumazab deruxitecan-nxki (Enhertu®; **14**) [Adapted from (Nakada et al. 2019)] and omacetaxine mepesuccinate (**15**)

myeloid leukemia, particularly for adult patients with resistance or intolerance to two or more tyrosine kinase inhibitors (Alvandi et al. 2014).

Thus, higher plants have been shown as outstanding and sustainable sources of therapeutically useful cancer chemotherapeutic agents and hence may be expected to continue to provide new drug leads for the development of new anticancer drugs in the future.

17.3 Examples of Plant-Derived Anticancer Agents Undergoing Clinical Evaluation

In the paragraphs following, a selection of compounds isolated from higher plants and their derivatives that are undergoing human clinical studies is covered (Table 17.1 and Fig. 17.3). These are included in a relevant database of the US National Cancer Institute (NCI) (National Cancer Institute 2020).

A plant-derived natural product that has reached both phase III and IV level clinical trials is the camptothecin derivative karenitecin (**16**). Karenitecin is a topoisomerase I inhibitor intended for the potential treatment of melanoma (Munster and Daud 2011). The tubulin-binding agent combretastatin A-4 analog, fosbretabulin (**17**), has also reached both phase III and IV level clinical trials for the treatment of

Table 17.1 Potential plant-derived anticancer agents in ongoing clinical trials[a]

Chemotherapeutic agent	Compound type[b]	I[c]	II[c]	III[c]
Karenitecin (**16**)	NP-derived	X	X	X (IV)
Fosbretabulin (**17**)	SS NP		X	X (IV)
Combretastatin A-1 (**18**)	SS NP	X	X	
BNC105 (**19**)	NP-derived		X	X (IV)
AT-101 (**20**)	NP-derived	X	X	
Picropodophyllotoxin (**21**)	NP	X	X	
trans-Resveratrol (**22**)	NP	X	X (IV)	
ME-344 (**23**)	NP-derived	X (IV)	X	X
ARQ 761 (**24**)	NP-derived	X (IV)		
TPI 287 (**25**)	NP-derived	X	X	X (IV)
Ortataxel (**26**)	NP-derived	X	X	X (IV)
Riviciclib (**27**)	NP-derived	X	X (IV)	
Minnelide (**28**)	NP	X (IV)		

[a]Adapted from a recent review article and https://www.cancer.gov/about-cancer/treatment/clinical-trials/search (Butler et al. 2014; National Cancer Institute 2020)
[b]NP = natural product; SS = semi-synthetic
[c]I, II, III (IV) correspond to phase I, phase II, and phase III trials; phase IV trials are a continuation of phase III trials on a larger population

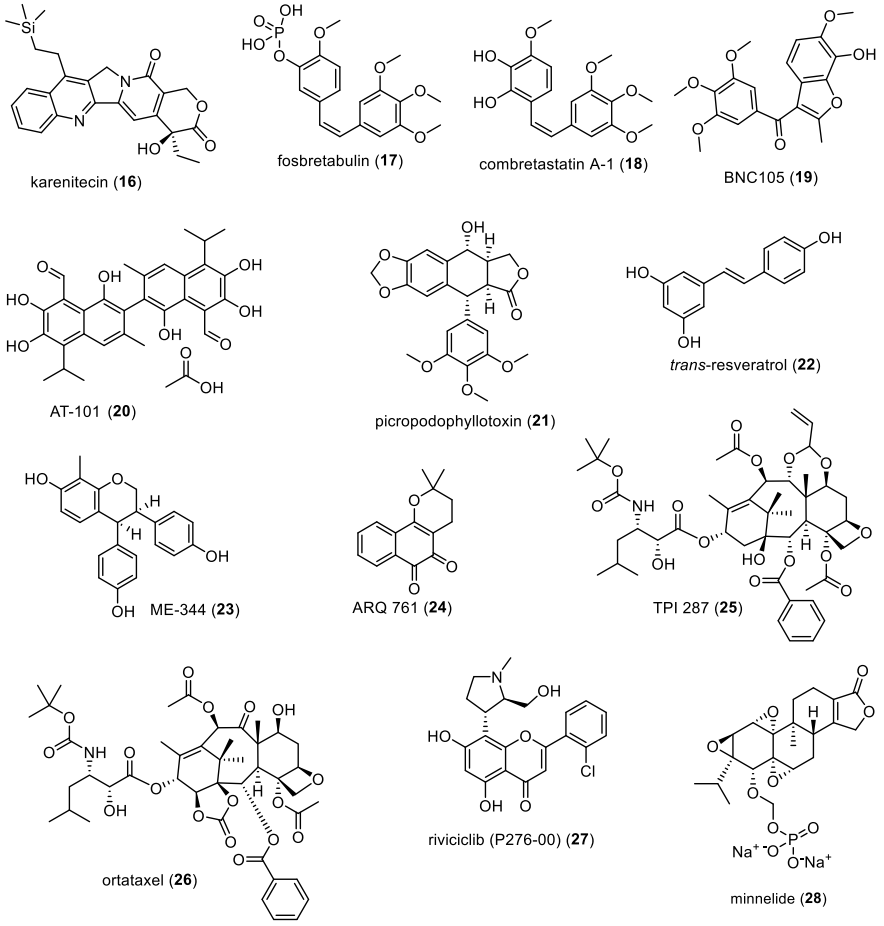

Fig. 17.3 Structures of some plant-derived anticancer agents undergoing clinical trials. [Adapted from (Butler et al. 2014)]

leukemia (Siemann et al. 2009). The structurally related compounds, combretastatin A-1 (**18**) and an additional derivative of combretastatin A-4, BNC105 (**19**), have also reached clinical trials (Butler et al. 2014; Rischin et al. 2011). AT-101 (**20**), a gossypol analog and a Bcl-2 inhibitor, has completed a phase II clinical trial for small cell lung carcinoma (Baggstrom et al. 2011). An insulin-like growth factor 1 receptor pathway modulator, picropodophyllotoxin (**21**), also known as AXL1717, has completed phase II trials against local and metastatic non-small cell lung cancer (Bergqvist et al. 2017; Butler et al. 2014). A naturally occurring plant stilbenoid, *trans*-resveratrol (**22**), has successfully completed phase II trials for relapsed multiple myeloma patients (Tomé-Carneiro et al. 2013).

ME-344 (**23**), a genistein derivative, is being studied as a mitochondrial inhibitor in phase I and IV clinical trials (Butler et al. 2014; Diamond et al. 2017; Scarfò and

Ghia 2013). ARQ-761 (**24**), a beta-lapachone derivative, has entered phase I and IV clinical trials as a reversible Bruton's tyrosine kinase (BTK) inhibitor. It has also been in phase I clinical trials for NAD(P)H:quinone oxidoreductase 1 (NQO1) cancer cell necrosis (Butler et al. 2014; Gerber et al. 2018). The tubulin-stabilizing agents, TPI 287 (**25**) and ortataxel (**26**), are paclitaxel derivatives that have been in phase II and IV clinical trials for glioblastoma (Butler et al. 2014; Khazir et al. 2014; McQuade et al. 2016; Silvani et al. 2019). Riviciclib (P276-00) (**27**), a rohitukine derivative, is under study in phase II and IV as a cyclin-dependent kinase modulator (Joshi et al. 2007). The transcriptional activator, minnelide (**28**), is currently in phase I and IV trials for non-small cell lung carcinoma as a prodrug of triptolide (Rousalova et al. 2013).

Therefore, from the examples given, plant-derived compounds representative of quite wide structural diversity are presently being investigated in human clinical trials as potential anticancer agents.

17.4 Production Methods for Selected Plant-Derived Cancer Chemotherapeutic Agents

In this section, the role of biotechnological methods in solving the supply issue of plant-derived anticancer natural products is presented. Discussion is limited to three examples of anticancer drugs representing three different structural classes of plant secondary metabolites: monoterpene bisindole alkaloids (Vinca alkaloids), a lignan (podophyllotoxin), and a diterpenoid (paclitaxel). Furthermore, emphasis is given on either a lead compound (podophyllotoxin) that subsequently was modified by chemical synthesis, or on those natural products directly used as drugs themselves without modification (vinblastine and vincristine, and paclitaxel).

17.4.1 Vinca Alkaloids

17.4.1.1 Overview and Problems with Supply

The Madagascar periwinkle *Catharanthus roseus* (L) G. Don (Apocynaceae) is currently the only commercial source of the anticancer monoterpene bisindole alkaloids, vinblastine (formerly vincaleukoblastine, **1**), and vincristine (formerly leurocristine, **2**) (Duge de Bernonville et al. 2015). These were isolated, respectively, in 1957 (vinblastine, **1**) and 1961 (vincristine, **2**) from the leaves of *C. roseus* by two independent research groups (Noble et al. 1958; Svoboda 1961). Biosynthetically, these alkaloids are heterodimers produced by the oxidative coupling of catharanthine and vindoline, which each in turn arise from the coupling of tryptamine and secologanin (Fig. 17.4). While tryptamine is biosynthesized from the amino acid trypto-

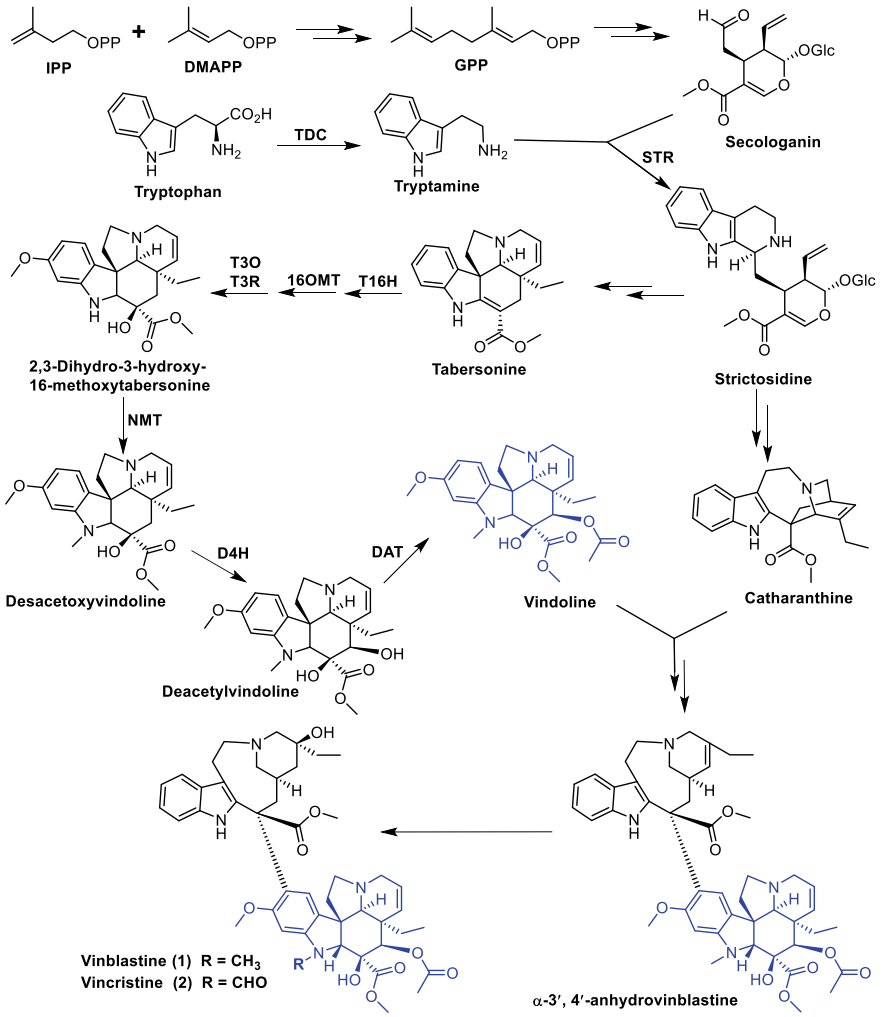

Fig. 17.4 Biosynthesis of vinblastine (**1**) and vincristine (**2**) [TDC: tryptophan decarboxylase; STR, strictosidine synthase; T16H: tabersonine 16-hydroxylase; 16OMT: 16-*O*-methyltransferase; T3O: tabersonine 3-oxygenase; T3R: tabersonine 3-reductase; NMT: *N*-methyltransferase; D4H: desacetoxyvindoline 4-hydroxylase; DAT: acetyl CoA:deacetylvindoline-4-*O*-acetyltransferase; Adapted from (Courdavault et al. 2014; Duge de Bernonville et al. 2015)]

phan in the leaf epidermis, the iridoid secologanin is derived from the monoterpenoid geranyl diphosphate (GPP) via several reactions in the internal phloem-associated parenchyma and epidermis cells (Pan et al. 2016a). Despite their very close structural similarity, these two compounds show remarkably different tumor specificities and toxicities (Noble 1990). Even though these bisindole alkaloids were found to be very effective in the anticancer chemotherapy field and were useful molecular probes in the

elucidation of the microtubule inhibitory mechanism of action of anticancer drugs, their amounts obtainable from their producing plant are extremely small (Ishikawa et al. 2009). Thus, a series of studies have been reported aimed at solving the supply problems of vinblastine (**1**) and vincristine (**2**) via various methods such as synthesis and biotechnological methods.

Factors Affecting Yield

As mentioned above, vinblastine (**1**) and vincristine (**2**) occur in the plant in very small concentration levels. For example, it required nearly 485 kg of dried leaves of *C. roseus* to provide approximately 1 g of vinblastine, representing a yield of 0.0002% dry weight (Noble 1990). This has led to major accessibility issues and, because of this, the prices of these drugs are very high. In addition, approximately 300 tons of *C. roseus* leaves are required to provide 3 kg of these drugs to meet their global demand (Newman and Cragg 2020b). These low yields result from an interplay of environmental, genetic, geography, developmental stage, plant part, and age factors. Most importantly, only the aerial parts of *C. roseus*, mainly the leaves, are able to produce the monoterpene bisindole alkaloids. These are not produced by the roots, as this organ lacks some of the essential enzymes involved in the biosynthesis of vindoline, one of the monomers in the bisindole alkaloids (Mahroug et al. 2007). Additionally, the biosynthesis of these and other monoterpenoid indole alkaloids is under strict regulation and involves more than 30 catalyzed steps with more than 35 intermediates, at least four cell types and at least five cellular compartments (Pan et al. 2016a). For example, Dutta et al. (2005) showed that differential expression levels of three important enzymes involved in the biosynthesis of catharanthine, vindoline, vinblastine, and vincristine correlated with the levels of these alkaloids in different parts, cultivars, mutants, and varieties of *C. roseus*. Plant age and leaf maturation are two of the other factors that affect the content of these alkaloids in the leaves. As an example, the content of α-3′,4′-anhydrovinblastine, the precursor of both vinblastine and vincristine, was found to be dependent on leaf maturation, which increased with leaf age and wounding. However, the content of the monomers decreased with aging (Naaranlahti et al. 1991). Similar trends were also observed for vinblastine, which increased with leaf age and plant maturation (Pan et al. 2016b). In addition, extraction methods, conditions, and solvents used as well as the isolation and purification procedures utilized also affect the yields of these alkaloids. For example, the use of ultrasound-assisted extraction with the ionic liquid 1-allyl-3-methylimidazolium bromide resulted in a higher extraction efficiency of vinblastine from the leaves of *C. roseus* compared to the conventional extraction solvents (85% ethanol and 0.15% sulfuric acid in 50% methanol) and extraction methods (heat reflux extraction and maceration) (Yang et al. 2011).

17.4.1.2 Biotechnological Methods to Improve the Supply of the Vinca Alkaloids

Cultivation and In Vitro Propagation

Catharanthus roseus can be propagated either from seeds or by in vitro propagation methods such as organ formation by either direct or indirect methods and somatic embryogenesis from various explants (Das et al. 2020). In each case, several factors determine the biosynthesis and thus the yields of the monoterpene indole alkaloids, including both the monomers and the bisindoles. For example, the cotyledons and hypocotyls of eight-day seedlings were found to contain variable levels of vindoline and catharanthine when compared to young leaves collected from 15-week old plants. The content of vindoline was also affected by the light-cycle used for cultivation (Magnotta et al. 2006). Production can also be improved using elicitors and physiological stressors. As an example, *C. roseus* plants inoculated with or without arbuscular mycorrhizal fungi and challenged with NaCl and $KHCO_3$ alone or in combination were studied in terms of the accumulation of vinblastine (**1**) and other metabolites. Significant improvement in vinblastine accumulation was observed in those plants inoculated with arbuscular mycorrhizal fungi without any stressors and from plants stressed with $KHCO_3$ alone. NaCl alone or in combination with $KHCO_3$ had no effect on the accumulation (De la Rosa-Mera et al. 2011). Vinblastine production was also shown to be elevated when *C. roseus* was supplemented with nitrate and then irradiated with ultraviolet B (UVB) light (Guo et al. 2014), and when grown under red light and irradiated with ultraviolet A (UVA) light (Fukuyama et al. 2017), and when supplemented with an equal mixture of nitrate and ammonium (Guo et al. 2011).

Plant Cell Culture

One of the problems encountered with the use of undifferentiated plant cell and hairy root cultures for improving the production of vinblastine (**1**) and vincristine (**2**) is the failure of these systems to produce these alkaloids reliably. This arises as their biosynthesis involves both the roots and above-ground parts, particularly since the biosynthesis of vindoline only occurs in the aerial parts of *C. roseus* (Hisiger and Jolicoeur 2007; Kidd et al. 2019). More specifically, the pathway that converts the branching intermediate tabersonine to vindoline is absent in these systems and thus represents a major obstacle in the production of vinblastine and vincristine in cell and hairy root cultures (Sun et al. 2018). These failures of undifferentiated plant cell cultures to biosynthesize vindoline have been correlated with the lack of expression and activity of the enzymes *N*-methyltransferase (NMT), desacetoxyvindoline 4-hydroxylase (D4H), and acetyl CoA:deacetylvindoline 4-*O*-acetyltransferase (DAT), the last three enzymes involved in the vindoline pathway (Fig. 17.4) (St-Pierre et al. 1998; Vázquez-Flota et al. 2002). Furthermore, these enzymes are localized in a cell- (e.g., D4H and DAT in the cytosols of laticifer and idioblast cells) and organelle- (e.g., NMT in thylakoids) specific manner in only the aerial parts and are under strict control (e.g., light is needed to activate *D4H* and *DAT* genes), explaining the lack

of accumulation of vindoline and the bisindoles in plant cell cultures (Salim and De Luca 2013; St-Pierre et al. 1998).

However, some reports have indicated the accumulation of vindoline and vinblastine (**1**) in various systems of *C. roseus* plant cell cultures such as vindoline in transformed (O'Keefe et al. 1997) and elicited (Ramani and Jayabaskaran 2008) cells and vinblastine in cell cultures containing elicitors and inhibitors (Guo et al. 2012). In addition, cell suspension cultures of *C. roseus* have been shown to biotransform vinblastine to vincristine (Hamada and Nakazawa 1991). Furthermore, cambial meristematic cells treated with several methods such as biotic (*Aspergillus flavus*) (Liang et al. 2018) and abiotic (β-cyclodextrin and methyl jasmonate) (Zhou et al. 2015b) elicitors are able to accumulate vindoline.

Plant Tissue and Organ Cultures

As indicated above, the plant roots as well as hairy root cultures of *C. roseus* fail to accumulate vindoline, vinblastine (**1**), and vincristine (**2**). This has been shown due to an alternative pathway that transforms tabersonine to other metabolites such as lochnericine, hörhammericine, and catharanthine instead of vindoline (Facchini and De Luca 2008; Magnotta et al. 2007; Rodriguez et al. 2003). Particularly, the *D4H* and *DAT* genes, the last two genes in the vindoline pathway, are not expressed in either the roots of the intact plant or in hairy root cultures. Furthermore, vindoline biosynthesis involves three different cells in the aerial parts, and these coupled with the light dependence of vindoline biosynthesis explains why these two systems are unable to produce vindoline, vinblastine, and vincristine (Mahroug et al. 2007; Thamm et al. 2016). Additionally, some hairy root metabolic engineering efforts by overexpression of certain of the enzymes involved in the vindoline and bisindole alkaloids biosynthesis have failed to accumulate these alkaloids. For example, even though *A. rhizogenes* transformed hairy roots of *C. roseus* overexpressing either the geraniol 10-hydroxylase (G10H) gene alone or with the ORCA3 transcription factor were able accumulate catharanthine, they were unable to accumulate vindoline, vinblastine, and vincristine (Wang et al. 2010). To add one more example, *C. roseus* hairy root metabolic engineering by overexpressing tabersonine 16-hydroxylase (T16H) and 16-hydroxytabersonine-*O*-methyltransferase (16OMT), the first two enzymes in the tabersonine to vindoline pathway, along with the regulators, failed to produce vindoline (Sun et al. 2018). However, similar to plant cell cultures, there are a few reports (e.g., Hanafy et al. 2016) that indicate the production of vincristine and vindoline in hairy root cultures.

As opposed to hairy root cultures, organ cultures of the above-ground parts such as shoots have been shown to produce vindoline, vinblastine (**1**), and vincristine (**2**) (Wink et al. 2007), depending on several factors. Thus, near-ultraviolet light stimulation was shown to increase the production of vinblastine in multiple shoot cultures of *C. roseus* (15 μg/g fresh weight) (Hirata et al. 1992). In embryos and leaves regenerated from protoplasts, yeast extract elicitation was found to improve the production of vinblastine and vincristine. The best result (15.5 and 4.1 μg/g dry weight, respectively), however, was obtained with 1.5 g/L of yeast extract from protoplast-regenerated leaves (Maqsood and Abdul 2017). In addition, different development

stages of somatic embryos of *C. roseus* initiated from embryogenic callus, and which were elicited by the fungus *Aspergillus flavus*, were shown to accumulate different levels of vinblastine and vincristine, with the most enhanced being observed in the maturation and germination stages (Tonk et al. 2016). Multiple shoot cultures treated with various elicitors and precursors were also able to accumulate vinblastine, with the highest being observed (approximately 0.03% DW), when supplied with the precursor tryptamine (100 mg/L). However, calli induced from the leaves of in vitro grown *C. roseus* were unable to produce vinblastine (Sharma et al. 2019).

Metabolic Engineering

Studies to improve the yields of monoterpene indole alkaloids using metabolic engineering nowadays are being facilitated by continuous elucidation of the biosynthetic machinery in *C. roseus* (Pan et al. 2016a). Such metabolic engineering is performed either in homologous (*C. roseus*) or heterologous (such as *Escherichia coli*, *Saccharomyces cerevisiae,* and *Nicotiana benthamiana*) systems, not only for the biosynthetic genes, but also for the transcription factors that regulate such genes (Sharma et al. 2020). Clearly, success with these approaches has been facilitated greatly by the ever-increasing number of research groups reporting the elucidation of genes, enzymes, regulatory mechanisms, and cellular and organ compartments of monoterpene indole biosynthesis (Caputi et al. 2018; Guirimand et al. 2020; Miettinen et al. 2014; Qu et al. 2015, 2018, 2019). Relevant technical progress made has been reviewed recently (Courdavault et al. 2014; De Luca et al. 2014; Duge de Bernonville et al. 2015; Thamm et al. 2016). Based on this accumulated knowledge of the monoterpene indole biosynthetic pathways, attempts have been made to improve the yields of vinblastine (**1**), vincristine (**2**), vindoline, catharanthine, and other monoterpene indole alkaloids. For example, Kumar et al. (2018) studied the effect of overexpression of geranyl(geranyl) diphosphate synthase [G(G)PPS] and its co-expression with geraniol synthase on the accumulation of the monomers and vinblastine using *Agrobacterium tumefaciens*-transformed *C. roseus* whole plants. Even though both systems showed improvement in the accumulation of the monomers, it was found that only those overexpressing G(G)PPS resulted in the improvement of vinblastine accumulation in the transgenic leaves (Kumar et al. 2018). In another study, the effects of *A. tumefaciens*-mediated overexpression of tryptophan decarboxylase and strictosidine synthase were studied in leaf-regenerated intact *C. roseus* plants. This led to the generation of four transgenic plants showing better accumulation of vinblastine [maximum 0.014% dry weight compared to the control (0.003% dry weight)] (Sharma et al. 2018).

In addition to the above and other homologous expression studies, several heterologous expression experiments with suitable hosts have been studied with respect to their ability to produce the target compounds, particularly the production of some important intermediates in the biosynthesis of the bisindole alkaloids. For example, Qu et al. (2015) studied the heterologous expression of the seven genes involved in the biosynthesis of vindoline from tabersonine in a yeast. They found that this expression was able to biotransform successfully tabersonine into vindoline, producing vindoline at a rate of 0.092 mg/L/h. The authors were able to achieve this by first

identifying the remaining two enzymes [tabersonine 3-oxygenase (T3O) and tabersonine 3-reductase (T3R)] involved in the tabersonine-to-vindoline pathway (Qu et al. 2015). To add a further example of a heterologous expression study, Brown et al. (2015) produced strictosidine in a yeast through the introduction of 21 genes and the deletion of three genes. After several optimization experiments, this resulted in the production of 0.5 mg/L of strictosidine (Brown et al. 2015).

17.4.2 Podophyllotoxin

17.4.2.1 Overview and Problems in Supply

The aryltetralin lignan podophyllotoxin (**29**) was isolated in 1880 from Podophyllin, a water-insoluble resin extracted by alcohol from the rhizomes of several species of *Podophyllum* (Berberidaceae), mainly *Podophyllum hexandrum* Royle [Himalayan mayapple, Indian mayapple; synonym: *Sinopodophyllum hexandrum* (Royle) T.S. Ying, *Podophyllum emodi*] and *Podophyllum peltatum* L. (American mandrake, American mayapple) (Stähelin and von Wartburg 1989). Its structure was completely elucidated in 1951 (Hartwell and Schrecker 1951). Podophyllotoxin (**29**) serves as the starting material for the semi-synthesis of etoposide (**6**), etopophos (**7**), and teniposide (**8**) (Fig. 17.5), which were developed in order to synthesize more potent and less toxic derivatives of podophyllotoxin (Stähelin and von Wartburg 1991). Still today, the supply of etoposide (**6**) and other analogs is met by partial synthesis from podophyllotoxin extracted from the Himalayan mayapple (*P. hexandrum*) (Schultz et al. 2019). However, this species is considered to be endangered due to the uncontrolled harvesting of the wild plants, requiring alternative methods of supply of this WHO-listed essential medicine (Li et al. 2018b; Schultz et al. 2019). To circumvent this problem, other viable options such as large-scale farming, which is based on the

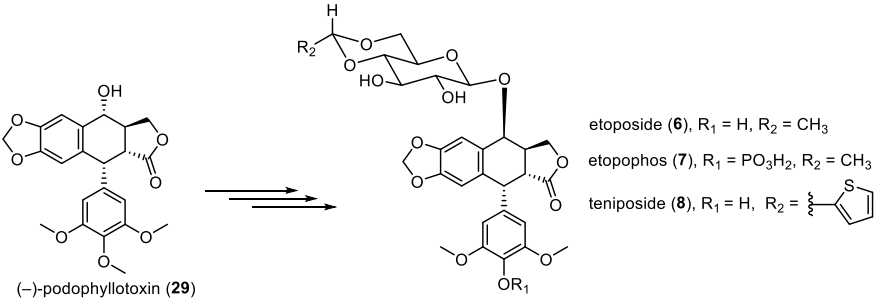

Fig. 17.5 Chemical structures of podophyllotoxin (**29**) and its derivatives [Adapted from (Stähelin and von Wartburg 1991)]

fact that wild varieties can provide high yields of podophyllotoxin, and biotechnological methods, such as those based on plant cell culture, have been suggested (Li et al. 2018b).

Factors Affecting Yield

In a similar manner to other secondary metabolites, the yield of podophyllotoxin (**29**) is affected by several factors. One of these is the plant source used for the isolation of this lignan. Podophyllotoxin has been isolated from several genera belonging to different plant families. Some of these, in addition to *Podophyllum* (Berberidaceae), include *Linum* (Linaceae) (Broomhead and Dewick 1990a), *Juniper* (Cupressaceae) (Renouard et al. 2011), *Diphylleia* (Berberidaceae) (Broomhead and Dewick 1990b), and several other genera (Newman and Cragg 2020b). Furthermore, each botanical source provides different amounts of podophyllotoxin. As an example, among the 12 species of Cupressaceae belonging to five genera (*Chamaecyparis*, *Cryptomeria*, *Cupressus*, *Juniperus*, and *Thuja*) studied for podophyllotoxin content, the needles (leaves) of Eastern red cedar (*Juniperus virginiana* L. "Canaertii") were found to contain the largest yield (0.34% dry weight) (Cantrell et al. 2013). Within the genus *Podophllyllum*, two plants are recognized as the major of sources of podophyllotoxin, viz., *P. hexandrum* and *P. peltatum* (Malik et al. 2014). The roots/rhizomes of the former have provided the largest amount of podophyllotoxin compared to the latter (e.g., 4.3% vs. 0.25% dry weight, respectively) (Broomhead and Dewick 1990b). Other *Podophyllum* species such as *P. sikkimensis* R. Chatterjee and Mukherjee (Paul et al. 2013) and *P. versipelle* Hance (0.32% dry weight) (Broomhead and Dewick 1990b) are usually found to contain lower levels of podophyllotoxin compared to *P. hexandrum*. However, the leaves of *P. peltatum* have been demonstrated to accumulate large amounts of podophyllotoxin, up to 5.6% dry weight (Moraes et al. 2000).

As mentioned above, the current major source of podophyllotoxin (**29**) is *P. hexandrum*, but this plant has been shown to contain variable amounts of podophyllotoxin dependent on several factors. For example, a study on the effects of altitude, the number of leaves, plant age, and collection season within the same collection region on podophyllotoxin production showed that higher yields were observed at elevated altitudes, and in those plants containing only one leaf rather than four, and in aged plants (four years), and those collected during May and June (Purohit et al. 1999). The effect of altitude on podophyllotoxin production has also been shown in other several studies. For example, the podophyllotoxin content of the roots of *P. hexandrum* collected from the Lahaul forest (4300 m) was higher (8.8 to 9.5% dry weight vs. 3.0 to 4.7% dry weight) than those collected from the Parvati forest (1300–1500 m) (Naik et al. 2010). The combined effects of several ecological factors such as temperature, soil pH, altitude, rainfall, sunshine, and nutrients have been shown to affect the content of podophyllotoxin. Thus, among eight sampling sites from seven provinces of mainland China studied for podophyllotoxin and other "active" substances produced by the roots and rhizomes of *P. hexandrum*, those collected from Jingyuan (Ningxia province) provided the largest amount (6.71% dry weight) followed by Yongdeng (Gansu province) and Huzhu (Qinghai province) (Liu et al. 2015). Genetic variability coupled with geographical and environmental

factors might contribute to these differences (Sultan et al. 2008, 2010). In addition to the above environmental and genetic factors, extraction methods can also affect the yield of podophyllotoxin. For example, Canel et al. (2001) found that extraction of the roots, rhizomes, and leaves of *P. peltatum* with an aqueous solvent increased the yield of podophyllotoxin by several times than when extracted with ethanol. This was due to the β-glucosidase enzymes present in tissues that catalyzed the conversion of podophyllotoxin 4-*O*-β-D-glucopyranoside to podophyllotoxin, which usually occurs when the tissues are damaged or treated with aqueous solvents (Canel et al. 2001).

17.4.2.2 Biotechnological Methods to Improve the Supply of Podophyllotoxin

Cultivation and In Vitro Propagation

In large-scale cultivation procedures, several factors may be varied, including temperature, soil pH, elevation, and nutrients, to enhance the biosynthesis and thus the resultant yield of podophyllotoxin (**29**). In one ex situ cultivation study, for example, plant age and hence collection time were the most significant factors to affect the yield of podophyllotoxin, when compared to other factors (Kushwaha et al. 2012). In another investigation, a higher production of podophyllotoxin was observed when *P. hexandrum* was cultivated at 3300 m than when compared with 2300 m, both in the leaves and rhizomes (Li et al. 2018a). Furthermore, the effect of elicitors on podophyllotoxin production has been studied. For example, statistically significant results were obtained when *P. hexandrum* leaves were treated with 3 mM methyl jasmonate, resulting in a 21% increase in podophyllotoxin production in the roots (Seegers et al. 2017). A temperature dependence of podophyllotoxin production has also been shown, with relatively higher accumulations obtained at lower temperatures. This was accompanied by changes in the expression levels of enzymes thought to be involved in the biosynthesis of podophyllotoxin, as determined by transcriptome analysis (Kumari et al. 2014). Micropropagation from various parts of podophyllotoxin-producing plants has been shown also to be successful for such plants that grow slowly and has endangered; one such example is for *Dysosma versipellis* (Hance) M. Cheng (Jiang et al. 2011).

Plant Cell Culture

Plant cell cultures, either using callus and/or suspension cultures, have been studied to optimize the production of podophyllotoxin (**29**). These investigations were performed on several plant species through the consideration and optimization of various factors such as biotransformation, elicitation, growth conditions, nutrients, and solvent extraction methods, in order to increase the yield. For example, when biotransformation using desoxypodophyllotoxin as the substrate was utilized to improve the yield of podophyllotoxin in *P. hexandrum* and *Linum flavum* cell suspension cultures, the highest conversion was observed in cell cultures of *P. hexandrum*

(van Uden et al. 1995). Furthermore, improvement of the yield of podophyllotoxin through co-culture of the hairy roots of *L. flavum* and cell suspension cultures of *P. hexandrum* has been observed. This was due to the uptake of coniferin produced by the hairy roots of *L. flavum* by cell suspension cultures of *P. hexandrum*, which used it to biosynthesize podophyllotoxin (Lin et al. 2003). In addition, improved production of podophyllotoxin in cell cultures derived from various plant species has been demonstrated by the use of elicitors. For example, salicylic acid was shown to increase the biosynthesis of podophyllotoxin (333 μg/g dry weight after 72 h elicitation with this compound) in cell cultures of *L. album*, despite showing non-variable outcomes on the growth, survival, and dry mass of the elicited cells, when compared to a control (Yousefzadi et al. 2010). Both a 15-and a 3.5-fold improvement in podophyllotoxin production were observed when callus cultures of *Juniperus chinensis* were treated with chito-oligosaccharide and laminaran enzyme-hydrolyzate elicitors, respectively (Muranaka et al. 1998).

Despite its lower podophyllotoxin (**29**) content compared to *Podophyllum hexandrum* (Broomhead and Dewick 1990b), *P. peltatum* has also been used to improve the yield of podophyllotoxin using cell cultures. Since an initial report (Kadkade 1981), several studies have shown this species can be used as an alternative to increase the yield of podophyllotoxin. For example, the effect of growth regulators, natural growth factors, carbon sources, callus age, light quality, and plant part used for callus induction was studied in callus cultures of *Podophyllum peltatum*. The highest production of podophyllotoxin was observed when 2,4-dichlorophenoxyacetic acid (2,4-D), casamino acids, sucrose, a duration of eight weeks, red light, and rhizomes and roots, respectively, were used (Kadkade 1982). Cell and adventitious root cultures were also shown to increase the yield of podophyllotoxin from *P. peltatum* by varying medium conditions (e.g., half *vs.* full Murashige and Skoog medium), hormones (e.g., indole-3-butyric acid) and elicitors (e.g., methyl jasmonate) (Anbazhagan et al. 2008).

Plant Tissue and Organ Cultures

Hairy root cultures induced by various strains of *Agrobacterium rhizogenes* have been studied to improve the production of podophyllotoxin (**29**) from various plants such as several species belonging to the genus *Linum* (Malik et al. 2014), *Hyptis suaveolens* (L.) Piot. (Lamiaceae) (Bazaldua et al. 2019), and *P. hexandrum* (Giri et al. 2001). With these systems, several factors such as the use of exogenous hormones (Farkya and Bisaria 2008), biotic elicitors (Bahabadi et al. 2014; Tashackori et al. 2016), and precursor feeding (Chashmi et al. 2016) were adjusted to improve the yield of podophyllotoxin. For example, of the various exogenous phytohormones (auxins, cytokinins and gibberellins) and their combinations tested, the highest production levels of podophyllotoxin, 14.9 and 15.0 mg/g dry weight, were obtained from a medium supplied with a specified concentration, 2 and 3 mg/L, respectively, of indole-3-acetic acid (IAA) (Farkya and Bisaria 2008). Furthermore, yields were improved by manipulation of extraction methods, culture composition, the addition of vitamins, and the use of various strains of *A. rhizogenes* or a combination of the aforementioned factors (Bazaldua et al. 2019; Chashmi et al. 2013; Renouard et al.

2018; Samadi et al. 2014). Recently, tetraploidy induction was used to increase the yield of podophyllotoxin in the shoots derived from *L. album* (Javadian et al. 2017). Furthermore, the role of plant growth regulators, carbon and nitrogen sources at different concentrations/ratios, culture medium strength, pH, and phosphate ratio on podophyllotoxin production in adventitious root cultures derived from the roots of *P. hexandrum* has been studied (Rajesh et al. 2014). In addition, roots derived from the callus and root explant of *P. hexandrum* were shown to produce similar (10.5% and 11.6% dry weight, respectively) amounts of podophyllotoxin compared to that produced by the roots and rhizomes of the original plant (9.3% dry weight) (Sagar and Zafar 2008).

Metabolic Engineering

Recently, Schultz et al. (2019) reported the use of metabolic engineering in *Nicotiana benthamiana* to increase the production of (−)-deoxypodophyllotoxin, a precursor of the etoposide aglycone (–)-4′-desmethylepipodophyllotoxin. (–)-4′-Desmethylepipodophyllotoxin is a more direct precursor of etoposide (**6**) than podophyllotoxin (**29**) and, thus, can be directly synthetically derivatized to the glycoside etoposide (Fig. 17.6) (Lau and Sattely 2015). Schultz et al. (2019) achieved this by the *Agrobacterium tumefaciens*-facilitated introduction of 16 genes involved in the transformation of phenylalanine → coniferyl alcohol → (–)-4′-desmethylepipodophyllotoxin in the leaves of *N. benthamiana*. This provided initially 3.5 mg/g dry weight of (−)-deoxypodophyllotoxin, which was 680 times higher than

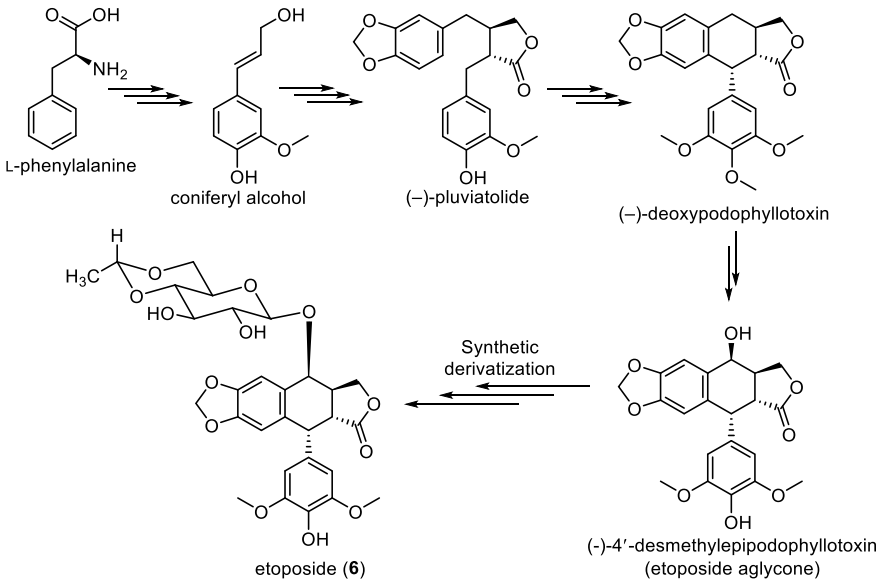

Fig. 17.6 Biosynthesis of the etoposide aglycone (–)-4′-desmethylepipodophyllotoxin and its synthetic derivatization to etoposide (**6**) [Adapted from (Lau and Sattely 2015; Schultz et al. 2019)]

the control (5.2 μg/g dry weight). Further optimization by varying the level of *A. tumefaciens* and harvest time (the optimum being *A. tumefaciens* OD_{600} of 3 and harvest time of 7–9 days, respectively), resulted in a (−)-deoxypodophyllotoxin yield of 4.3 mg/g dry weight. A scale-up procedure using 15–20 plants based on these optimized conditions led to the isolation of 0.7 mg/g (dry weight) of (−)-deoxypodophyllotoxin (Schultz et al. 2019). This is based on the results of the same group who identified the six enzymes that catalyze the conversion of (–)-pluviatolide to the etoposide aglycone (–)-4′-desmethylepipodophyllotoxin (Fig. 17.6) (Lau and Sattely 2015).

17.4.3 Paclitaxel

17.4.3.1 Overview and Problems in Supply

The isolation of the diterpene paclitaxel (**9**, Taxol®, Bristol-Myers Squibb) from the stem of the bark of the Pacific yew or western yew (*Taxus brevifolia* Nutt., Taxaceae) was reported in 1971 (Wani et al. (1971). In their report, the authors indicated that "*Taxol has potent antileukemic and tumor inhibitory properties…*" (Wani et al. 1971) and this was confirmed by several reports both in preclinical and clinical settings as reviewed by several authors (Foa et al. 1994; Kohler and Goldspiel 1994; Rose 1992; Rowinsky et al. 1990; Rowinsky and Donehower 1995), including its unique mechanism of action on tubulin first reported in 1979 by Horwitz and colleagues (Schiff et al. 1979). These and other factors made paclitaxel (**9**) of major interest, which drew attention from several diverse groups including physicians, environmentalists, and organic chemists, of whom the latter embarked on the partial and total synthesis of the drug (Nicolaou and Guy 1995). However, even though paclitaxel was the first taxane diterpenoid to demonstrate antitumor (Wani et al. 1971) and broad-spectrum anticancer activity, more than 20 years elapsed before this natural product was approved by the US FDA (December 1992), as a drug for the treatment of ovarian cancer. One of the factors for such a delay was the extremely small amount of the drug present naturally in the plant source, which required the need for a tedious large-scale isolation procedure (Cragg et al. 1993).

Biosynthetically, as a derivative of the isoprenoid class of natural products, the taxane diterpene skeleton of paclitaxel (**9**) is derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These undergo several reactions to form the diterpene precursor geranylgeranyl diphosphate (GGPP). Starting from GGPP, there are at least 19 steps involved in the biosynthesis of paclitaxel that include the formation of the first committed product, taxa-4(5),11(12)-diene, from GGPP catalyzed by the enzyme taxadiene synthase (TS) (Croteau et al. 2006). Taxa-4(5),11(12)-diene then undergoes several oxidation steps, including hydroxylation, acylation, benzoylation, and oxetane ring formation, to provide 10-deacetylbaccatin III, which can be acetylated by the enzyme 10-deacetylbaccatin III-10β-*O*-acetyltransferase (DBAT), to produce baccatin III. The latter then condenses

with β-phenylalanoyl-CoA derived from α-phenylalanine via β-phenylalanine to form paclitaxel via several reactions including the last benzoylation step (Fig. 17.7) (Croteau et al. 2006; Yu et al. 2017).

Factors Affecting Yield

In the initial structure elucidation report, paclitaxel (**9**) was isolated with a yield of 0.02% (Wani et al. 1971). Such a low concentration level, along with slow growth of the producing plant, posed a major restriction on the early development of paclitaxel. Initially, the supply issue was circumvented by the large-scale collection of *T. brevifolia*, but this raised issues with regard to the possible extinction of this plant

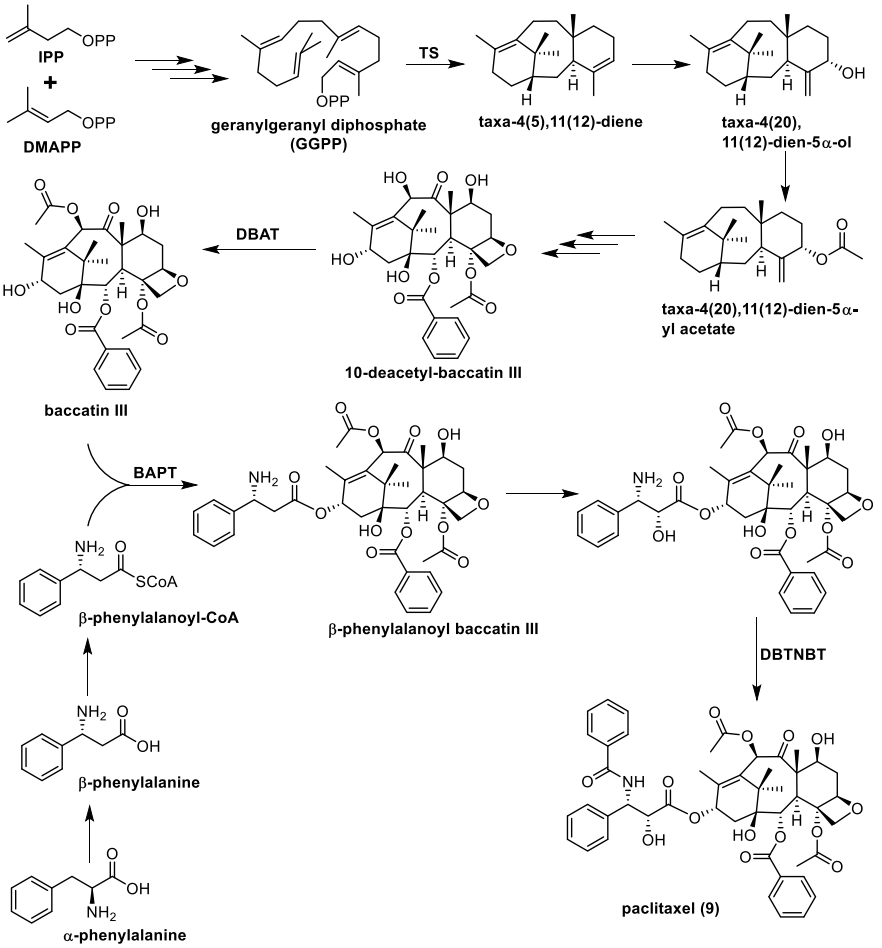

Fig. 17.7 Biosynthesis of paclitaxel (**9**) [TS: taxadiene synthase; DBAT: 10-deacetylbaccatin III-10β-*O*-acetyltransferase; BAPT: baccatin III 13-*O*-(3-amino-3-phenylpropanoyl) transferase; DBTNBT: 3′-*N*-debenzoyl-2′-deoxytaxol-*N*-benzoyltransferase. Adapted from (Liu et al. 2016)]

(Kingston and Newman 2007). As a result, paclitaxel has been the subject of many efforts to solve the supply crisis via various methods such as partial and total synthesis (Nicolaou and Guy 1995), plant tissue culture, and the search for high-yielding *Taxus* species/cultivars or other sources. For example, one of the methods used to solve the paclitaxel supply issue in early work was by semi-synthesis of paclitaxel from the more abundant (e.g., 1 g/kg) precursor 10-deacetylbaccatin III (Denis et al. 1988; Kingston and Newman 2007).

Similar to podophyllotoxin (**29**) and as discussed in the previous section, paclitaxel (**9**) has been isolated from other plant sources, in addition to species of the Taxaceae, with each providing a variable yield dependent on several factors. Even though paclitaxel is biosynthesized by all *Taxus* species that have been investigated, its concentration levels are dependent on several factors such as species, season, and plant part (Croteau et al. 2006). For example, the needles of *T. brevifolia* were shown to yield lower amounts of paclitaxel compared to the bark (Cragg et al. 1993). However, the needles of other *Taxus* species were found to contain similar amounts of paclitaxel (0.008–0.01%) to the bark of *T. brevifolia* (0.01%) (Witherup et al. 1990). This variation of paclitaxel (**9**) with respect to plant part has been shown for other *Taxus* species as well. For example, the roots of *T. mairei* contain larger amounts of paclitaxel (0.19%) than the stem bark, heartwood, twigs, and needles (Liu et al. 2001). In another study, the bark of the Himalayan yew (*T. wallichiana*) was found to contain the largest amounts of paclitaxel when compared to the stems and needles (Mukherjee et al. 2002). This variation in tissue accumulation has been shown to be due to the differential expression of some of the important biosynthetic genes and enzymes in the tissues (Mubeen et al. 2018).

Another important factor is the variability of paclitaxel (**9**) across different species of *Taxus*, which was confirmed in several phytochemical studies (ElSohly et al. 1997a; Mattina and Paiva 1992; Poupat et al. 2000; van Rozendaal et al. 2000; Zhou et al. 2019). For example, of the ten *Taxus* species needles that were studied for paclitaxel and other taxanes, the highest amount of paclitaxel was obtained from *T. floridana* (516 μg/g) and *T. globosa* (433 μg/g). However, the needles of some of the cultivars of the same species (e.g., *T. baccata* "Imperialis," *T. cuspidata* "Henry" and *T.* × *media* "Dutweileri") were devoid of any paclitaxel content (van Rozendaal et al. 2000). This variability of paclitaxel between species might result from genetic diversity related to biosynthetic enzymes and regulators (Yu et al. 2017). For example, the variation in paclitaxel and 10-deacetylbaccatin III observed between *T.* × *media* and *T. mairei* was accompanied by fluctuations in some of the important enzymes involved in the biosynthesis of these taxanes, as revealed by transcriptome analysis (Yu et al. 2017). In addition to their interspecific variation, cultivars of the same species are known to accumulate variable amounts of paclitaxel. For example, of the 17 cultivars of *T.* × *media* with different growth characteristics, needles of the cultivars "Coleana," "Hicksii," and "Stovekenii" contained the highest amount of paclitaxel (378, 322 and 309 μg/g dry weight, respectively) (Wang et al. 2006).

The content of paclitaxel has also been shown to depend on plant age (Mukherjee et al. 2002; Nadeem et al. 2002). As an example, old trees of *T. baccata* (126–161 years) contained more paclitaxel on average than young (40–57 years) and

mature trees (96–108 years) (0.1 vs. 0.05 and 0.04%, respectively) (Nadeem et al. 2002). Environmental factors such as altitude and temperature also affect the content of paclitaxel (Ballero et al. 2003; Mukherjee et al. 2002; Wheeler et al. 1992; Xi et al. 2014). For example, the needles of wild *T. baccata* collected from various altitudes (700–1200 m) in Sardinia were devoid of paclitaxel (Ballero et al. 2003). The collection time and season are other important factors that affect paclitaxel yield (ElSohly et al. 1997b; Glowniak et al. 1999; Veselá et al. 1999; Wheeler et al. 1992). Finally, the selection of extraction methods and of other parameters (e.g., temperature and extraction time) might have significant effects on the overall yield and time required for extraction of paclitaxel and other important taxanes (Kawamura et al. 1999; Talebi et al. 2004).

In addition to the Taxaceae, which are gymnosperms, paclitaxel has also been isolated from angiosperms of the family Betulaceae, in particular from the bark, shells, and leaves of *Corylus avellana* (hazelnut tree). However, the amounts obtained have been usually smaller than those found in *Taxus* species (Gallego et al. 2017). Furthermore, compound yields vary with, for example, plant part (Hoffman and Shahidi 2009) and collection site (Ottaggio et al. 2008). Another important potential general paclitaxel source that has attracted much attention is endophytic fungi. Starting from the first report in 1993 by Stierle et al. (1993), paclitaxel has been shown to be produced by more than 200 endophytic fungi, even those obtained from plants that do not produce paclitaxel, and this topic has been reviewed recently (Newman and Cragg 2020b).

17.4.3.2 Biotechnological Methods to Improve the Supply of Paclitaxel

Cultivation and In vitro Propagation

To meet the increasing global demand and to avoid the extinction of Pacific yew trees as a result of the collection of their bark, much attention has been given to the cultivation of *Taxus* plants in nurseries in several geographic regions. Furthermore, preference has been given to paclitaxel (**9**) extraction from the aerial plant parts (twigs/needles) to avoid damaging these slowly growing trees (Liu et al. 2016). Thus, from the above discussion, any cultivation and in vitro propagation efforts should take these and other factors into consideration in order to succeed as an alternative source of paclitaxel. For example, a negative correlation was observed between minimum and maximum temperature and paclitaxel production in *T. wallichiana* var. *mairei* grown and collected in Ningbo, China (Yang et al. 2016). In another study, bark samples collected from *T. brevifolia* grown under shade conditions contained more paclitaxel than those collected from sun-exposed trees (Kelsey and Vance 1992). Furthermore, several in vitro propagation methods have been studied in terms of enhancing paclitaxel and other taxane production and the germination, regeneration, and conservation of yew trees (Majada et al. 2000; Tafreshi et al. 2011). For example, for one-year old in vitro grown plantlets of *T. baccata*, the aerial parts contained more total taxanes than the roots. However, the roots contained more paclitaxel

and other taxane derivatives with ester side chains than the roots. In both cases, the amount of each type obtained was plant age-dependent. These differences were attributed to differences in the expression of some of the early and late biosynthetic genes, where some were found to be rate-limiting (Onrubia et al. 2011). In another example, the roots of various hydroponically grown cultivars and species of *Taxus* were shown to contain higher or similar amounts of paclitaxel compared to the aerial parts (Wickremesinhe and Arteca 1994), and the concentration levels attained could be manipulated by the use of plant growth regulators (Wickremesinhe and Arteca 1996). Finally, since yew trees require large areas and a long time to grow, which makes their collection labor intensive, cultivation efforts need to take these factors into account (Anterola et al. 2009).

Plant Cell Culture

Several species of *Taxus* such as *T. cuspidata*, *T. chinensis, T. baccata, T. globosa, T. media,* and *T. wallichiana* have been studied for the production of paclitaxel (**9**) in plant cell cultures (Malik et al. 2011; Navia-Osorio et al. 2002a, 2002b; Osuna et al. 2015; Roberts et al. 2003; Tabata 2006; Zhang and Fevereiro 2007). One of the effective methods used to enhance the production of paclitaxel and other taxanes in these systems has been the use of various elicitors such as coronatine, jasmonic acid, methyl jasmonate, and cyclodextrins (Cusido et al. 2014). For example, Sabater-Jara et al. (2014) showed synergistic effects of the combined use of various forms of cyclodextrins and methyl jasmonate on paclitaxel and other taxane derivative production in suspension cultures of *T.* × *media.* The combined use of both elicitors led to higher accumulations of paclitaxel compared to when either one was used alone (65.0 mg/L *vs.* 5.9 and 13.9 mg/L, respectively). This was accompanied by increased levels of the genes involved in the biosynthesis (*TXS*, *T7βOH*, *DBAT*, *BAPT,* and *DBTNBT*) and the ABC genes involved in the transport of paclitaxel (Sabater-Jara et al. 2014).

In addition to elicitation, numerous other methods such as immobilization (Bentebibel et al. 2005; Bonfill et al. 2007), two-stage systems (Khosroushahi et al. 2006), media optimization (Kajani et al. 2012), precursor feeding (Syklowska-Baranek and Furmanowa 2005), and two-phase systems (Wang et al. 2001) have been shown to enhance the production of paclitaxel (**9**) and other taxanes in *Taxus* plant cell cultures. Furthermore, Lee et al. (2010) developed cambial meristematic cells that overcome several problems associated with dedifferentiated plant cells and hence enhance the production of paclitaxel. Along with the use of elicitors and precursors, cambial meristematic cells showed a superior performance when compared to dedifferentiated cells in both small-scale (125 mL) flasks and large-scale (e.g., 3 and 20 L) bioreactors. For example, while dedifferentiated cells derived from needles or embryos produced 23 mg/kg or 39 mg/kg fresh weight of paclitaxel, respectively, cambial meristematic cells were able to produce 102 mg/kg fresh weight of this diterpenoid (Lee et al. 2010).

In fact, production of paclitaxel (**9**) by plant cell culture represents one of the success stories through the use of this biotechnological method in the production of a valuable pharmaceutical. Using Chinese yew (*T. chinensis*) cell cultures in 75,000 L

capacity bioreactors, Phyton Biotech, Inc., now a subsidiary of DFB Pharmaceuticals, produces paclitaxel commercially. Another company that uses cell culture to supply paclitaxel (Genexol®) for the global market is the Korean company Samyang Genex (Leone and Roberts 2013; Wilson and Roberts 2012).

As mentioned in the preceding section, paclitaxel (**9**) and other taxanes have also been isolated from hazelnut tree (*C. avellana*) and thus, several *C. avellana* cell cultures have been studied for enhancing paclitaxel production, as recently reviewed (Gallego et al. 2017).

Plant Tissue and Organ Cultures

In addition, plant cell cultures, hairy root cultures of a number of *Taxus* species have been evaluated for their ability to produce and enhance the production of paclitaxel (**9**). For example, Furmanowa and Syklowska-Baranek (2000) studied paclitaxel production in hairy root cultures of *T.* × *media* var. *hicksii* Rehd. induced by *A. rhizogenes* strain LBA 9402. They noted that elicitation with 100 μM methyl jasmonate produced the highest paclitaxel levels when compared with non-elicited hairy roots (210 vs. 69 μg/g dry weight, respectively) (Furmanowa and Syklowska-Baranek 2000). The same research group increased the production of paclitaxel by the use of various concentrations of the precursors L-phenylalanine and *p*-aminobenzoic acid supplemented alone or with 100 μM methyl jasmonate. The highest paclitaxel production was observed when 100 μM of either of these precursors was used in combination with 100 μM methyl jasmonate [319.7 μg/g (568.2 μg/L) and 130.5 μg/g (221.8 μg/L), respectively] (Syklowska-Baranek et al. 2009). In addition to the above species, hairy root cultures of other *Taxus* species such as *T. cuspidata* (maximum of 52.2 mg/L with 100 μM methyl jasmonate) (Kim et al. 2009) and *T. brevifolia* (the highest being 0.48 mg/g dry weight) (Huang et al. 1997) were shown to produce paclitaxel.

Furthermore, transgenesis and two-phase systems are additional recent methods studied for enhancing the production of paclitaxel (**9**) in hairy root cultures of *T.* × *media* var. *hicksii* (Syklowska-Baranek et al. 2015a, 2015b, 2018). For example, Syklowska-Baranek et al. (2019) examined the effect of several parameters (such as with or without the *TXS* gene, with or without and single *vs.* twice-elicitation, single *vs.* two-phased system) on paclitaxel production and some of its biosynthetic gene profiles in the hairy root cultures of *T.* × *media* var. *hicksii*. They found that the hairy root line containing the *TXS* gene and elicited with a single methyl jasmonate treatment produced a higher paclitaxel concentration level than a line without the *TXS* gene, even with elicitation (maximum of approximately 2.5 and 0.5 mg/g dry weight, respectively). These differences were accompanied by differences in the expression profiles of the *TXS*, *BAPT,* and *DBTNBT* genes (Syklowska-Baranek et al. 2019).

Metabolic Engineering

In similar work on other effective plant-derived anticancer agents, one of the limitations of the application of metabolic engineering procedures in reconstructing the whole paclitaxel (**9**) biosynthetic pathway in heterologous hosts is the fact that not of all of the biosynthetic enzymes of taxanes are yet discovered. However, several

engineering attempts have made to enhance the production of early intermediates and precursors (Courdavault et al. 2020), using numerous heterologous hosts, such as *Arabidopsis thaliana* (Besumbes et al. 2004), *Saccharomyces cerevisiae* (a yeast) (Dejong et al. 2006), *Escherichia coli* (Huang et al. 2001), tomato (Kovacs et al. 2007), a species of moss (Anterola et al. 2009), an endophytic fungus (Bian et al. 2017), and *Bacillus subtilis* (Abdallah et al. 2019). For example, Ajikumar et al. (2010) were able to produce approximately 1 g/L of taxadiene in *E. coli* using a method they developed called "multivariate modular pathway engineering." Using this procedure, they divided the taxadiene biosynthetic pathway into two separate modules, which allowed them to identify factors that affected metabolic flux and thus optimize these toward increasing the yield of taxadiene (Ajikumar et al. 2010). In another study, Zhou et al. (2015a) took metabolic engineering further by co-culturing engineered *E. coli* and *S. cerevisiae* to overcome several limitations of engineering both microbes alone, to produce ferruginol, nootkatone and precursors of paclitaxel. In this microbial consortium, for example, taxadiene produced by engineered *E. coli* was used by *S. cerevisiae* to produce oxygenated taxanes (e.g., taxadien-5α-ol) and a monoacetylated dioxygenated taxane putatively assigned as taxadien-5α-acetate-10β-ol. After several optimization experiments (e.g., changing the carbon source from glucose to xylose), this team of investigators was able to produce 33 and 1 mg/L of taxanes, respectively (Zhou et al. 2015a).

As alternatives to these microbes, plants have been studied as heterologous hosts as they possess some significant advantages over microbial hosts, such as improved cytochrome P450 chemistry (Li et al. 2019). For example, expression of taxadiene synthase in yellow-fruited tomato resulted in the production of taxadiene at a yield of 471 μg/g dry weight (Kovacs et al. 2007). In another more recent example, Li et al. (2019) were able to produce taxadiene and taxadiene-5α-ol (56.6 and 1.3 μg/g fresh weight, respectively), in the leaves of engineered *N. benthamiana.* They achieved this by increasing the availability of isoprenoid precursors such as geranylgeranyl diphosphate through the engineering of the corresponding enzymes. More importantly, the initial failure of the conversion of taxadiene to taxadiene-5α-ol was overcome by chloroplast compartmentalization of the enzymes taxadiene synthase, taxadiene-5α-hydroxylase, and cytochrome P450 reductase (Li et al. 2019).

17.5 Conclusions

Scientific interest in plant secondary metabolites to treat various forms of human cancer has continued unabated for nearly 60 years since the two bisindole alkaloids, vinblastine (**1**), and vincristine (**2**) became approved oncolytic agents. Such interest was heightened after the discovery of the diterpene derivative, paclitaxel (**9**), due in large part to its unprecedented mode of cellular action on tubulin, and its subsequent very wide clinical use on approval. It is not unreasonable that new examples of plant-derived derivatives with potential anticancer activity still remain to be discovered, so there is a worldwide search for such compounds, as exemplified

by relevant studies by our own research team (e.g., Henkin et al. 2018). A number of plant-derived compounds are in clinical trials as potential cancer chemotherapeutic agents, and these represent a quite diverse range of structural types. A number of ingenious methods have been applied to enhancing the available supply of plant-derived anticancer agents already on the market, including procedures for enhanced cultivation and in vitro propagation. Also, biotechnological methods such as plant cell tissue and organ culture methods have been examined in detail, in addition to metabolic engineering. Future refinement of all of these methods may be anticipated in the future.

Acknowledgements Research conducted in our own laboratory directed toward the discovery of new anticancer agents from higher plants and other organism has been funded through program project P01 CA125066 by the U.S. National Cancer Institute, NIH, Bethesda, MD, USA.

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