

Ethnobotany and Medicinal Plant Biotechnology: From Tradition to Modern Aspects of Drug Development*

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ABSTRACT

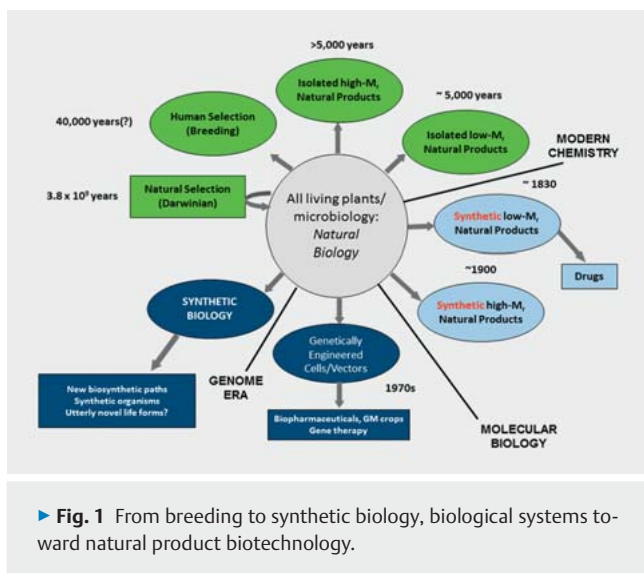
Secondary natural products from plants are important drug leads for the development of new drug candidates for rational clinical therapy and exhibit a variety of biological activities in experimental pharmacology and serve as structural template in medicinal chemistry. The exploration of plants and discovery of natural compounds based on ethnopharmacology in combination with high sophisticated analytics is still today an important drug discovery to characterize and validate potential leads. Due to structural complexity, low abundance in biological material, and high costs in chemical synthesis, alternative ways in production like plant cell cultures, heterologous biosynthesis, and synthetic biotechnology are applied. The basis for any biotechnological process is deep knowledge in genetic regulation of pathways and protein expression with regard to today's "omics" technologies. The high number genetic techniques allowed the implementation of combinatorial biosynthesis and wide genome sequencing. Consequently, genetics allowed functional expression of biosynthetic cascades from plants and to reconstitute low-performing pathways in more productive heterologous microorganisms. Thus, *de novo* biosynthesis in heterologous hosts requires fundamental understanding of pathway reconstruction and multitude of genes in a foreign organism. Here, actual concepts and strategies are discussed for pathway reconstruction and genome sequencing techniques cloning tools to bridge the gap between ethnopharmaceutical drug discovery to industrial biotechnology.

Ethnopharmacy

For centuries, the history of pharmacy and drug discovery has been identical with the history of pharmacognosy and ethnobotany. Based on the story of curare, quinine, and later morphine or artemisinin, plenty of natural products and plant extracts have been used in ethnopharmacology [1]. According to Heinrich et al., ethnopharmacology by its definition draws its attention to the scientific study of indigenous drugs but does not explicitly address the issue of searching or even cultivating and breeding for an enhanced yield. As stated by the World Health Organization (WHO) [2], medicinal plants are an important element of indigenous medical systems in many parts of the world. About 80% of the world's population relies on herbal medicinal products due to

limited access to Western medicine with regulated mostly synthetic or biotechnological drugs [3]. However, not only so-called low-income countries but also Europe have benefited from the close sharing of knowledge with other mostly tropical countries. Many of today's modern drugs like local anesthesia (e.g., cocaine), pain killer (e.g., morphine), and antineoplastic drugs (e.g., podophyllotoxin, camptothecin) have their structural skeleton in a natural product analogue. According to Newman and Cragg, about 40% of all clinically used drugs originate from natural products

* Dedicated to Professor Dr. Robert Verpoorte in recognition of his outstanding contribution to natural products research.



[1, 4, 5]. The role of traditional medicine is to provide medicinal active plants in the drug discovery process [6–8].

Even though our ancestors did not have any detailed knowledge about chemical structures, natural products formed the basis of many medicines. The reasons for this are manifold, but it is likely that the ability of nature to create fantastic complex and structurally diverse molecules is the most convincing argument that natural products show very broad biological activity. Besides of the history of natural products based on plants, we should not forget that microorganisms are the real chemists that provide an unbelievably high number of secondary natural products [9]. About 250,000 higher plants are known and about 120,000 isolated and structure elucidated compounds have been published. A closer look at the biodiversity of microorganisms shows that we even do not know how many of them exist on this planet. Some estimates suggest that we know roughly only about 1% of living microorganisms and we have just started to understand the chemical diversity of a few thousand molecules.

In its primitive form, plant breeding started after the invention of agriculture. Breeding of crops as wild strains from nature in pre-historic times was also the starting point of ancient plant breeders. Depending on classical tools to develop new and improved strains for food, they focused mainly on crop plants like maize and corn [10, 11]. As depicted in ► **Fig. 1**, plant breeders simply selected food plants with desirable characteristics and applied crossing techniques. Later, Gregor Mendel’s experiments on hybridization [12] had a strong impact, but with the advent of biotechnology, breeders were now in the position to incorporate molecular tools in their breeding work. Optimization of medicinal plants and natural product yield by breeding played no major role and started with the 20th century. New opportunities in gene technology paved the road to metabolic engineering, and in continuation with metabolomics tools [13], synthetic biology is the most recent milestone in this development. Integration of life science disciplines will have a new potential we cannot fully oversee today. Heterologous assembly of artificial pathways, crossing species borders, and redesigning cellular metabolism are only some

examples and will be briefly introduced [14]. Today, secondary natural products like morphine [15], vanillin [16], resveratrol [17], and tetrahydrocannabinolic acid [18] are already biosynthesized in microorganisms.

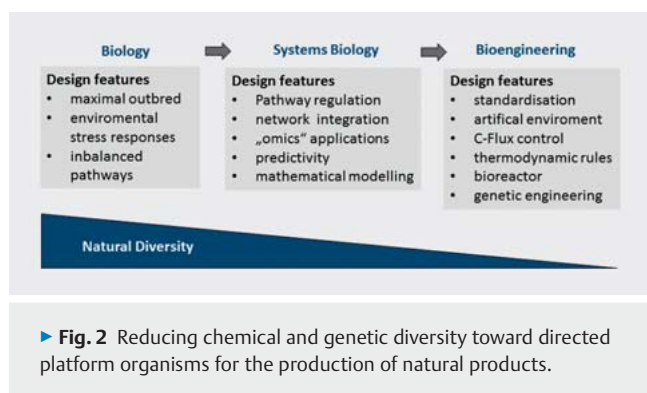
In various online databanks information on the genome, transcriptome, and metabolome is accessible. For instance, transcriptome data of 1000 (medicinal) plants from the 1KP project are ready to use [19, 20]. In the times of “omics” with floods of genome and transcriptome, data are coming over us and scientists do not fully understand how to dig for new drug leads and how to use all data for a smart and lean drug discovery process [21]. In principal, biologists, and physicians have accumulated an enormous amount of digital information and application of omics tools belongs to standard operation methods in the context of quality control and identification of natural products [22, 23]. Since the finalization of the human genome project and other related projects to explore human proteome, transcriptome, or epigenome, the pharma industry has not released one new drug entity that has kept the promise of the uprising genome era. On the contrary, new drug entities still come from classical drug discovery processes including natural products [24]. The main reason for this is that creative nature still provides extremely complex chemical skeletons that chemists cannot imagine in their labs. As chemists, pharmacists, and physicians, we find molecules fascinating with overbridging ring systems and stereochemistry as known for paclitaxel, quinine, or artemisinin.

We acknowledge the beauty of these molecules, but we mostly do not see the enormous problems we face when we try to get these compounds as clinical drugs in their final dosage form to the patient. Natural products are of high interest because of high price ► **Table 1**; they are considered “orphan drugs” by pharmaceutical industry. Mostly they cannot be obtained in a sufficient amount from plant source; even emerging outdoor cultivation (e.g., scopolamine), production in plant cell cultures (e.g., shikonin, paclitaxel), or collection from the wild (e.g., podophylotoxine) are making big challenges why the pharma industry is reluctant to develop pure natural products as drug candidates. Other alternatives to produce are in general costly and hard to translate into the pharmaceutical industry. As an example, outdoor production of *Duboisia myoporoides* R.Br. (Solanaceae) to extract scopolamine is costly and demands high logistic effort [25]. Plant cell callus cultures do not provide high yields and deliver yields in low range of mg/L (taxanes: 5–10 mg/L; hyoscyamine: 5 mg/L; ginsenosides: 28–145 mg/L) [26]. With the exception of paclitaxel [27], no drug was ever produced in a commercial-scale in plant suspension cultures. In the last 40 years, shikonin as cosmetic has been developed and entered for a short time the market only [28].

Impact of metabolic engineering and synthetic biology changed the rules of the game and microorganisms became interesting for implementation of full plant pathways expression. Starting from genome sequencing of medicinal plants to describe and formally explain and document physiological conditions to systems biology and bioengineering, chemical and genetic diversity was reduced to create a minimal cell as a high-performer platform organism ► **Fig. 2**. Applying engineering principles like mathematical modelling, statistical calculations for C-flux optimi-

► **Table 1** Plant-derived natural products of importance for the pharmaceutical industry.

Compound	Plant species	Need (to/y)	Price USD\$/kg	Cultivation in ha	Reference
Artemisinin	<i>A. annua</i>	50–60	100	29,000	[42, 45]
Paclitaxel	<i>T. brevifolia</i>	0.5	26,000–38,000	Wild collection (750,000 yew trees/year)	[57]
Docetaxol	<i>T. brevifolia</i>	0.3	8200–43,200	Semisynthesis	[57]
Resveratrol	<i>V. vinifera</i>	10,000	600	Fermentation, synthesis	[58]
Ajmalicine	<i>R. serpentina</i>	0.3	1500	Not known	[59]
Anthocyanins	<i>V. vinifera</i>	2.0	2000	Not known	[60, 61]
Vincristine	<i>V. minor</i>	0.8	350,000	Not known	[57, 60, 61]
Colchicine	<i>C. autumnale</i>	5.0	6000	Not known	[60, 61]



zation, and optimization of energy conversion (ATP, NAD[P]H) [29] were applied as we know by definition for the discipline of bioengineering (► **Fig. 2**).

Two natural products have heavily impacted pharmaceutical research and bioengineering in the last 20 years: paclitaxel and artemisinin, which were both game-changers in medicinal plant biotechnology and synthetic biology. Because of the economic importance and impact on bioengineering, both are outlined here and the development from first discovery to the final industrial production explained.

Bioengineering of Paclitaxel Production

In 1962, first samples of the Pacific yew tree (*Taxus brevifolia* Nutt., Taxaceae) were collected and tested by the National Cancer Institute for antineoplastic activity. After a lengthy development process, clinical trials started in 1984 and market authorization followed in 1994 [30, 31]. Besides of difficulties in structure elucidation and determination of the pharmacological profile, a main obstacle was supply of the pure active ingredient. First, extraction of bark material from the at least 50-year-old trees from the wild was most appropriate and problematic at the same time. *T. brevifolia* is a rather slow-growing tree producing paclitaxel in very low amounts (10 µg/kg) [30]. To treat all women in the United States suffering from ovarian cancer within a year, the whole population of the tree has to be cut down in the United States, which would lead to its full extinction. Meeting the annual therapeutic require-

ments of patients with ovarian cancer requires 15–20 kg of the drug. Extending the indication to other cancer types, the demand will increase to up to 250–300 kg/year, an amount that would need to be isolated from 145,000 tons of bark. Harvesting such an amount is unrealistic and unsustainable. The strategy for obtaining pure active ingredients shifted from plant extraction (1975–1990) to the commercial silvicultural production of baccatin II as biosynthetic precursor from *Taxus baccata* L., Taxaceae (1990–2002) to current (2003) *in vitro* fermentation technology [32–34]. The first study to develop and optimize a cell-based bioprocess started at the beginning of the 1990s and demonstrated that calli of *Taxus* sp. were able to produce paclitaxel and its precursors at least as efficiently as the plant [32]. Successful strategies to enhance yield of paclitaxel derived later from bioengineering, improved media composition, high producer cell lines, elicitors, phytohormones, and bioreactor design [35, 36]. At the beginning of 2010s, omics technologies pushed plant cell fermentation to rational approaches [37]. The primary task was to identify biosynthetic genes, enzymes and regulating factors, and systems biotechnology information. In the postgenomic age, high-throughput methods like transcription profiling, microarrays, modern PCR techniques, and proteomics will accelerate and catalyze discovery of new genes and proteins for creating either new biosynthetic pathways or to improve the catalytic rate of enzymes to overcome bottlenecks in the carbon flux. Since 2000, several studies have taken an empirical approach to the elucidation of gene regulatory mechanisms related to paclitaxel biosynthesis in different *Taxus* species. As an example, increase of geranyl diphosphate as central precursor for formation of taxadiene as first committed metabolite toward taxanes and upregulation of the limiting enzyme taxadiene synthase was groundbreaking finding from postgenomics. Later on, more biosynthetic steps were analyzed and enzymes either upregulated, modified by protein engineering, or fully substituted to increase production yield of paclitaxel 40-fold in fermentation cultures [38, 39].

Bioengineering of Artemisinin Production

The second model case of interest is artemisinin. This molecule is by structure, clinical indication, and social importance one of its kind. In 2016, Youyou Tu was awarded for her discoveries concerning a novel therapy against malaria, but also for discovery and

structure elucidation of artemisinin from *Artemisia annua* L., Asteraceae [40]. This plant, whose Chinese name is *qinghao*, was described vividly in the fourth century the book *Zhouhou Beiji Fang (The Handbook of Prescriptions for Emergencies)* by the Chinese physician Ge Hong as a treatment for malarial fever [41]. Drugs based on artemisinin have led to the survival and improved health of millions of people. But the story is much more exciting. Artemisinin can be considered as the first secondary natural product paving the road for plant based molecules toward metabolic engineering and synthetic biology. Artemisinin is a sesquiterpene lactone with an overbridged trioxan ring system containing a peroxide. Besides this fact, artemisinin has seven stereocenters. For this reason, organic synthesis is expensive and not affordable for companies. As outlined for paclitaxel extraction, the yield of artemisinin of 0.4% is also very low in *A. annua*. In combined efforts by the Bill and Melinda Gates Foundation and the University of York, high-yield varieties (1.2–1.6%) have been produced by molecular breeding technologies. Nevertheless, the price of pure artemisinin has not decreased and at 150–1500 USD/kg remains still quite high [42]. Artemisinin and related combination therapies have become indispensable for most patients suffering from malaria because of resistance of *Plasmodium* parasites—transmitted by *Anopheles* flies—against classical quinine based drugs. In 2003, the University of California and the same above-mentioned foundation started a synthetic biology project to produce artemisinin in baker's yeast at lower cost. This ambitious challenge was supported by 40 million USD and led to the heterologous production of dihydroartemisinic acid [43]. Keasling et al. from the University of California identified all relevant genes and enzymes for the mevalonate pathway and early biosynthesis of sesquiterpene lactones by genome sequencing and high throughput gene design. A critical element of Keasling's work was the development of genetic tools to aid in the manipulation of microbial metabolism, particularly for low-value products that require high yields from sugar [44]. His laboratory developed single-copy plasmids for the expression of complex metabolic pathways. He constructed promoter systems that allow regulated control of transcription consistently in all cells of a culture and mRNA stabilization technologies to regulate the stability of mRNA segments. Furthermore, new protein engineering approach to attach several enzymes of a metabolic pathway onto a synthetic protein scaffold to increase pathway flux were also invented and applied [43]. The mevalonate pathway in baker's yeast was fully reconstructed, metabolic bottle necks identified, and carbon flux starting from glucose as substrate optimized to boost up farnesyl pyrophosphate as the main precursor for the first committed step to form amorpha-4,11-diene. Most of the time and efforts were spent on the elucidation of the early biosynthesis. In short, oxidation of the methyl group in position C12 by three cytochromes to artemisinic acid was a challenging task. All attempts to identify enzymes catalyzing artemisinic acid to artemisinin failed, and up to now, no enzyme has ever been detected. The question remains whether final synthesis is enzyme-based or simple photooxidation in the oil container of plant trichomes. After negotiations with the WHO, Novartis, and Sanofi, the heterologous biosynthesis of artemisinic acid was transferred to an industrial level [45]. In a semi-biotechnological step, a photochemical reaction step was coupled to produce arte-

► **Table 2** Recombinant secondary natural plant products from heterologous production.

Compound	Production organism	Titer	Reference
Resveratrol	<i>E. coli</i>	1.4 g/L	[58]
	<i>S. cerevisiae</i>	5 g/L	[64]
Vanillin	<i>S. cerevisiae</i>	45 mg/L	[16, 62]
Naringenin	<i>S. cerevisiae</i>	474 mg/L	[63]
Dihydroartemisinic acid	<i>S. cerevisiae</i>	100 mg/L	[43]
Artemisinic acid		25 g/L	[65]
Morphine	<i>S. cerevisiae</i>	131 mg/mL	[66]
Ginsenosides	<i>S. cerevisiae</i>	54–1189 mg/L	[52]

misinin in a classical chemical engineering environment. In 2013, Sanofi announced the launch of a production facility in Garessio, Italy, to manufacture the antiplasmodial drug on a large scale [46]. Sanofi produced 25 tons of artemisinin in 2013, ramping up the production to 55–60 tons in 2014 and supplying approximately one-third of the global annual need for artemisinin. The price per kg is 350–400 USD/kg, roughly the same as the botanical source.

Future Bioengineering of Cell Factories for Natural Products

What can we expect in the future from synthetic biology and bioengineering? Plant secondary metabolites exhibit a variety of biological activities and therefore serve as valuable therapeutics. As outlined, the small amounts isolated from plants still do not meet market demands. The term “metabolic engineering” was introduced 25 years ago to describe the application of recombinant DNA technology for improving metabolic processes in organisms [47]. Nowadays, the idea of genetic modification has fully changed to the employment of alternative routes with the same catalytic function but better performance, even from microorganisms that have never produced any plant metabolite [14, 48]. Based on next-generation sequencing data, pathways are not just reconstructed from plants but are composed of genes from various species in order to maximize efficiency. Metabolic engineering in plants involves the modification of endogenous pathways to enhance production of the compound of interest (► **Table 2**) to minimize biosynthesis of unwanted side products and to increase the biosynthetic rate by smart pathway design [14, 49]. It does not matter if genetic principles are applied either for plants or heterologous production platforms (e.g., *Escherichia coli*, *Saccharomyces cerevisiae*) [48]. Most strategies fit in a universal way: (i) *in silico* engineering (e.g., CellDesigner, e-cell [50, 51]) of single steps in a pathway to understand metabolic flux of metabolites from the primary to the secondary pathway; (ii) balancing the energetic flow of ATP, NADPH, and NADH; (iii) blocking competitive pathways; (iv) introducing pathway short cuts that divert meta-

bolic flux in a particular way [52]; and (v) identifying network topologies and modifying signaling cascades [14, 53].

However, all mentioned approaches have only limited value as single biochemical events. The power of metabolic engineering will become clear if multiple steps are affected in the same pathway. That requires a deep understanding of the biochemical and genetic underlying principles and mostly highly sophisticated computing and bioinformatics [54, 55]. The potential of bioengineering to improve secondary natural product biosynthesis in a rational directed way by modulating individual steps has been demonstrated in the past, and we can expect in the near future revolutionary concepts [56]. It will be no surprise to work with artificial or minimal cells that are *in silico* designed from scratch and we will choose our pathway of interest by copy and paste on an office computer.

Conflict of Interest

The author declares no conflict of interest.

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