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MINI-REVIEW

Taxanes: perspectives for biotechnological production

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Abstract Taxol is a valuable plant-derived drug showing activity against various cancer types. Worldwide efforts had been made to overcome the supply problem, because the supply by isolation from the bark of the slow-growing yew trees is limited. Plant cell cultures as well as chemical and biotechnological semisynthesis are processes, which are intensively investigated for the production of taxanes paclitaxel (Taxol) and docetaxel (Taxotere) in the last few years. This article provides a comparison of the current research on taxane biosynthesis and production in yew cell cultures.

Introduction

Medicinal plants are most promising sources for the development of drugs, and many types of active ingredients from plant resources have been studied. However, in some cases, the supply of active compounds can have some difficulties: limited quantity of active compounds in the plant, low plant growth rate, limited localization of active ingredients in the specific organs, and destruction of the natural resources. Therefore, the stable commercial supply of the compounds is very difficult. Paclitaxel, a very important anticancer drug commonly called Taxol, is the best-known example of an effective drug with such huge supply problems. Another taxane used as anticancer drug is docetaxel, commonly called Taxotere. The active ingredients in Taxol and Taxotere are mainly derived via chemical semisynthesis from the advanced taxoid 10-deacetylbaccatin III, which is readily available from the needles of the European yew tree, *Taxus baccata*.

Exciting progress has been made in the elucidation of the biosynthetic mechanism of paclitaxel in *Taxus* due to the fundamental works of Walker, Croteau, Chang, Jennewein, and others, especially in the past 10 years. Recently, in cell cultures of *T. cuspidata*, the expression profile of the paclitaxel biosynthetic pathway genes, as a time course after elicitation using methyl jasmonate, were instigated (Nims et al. 2006). Potential bottlenecks in the terminal steps were found, which led to accumulation of intermediates at high levels, whereas paclitaxel remains at low amounts. Targeting the late pathway steps and identification of absolute gene targets for metabolic engineering may be effective in enhancing paclitaxel accumulation in *Taxus* cell suspension cultures. Once these mechanisms are fully understood, developing superior strains for use in bioprocesses for paclitaxel synthesis should be possible.

Cell culture

The first successful mass cultivations of plant cell and tissue cultures date back to the period of time between 1956 and 1959. Decisive milestones in the context of the further development of this technology represent the commercialization of the large industrial production of shikonin, the first secondary metabolite produced commercially in 1983 as well as 1988, the admittance of the production of ginsenosides in Japan. Fourteen substances or products become produced commercially and semicommercially on the basis of plant cell cultures in bioreactors with 0.75 l to 75 m^3 of work volume at present (Eibl and Eibl 2002; Roja and Rao 2000).

Taxus cell culture has been considered as a promising tool to produce paclitaxel and has been extensively investigated

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(Christen et al. 1989, 1991; Mirjalili and Linden 1996; Ketchum et al. 1997; Nguyen et al. 2001; Tabata 2004, 2006; Khosroushahi et al. 2006). Studies to optimize the production rate show an enhanced content of paclitaxel by differently treated *Taxus* cell cultures compared with control. Paclitaxel concentration in the plant cells reached approximately 0.5% of dry weight by adding methyl jasmonate (Yukimune et al. 1996). Methyl jasmonate induces the up-regulation of secondary metabolic genes specifically involved in stress, wounding and pathogen ingress (Reymond et al. 2004).

The accumulation of paclitaxel in cells leads to feedback repression and product degradation (Wu et al. 1999). Therefore, the in situ removal of paclitaxel from the suspension cultures is essential for improvement in productivity (Yuan et al. 2001). A perfusion bioreactor incorporating a simple and effective cell/medium separation device was developed (De Dobbeleer et al. 2006). An external column containing polymeric resins extracts the secondary metabolites continuously. A stable cell/medium separation was obtained with *E. californica* suspension cells as model biological system. Studies with other plant species have to be conducted; preliminary assays performed with *Nicotiana tabaccum* seemed to confirm the applicability of the bioreactor to that cell suspension as well (De Dobbeleer et al. 2006). This principle may also be applied to the in situ removal of the taxanes from *Taxus* cell culture media.

The effect of the plant peptide regulator phytosulfokine-α, a small peptide with five amino acids showing mitogenic activity with plant cultures, on the growth and Taxol production from *Taxus* sp. suspension cultures was investigated (Kim et al. 2006). The response was cell line dependent. With *T. canadensis* (C93AD), a very strong synergistic response of phytosulfokine-α and methyl jasmonate elicitation was observed. This synergistic treatment was able to partially revive taxane production in cultures that have lost productivity due to extended time.

The influence of a short heat shock on suspension cultures of *T. yunnanensis* cells was investigated (Zhang and Fevereiro 2006). The heat shock reduced cell viability and growth but significantly induced paclitaxel production depending on the intensity and the physiological state of the cells. This work provides some novel insights into understanding the relationship between heat shock response and secondary metabolism in plant cells.

Paclitaxel storage in *Taxus* suspension cell cultures was studied through the simple use of cell wall digesting enzymes inducing a significant increase in the paclitaxel present in the extracellular medium (Roberts et al. 2003). Paclitaxel- and baccatin III-producing cells of *T. baccata* were immobilized within calcium-alginate beads (Bentebibel et al. 2005). Among the reactors tested, the stirred bioreactor was the most efficient in promoting immobilized cell production of paclitaxel. The paclitaxel productivity obtained in this study is one of the highest reported so far by academic laboratories for *Taxus* species cultures in bioreactors (Table 1).

The use of a mechanical stimulus, ultrasound, and a putative chemical elicitor, methyl jasmonate, combined with in situ solvent extraction (two-phase culture), to enhance paclitaxel production by *T. chinensis* cells in suspension culture, were investigated (Wu and Lin 2003). It was found that the enzyme activity of secondary metabolic pathways was stimulated, which was partially responsible for the enhanced paclitaxel production.

Taxoids are complex molecules that are chemically similar to paclitaxel. A highly efficient bioprocess strategy for the production of the taxoid taxuyunnanine C (Fig. 1) with *T. chinensis* was developed by repeatedly eliciting a fed-batch culture with a newly synthesized powerful jasmonate analog, 2,3-dihydroxypropyl jasmonate (Qian et al. 2005). A high productivity of 33.2 mg l^{-1} day^{-1} was achieved in a 1-L airlift bioreactor, higher than previous reports on taxuyunnanine C production in bioreactors. Furthermore, tandem mass spectrometry by electrospray ionization has been used for profiling of taxoids from the needles of *T. wallichiana* (Madhusudanan et al. 2002). This technique was shown to be a fast and reliable technique for

Table 1 Extracellular paclitaxel production rates by cell cultivation described in the literature (Zhong 2002 and this work)

Cell line	Production rate (mg l^{-1} day^{-1})	Reference
In flasks		
T. baccata	1.02	Khosroushahi et al. 2006
T. canadensis (C93AD)	1.68	Kim et al. 2006
T. chinensis	2.24	Kim et al. 2001
T. chinensis	3.27	Choi et al. 2000b
T. chinensis	3.64	Choi et al. 2000a
T. mairei	4.76	Mulabagal and Tsay 2004
T. cuspidata	5.32	Nguyen et al. 2001
T. media	7.86	Yukimune et al. 1996
T. canadensis	9.75	Ketchum et al. 1999
In bioreactors		
T. cuspidata	0.11	Son et al. 2000
T. x media var. Hicksii	0.31	Syklowska-Baranek and Furmanowa 2005
T. media	0.53	Cusido et al. 2002
T. wallichiana	0.75	Navia-Osorio et al. 2002
T. cuspidata	1.10	Pestchanker et al. 1996
T. chinensis	1.50	Wang et al. 2001
T. chinensis var. *mairei*	1.52	Yuan et al. 2001
T. yunnanensis	1.90	Zhang et al. 2002
T. baccata	2.71	Bentebibel et al. 2005

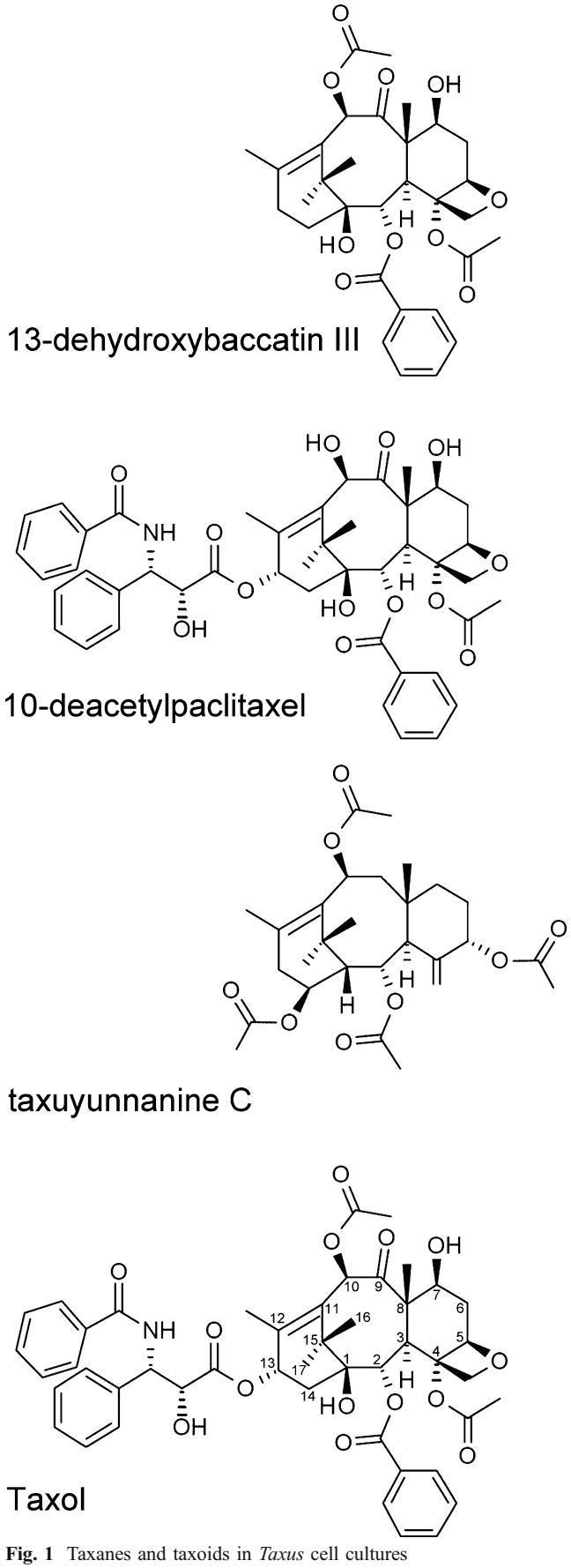

Fig. 1 Taxanes and taxoids in *Taxus* cell cultures

the analysis of taxoid samples. The taxane derivatives 13-dehydroxybaccatin III (13-DHB III) and 10-deacetylpaclitaxel (10-DAP), useful as semisynthetic precursors of paclitaxel, can be isolated during purification of paclitaxel from *T. chinensis* cell culture extracts. A simple and economical procedure, which requires only minimal additional expense for reagents or equipment, was developed (Pyo et al. 2006). Precipitation from organic solvents followed by chromatography with C18 octadecylsilane and silica-gel resins resulted in paclitaxel of 99.5% purity, 13-DHB of >99% purity, and 10-DAP of >90%.

The production has been scaled up, and presently, bioreactors of up to 75,000 l are being employed by ESCAgenetic (CA, USA), Phyton (NY, USA), Samyang Genex (Taejon, Korea), and Phyton Biotech (Germany; Zhong 2002; Wink et al. 2005). *Taxus* plant cell cultures are still limited for large-scale commercial use because of the low and unstable paclitaxel yield, as well as high production cost, low natural yields, and selectivity over unwanted byproducts. Additionally, cell cultures display a large degree of heterogeneity in secondary metabolite production capabilities. There have been few reports on this variability in plant cell cultures, particularly the long-term stability of cell suspensions to maintain high levels of productivity (Kim et al. 2004).

Enzymatic semisynthesis

The biosynthesis of the anticancer drug Taxol in yew (*Taxus*) species and the enzymes involved in this pathway have been basically elucidated. Many genes encoding these enzymes have been cloned and characterized (Table 2, Jennewein et al. 2004a; Nims et al. 2006). Expression of these enzymes in bacteria, yeasts, fungi, or plants and the development of adapted bioreactors can be a way to solve the supply problem of the drug Taxol.

Taxol-producing bacteria and fungi

The first report on the isolation of Taxol-producing fungi from the tree *T. brevifolia* was in 1993 (Strobel et al. 1993). In the following years, there had been a great deal of interest in finding other fungi that produce Taxol. Three strains of these fungi (HQD33, HQD48, and HQD54) were isolated from phloem of *T. cuspidata* (Zhou and Ping 2001). The strain HQD33 is an endofungus *Nodulisporium sylviforme* (Zhou et al. 2005). Its spore experienced a series of mutagenesis screening, and finally, a strain NCEU-1 was obtained, which was used as parent strain for raising the Taxol yield. Protoplast mutagenesis was done using ultraviolet (UV) radiation and combined treatment of UV and LiCl (Zhao et

al. 2005; Zhou et al. 2005), which has not been reported before. These studies have importantly practical significance to a biotechnological production of Taxol.

Three enzymes from bacteria isolated from soil facilitate the production of 10-deacetylbaccatin III. A strain of *Nocardioides albus* (SC13911) was found to produce an extracellular C-13 taxolase that specifically removed the C-13 side chain from paclitaxel, cephalomannine, 7β-xylosyltaxol, 7β-xylosyl-10-deacetyltaxol, and 10-deacetyltaxol (Hanson et al. 1994). A strain of *Nocardioides luteus* (SC13912) was found to produce an intracellular C-10 deacetylase that removed the 10-acetate from baccatin III and paclitaxel (Hanson et al. 1994). This enzyme was found to acetylate 10-deacetylbaccatin III with vinyl acetate to yield baccatin III without any protection (Patel et al. 2000). A strain *Moraxella* sp. was found to produce a xylosidase that removes the xylosyl group from 7-xylosyltaxanes, major components of the mixture found in the bark of the Pacific yew (*T. brevifolia*), thereby making the 7-xylosyltaxanes available as sources of 10-deacetylbaccatin III (Hanson et al. 1997). The enzyme activity was located in both the soluble and particulate fractions of the cell. Treatment of yew extracts with these three enzymes converted the complex mixture of taxanes primarily to 10-deacetylbaccatin III and increased the amount of this key precursor by 4–24 times (Hanson et al. 1994; Patel 1998). Several strains that contain enzyme activities able to 4-deacetylate 10-deacetylbaccatin III and baccatin III to 4-deacetyl-10-deacetylbaccatin III in 90% yield were isolated (Hanson et al. 2006). Taxanes based on this substance are promising candidates for second generation paclitaxel analogues (Fang and Laing 2005).

Table 2 The cloned genes involved in Taxol biosynthesis pathway in *Taxus* (Nims et al. 2006)

Enzyme	Reference
Geranylgeranyl diphosphate synthase	Hefner et al. 1998
Taxadiene synthase	Wildung and Croteau 1996
Taxane 5α-hydroxylase	Jennewein et al. 2004b
Taxa-4(20), 11(12)-dien-5α-ol-*O*-acetyltransferase	Walker et al. 2000c
Taxane 10β-hydroxylase	Schoendorf et al. 2001
Taxane 13α-hydroxylase	Jennewein et al. 2001
Taxane 2α-*O*-benzoyltransferase	Walker and Croteau 2000b
10-Deacetylbaccatin III-10-*O*-acetyltransferase	Walker and Croteau 2000a
Phenylalanine aminomutase	Walker et al. 2004
Baccatin III:3-amino-3-phenylpropanoyltransferase	Walker et al. 2002a
3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyltransferase	Walker et al. 2002b

Overexpressing enzymes of the paclitaxel pathway in bacteria and yeasts

Several oxygenases involved in the early hydroxylation steps of the pathway have been identified, and the corresponding genes have been cloned (Chau and Croteau 2004). Biosynthesis of taxadiene, the key intermediate of paclitaxel, in cell-free extracts of *Escherichia coli* by overexpressing genes encoding isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, and taxadiene synthase was reported (Math et al. 1992; Hahn and Poulter 1995; Wildung and Croteau 1996; Huang et al. 1998, 2001). cDNAs encoding a taxoid 5α-*O*-acetyltransferase and a taxoid-10β-*O*-acetyltransferase have been acquired from a recently isolated family of *Taxus* acyl/aroyltransferase clones and investigated with a range of polyhydroxylated taxoids as substrates (Chau et al. 2004a,b). A family of cytochrome P450 genes, obtained from a yew cell cDNA library, were functionally expressed and screened with taxusin as a surrogate substrate. The overexpressed enzyme also catalyzes the exchange of propionyl and *n*-butyryl from the corresponding CoA thioester to the hydroxyl group at C10. In addition, in vivo studies revealed that *E. coli* producing endogenous acetyl-CoA and overexpressing the recombinant acetyltransferase can convert exogenously supplied 10-deacetylbaccatin III to baccatin III (Loncaric et al. 2006).

The cDNA clone for this 10-deacetylbaccatin III-10-*O*-acetyltransferase has been isolated from *T. cuspidata* (Walker and Croteau 2000a). Expression in *E. coli* JM109 afforded a functional enzyme, which produces baccatin III from 10-deacetylbaccatin III and acetyl-CoA. The recombinant acetyltransferase is apparently regiospecific toward the 10-hydroxyl group of the taxane ring (Walker and Croteau 2000a).

Another key step in paclitaxel semisynthesis is the coupling of chiral C-13 paclitaxel side chain to baccatin III. To identify the side chain transferase of paclitaxel biosynthesis, a set of transacylases obtained from an enriched cDNA library was screened (Walker et al. 2002a). Identification of this clone completes acquisition of the five aroyl/acyltransferases involved in the biosynthesis of paclitaxel. Application of these transacylase genes in suitable host cells can improve the production yields of Taxol and could enable the preparation of second generation Taxol analogs possessing greater bioactivity and improved water solubility (Fang and Laing 2005).

In vivo feeding studies with *Taxus* tissues and characterization of the two transferases responsible for C13-side chain construction have suggested a sequential process in which an aminomutase converts α-phenylalanine to β-phenylalanine, which is then activated to the corresponding CoA ester and transferred to baccatin III to yield β-

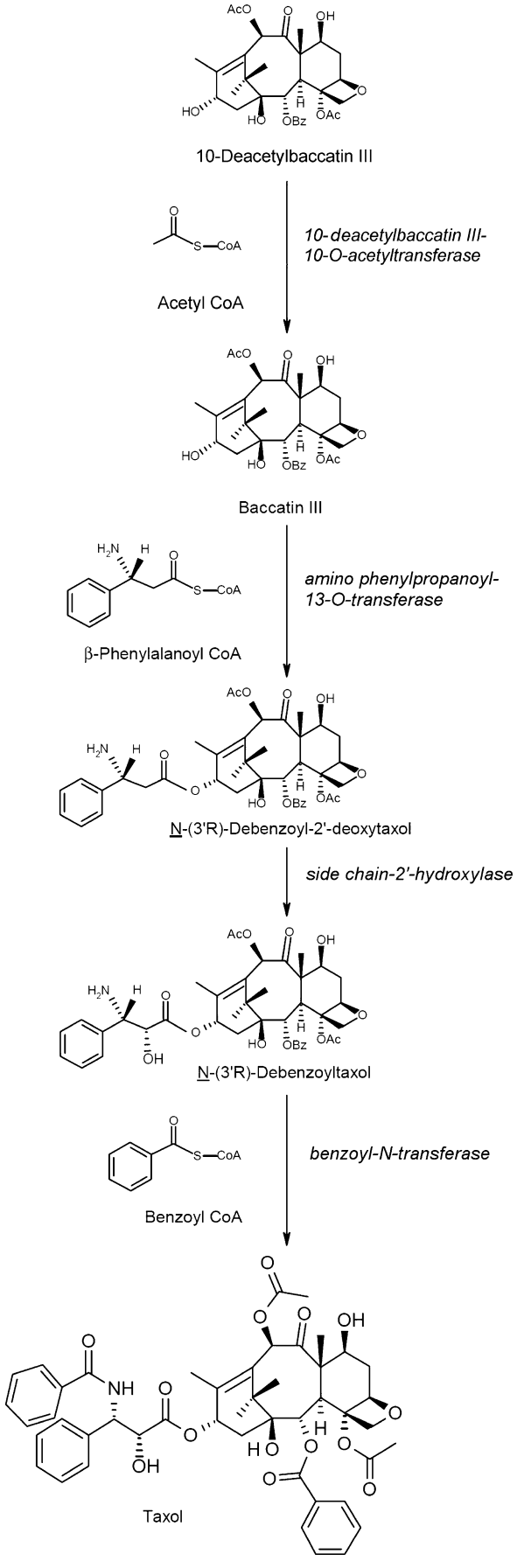

Fig. 2 Enzymatic semisynthesis of paclitaxel (Long and Croteau 2005)

phenylalanoyl-baccatin III that undergoes subsequent 2′-hydroxylation and *N*-benzoylation to afford paclitaxel (Fig. 2, Long and Croteau 2005). However, because the side chain transferase can utilize both β-phenylalanoyl-CoA and phenylisoserinoyl-CoA in the C-13-*O*-esterification of baccatin III, ambiguity remained as to whether the 2′-hydroxylation step occurs before or after transfer of the amino phenylpropanoyl moiety. Using cell-free enzyme systems from *Taxus* suspension cells, no evidence was found for the direct hydroxylation of β-phenylalanine to phenylisoserine; however, microsomal preparations from this tissue appeared capable of the cytochrome P450-mediated hydroxylation of β-phenylalanoyl-baccatin III to phenylisoserinoyl-baccatin III as the penultimate step in the formation of paclitaxel and related N-substituted taxoids. These preliminary results, which are consistent with the proposed side chain assembly process, have clarified an important step of paclitaxel biosynthesis and set the foundation for cloning the responsible cytochrome P450 hydroxylase gene (Long and Croteau 2005).

Recently, coexpression of *Taxus* cytochrome P450 reductase with cytochrome P450 oxygenases involved in paclitaxel biosynthesis in yeast, demonstrating that functional transgenic coupling of the *Taxus* reductase with a homologous cytochrome P450 taxoid hydroxylase, represents an important initial step in reconstructing paclitaxel biosynthesis in a microbial host (Jennewein et al. 2005). Eight taxoid biosynthetic genes of the baccatin III pathway (a sequence of 11 enzymatic steps from primary metabolism) were functionally expressed in *Saccharomyces cerevisiae* using three plasmids (DeJong et al. 2006).

Production in reactors

Zocher et al. (1996) reported the first cell-free acetylation of 10-deacetylbaccatin III in crude extracts from roots of *T. baccata* saplings using ^{14}C- or ^{3}H-labeled acetyl-CoA as the acetyl donor. For purposes of gene conservation, cuttings of several clones of *T. baccata* L. were harvested and selected for their capacity for root production and growth (Ewald et al. 2002). The amount of paclitaxel and the activity of the biosynthetic enzyme 10-deacetylbaccatin III-10-*O*-acetyltransferase in these newly formed roots were enhanced by treatments with chemical elicitors. The expression of the cloned cDNA of 10-deacetylbaccatin III-10-*O*-acetyltransferase from *T. baccata* in *E. coli* was investigated and optimized using three kinds of isopropyl-beta-D-thiogalactopyranoside (IPTG) inducible fusion expression systems (Fang and Ewald 2004). Unfortunately, the enzyme obtained

was not stable at room temperature, and the activity was drastically decreased during purification procedures.

Seedling cultures from autochthonous plant material in Eichsfeld, a landscape with a high population density of yew trees in Germany, as well as rooting cultures from the cutting propagation of Ewald et al. (2002) were established by our group. The contents of 10-deacetylbaccatin III as the main taxane varied from 200 to 4,000 μg per g of dried needles depending on the origin and age of the plants (Frense et al. 2005).

The enzyme 10-deacetylbaccatin III-10-*O*-acetyltransferase from the Zocher's group, which was expressed in *E. coli*, was used for the semisynthesis of baccatin III from 10-deacetylbaccatin III. A new enzyme membrane bioreactor, on the basis of a hollow fiber membrane, was developed by our group (Frense et al. 2004; Pflieger et al. 2006). It works as a membrane contactor and ensures a dispersion-free contact between a watery and an organic phase. The enzyme and substrate circulate in the inner side of the hollow fibers, the water-insoluble organic solvent on the outer-side. Membrane material and solvent were optimized regarding the selective extraction of baccatin III into the solvent, which reduces the purification effort. The integrated process control regulates the addition of the substrate according to the actual productivity and increases the process stability through this.

Baccatin III was produced in this membrane reactor in quantities between 1.6 and 2.4 g in 1 week corresponding to a productivity related to the volume of the enzyme extract of up to 500 mg l^{-1} day^{-1} (Frense et al. 2004). To our knowledge, this is the highest enzymatic production rate of taxanes in a bioreactor scale that has been described. Interestingly, the addition of the expensive cofactor acetyl-CoA was not necessary; only glucose had to be added. There must be acetyl-CoA in the enzyme raw extract used, as well as a still unknown cofactor regeneration system. Semisynthesis of the anticancer drug paclitaxel can be carried out by further chemical coupling of the C-13 paclitaxel side chain to this baccatin III as described previously.

Summary

New taxanes have been developed to overcome the clinical problems related to the use of taxanes and to extend the spectrum of agents against taxane-sensitive tumors. Among these agents, a newly developed taxane (BAY 59-8862) appears particularly interesting for the fact that it shows excellent oral bioavailability and activity in tumors with inherent resistance to paclitaxel (Ferlini et al. 2003).

Since the Food and Drug Administration's approval of Taxol (Bristol-Myers Squibb) and Taxotere (Rhone-Poulenc Rorer SA, now Aventis) as anticancer drugs, a number of new analogues have been prepared with the view of obtaining taxoids with better potency and bioavailability to targeted cells, as well as taxoids efficient in targeting multidrug resistant cancer or taxoids acting as modulators of multidrug resistance (Dubois et al. 2003).

Research into the synthesis of paclitaxel is still ongoing with a number of groups around the world carrying out work not only to develop newer and shorter semisynthetic routes to this natural product, but also with a view to create a range of structures based on paclitaxel that may be more biologically active and/or easier to synthesize (Patel 2004). In consideration of the market values of Taxol, the biotechnological production of this and related substances offers a sustainable and economically interesting alternative to extraction from plant material and to production by plant cell cultivation.

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