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REVIEW

# Combinatorial biosynthesis in plants: A (p)review on its potential and future exploitation

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Combinatorial biochemistry, also called combinatorial biosynthesis, comprises a series of methods that establish novel enzyme–substrate combinations *in vivo* and, in turn, lead to the biosynthesis of new, natural product-derived compounds that can be used in drug discovery programs. Plants are an extremely rich source of bioactive natural products and continue to possess a huge potential for drug discovery. In this review, we discuss the state-of-the-art in combinatorial biosynthesis methods to generate novel molecules from plants. We debate on the progress and potential in biotransformation, mutasynthesis, combinatorial metabolism in hybrids, activation of silent plant metabolism and synthetic biology in plants to create opportunities for the combinatorial biosynthesis of plant-derived natural products, and, ultimately, for drug discovery. The therapeutic value of two classes of natural products, the terpenoid indole alkaloids and the triterpene saponins, is particularly highlighted.



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## 1 Introduction

For thousands of years, mankind has made use of plant-derived natural products to treat diseases and injuries.[1] Today, these small molecules are still utilized in a variety of therapeutics and play an important role in drug discovery programs.[2–5] Nonetheless, there is a continuous demand for novel molecules with new or superior pharmaceutical activities, because of, among others, newly emerging diseases and the growing drug tolerance and resistance in microorganisms. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) causes around 19 000 deaths per year in the United States.[6,7]

Advances in functional genomics and application of multi-disciplinary approaches allow picking of the inherent bioactive molecules from medicinal plants, resulting in the discovery and development of new and important drugs, such as the recently approved plant-derived drugs elliptinium, galantamine and huperzine.[3,8] Chemical synthesis of natural compounds generates a better understanding of how the molecules function, and allows targeted design of novel compounds with improved or even new functions.[9] Unfortunately, due to their structural complexity, it is often commercially unfeasible to chemically synthesize the bioactive plant-derived molecules.[10] Therefore, development of novel drugs from natural plant sources by using present-day experimental tools remains a rational approach.

Traditionally, medicinal plant extracts had been the only source of plant-derived natural products used in pharmacognosy and their bioactive compounds had been discovered by traditional screening.[11] For instance, a large-scale screening for

antitumor agents, conducted by the National Cancer Institute, led to the discovery of paclitaxel from the bark of the Pacific Yew tree, *Taxus brevifolia*.[12] Yet, such traditional pharmacological screenings of medicinal plants are time-consuming and expensive, drawbacks, which might be partially overcome by high-throughput pharmacological screening of crude plant extracts.[13] Despite the potential of plant natural products, the pharmaceutical industry has shifted focus to the high-throughput screening of combinatorial chemistry libraries, of which the output in terms of new drugs is, until now, far below the initial expectations.[14–17]

An alternative approach to generate new molecules or scaffolds is combinatorial biosynthesis in which gene products from different organisms are combined to produce new bioactive compounds. In a broad sense, combinatorial biosynthesis can be practically achieved with (i) biotransformation, in which the compounds are modified chemically by biocatalysts; (ii) mutasynthesis or feeding of compounds to mutant biocatalysts; (iii) combinatorial metabolism in hybrids; (iv) activation of silent metabolism; and (v) synthetic biology or the combinatorial piling up of genes in heterologous (plant) hosts (Scheme 1).

In this review, we focus on the potential and the progress made in combinatorial biosynthesis in plants. The practical mechanisms available to date are discussed and a possible future outline is presented to use plant-based combinatorial biosynthesis platforms for novel drug discovery.

## 2 Combinatorial biosynthesis in microorganisms: setting the exemplar

When Hopwood and coworkers[18] introduced genes involved in the biosynthesis of the antibiotic actinorhodin into a medermycin-producing *Streptomyces* strain, the transformed strain produced a novel antibiotic, mederrhodin A. This compound had a structure similar to that of medermycin, but with an additional hydroxyl group typical of actinorhodin (Fig. 1).[18] This was the first report that established the feasibility and potential of interchanging and combining genes from different organisms as a manner to generate hybrid pathways, leading to the synthesis of 'hybrid' or 'new-to-nature' compounds. Since then, combinatorial biosynthesis has become a powerful platform for generating structural diversity in microbial natural products.[19–22]

In particular, combinatorial biosynthesis proved to be an excellent approach for the development of novel polyketides, a large family of natural products, which include many pharmaceuticals.[19–28] Polyketides are biosynthesized from acyl-CoA monomers of simple carboxylic acids by large, multifunctional proteins (megasynthases) that comprise several active catalytic sites. Each active site or module catalyzes one specific cycle of the polyketide chain elongation and the associated modification of the polyketide backbone.[19,26] The large natural diversity of polyketide structures is obtained by combinations of different catalytic modules (incorporation of different acyl-CoA residues) in a different sequence and a different number of modules. After the synthesis of the polyketide backbone, various cyclization and tailoring enzymes increase the structural variability. The

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**Alain Goossens**

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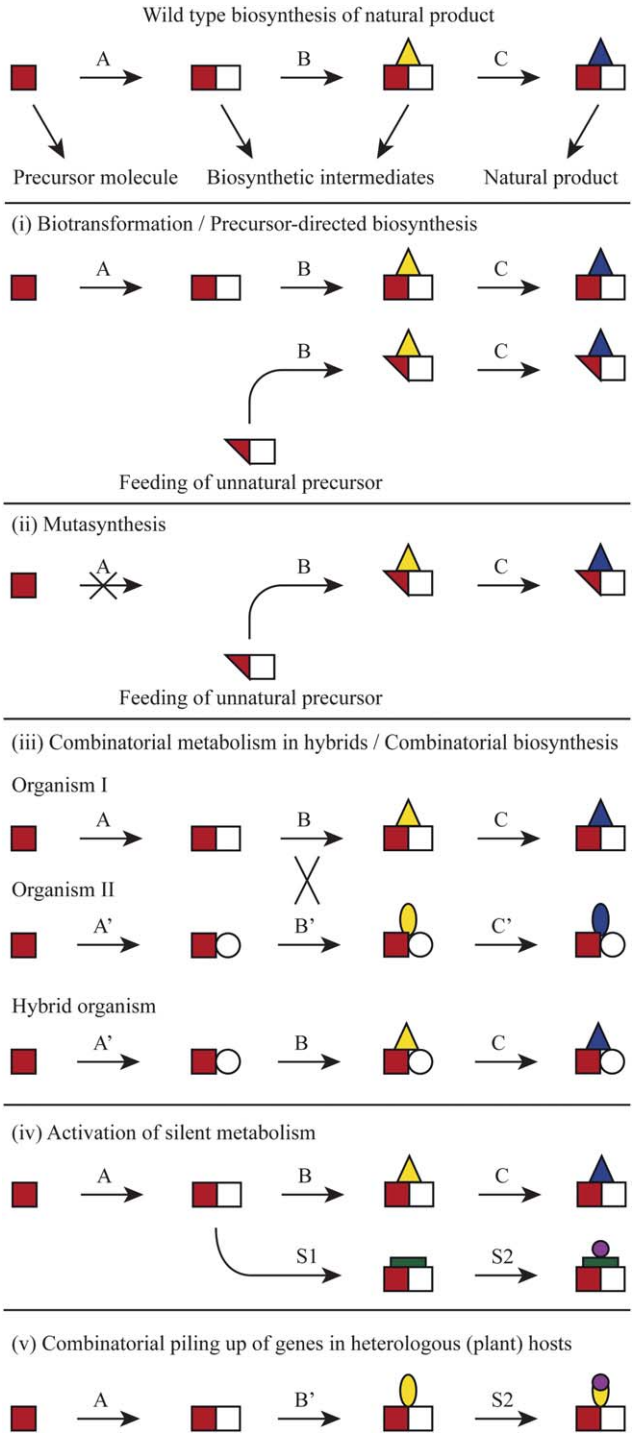


**Scheme 1** Various approaches for combinatorial biosynthesis of plant-derived natural products. A, B, C, S1, and S2, enzymes. Colored symbols represent small molecule scaffolds and modifications thereof.

tremendous combinatorial biosynthesis potential of polyketides was underscored by McDaniel and coworkers.[29] By engineering the multiple biosynthetic steps in a polyketide synthase that produced the macrolide ring of the widely used antibiotic erythromycin, a small combinatorial library of more than 50 novel polyketides was generated.[29] Later, the molecular nature of the polyketide synthesis allowed the establishment of a modular

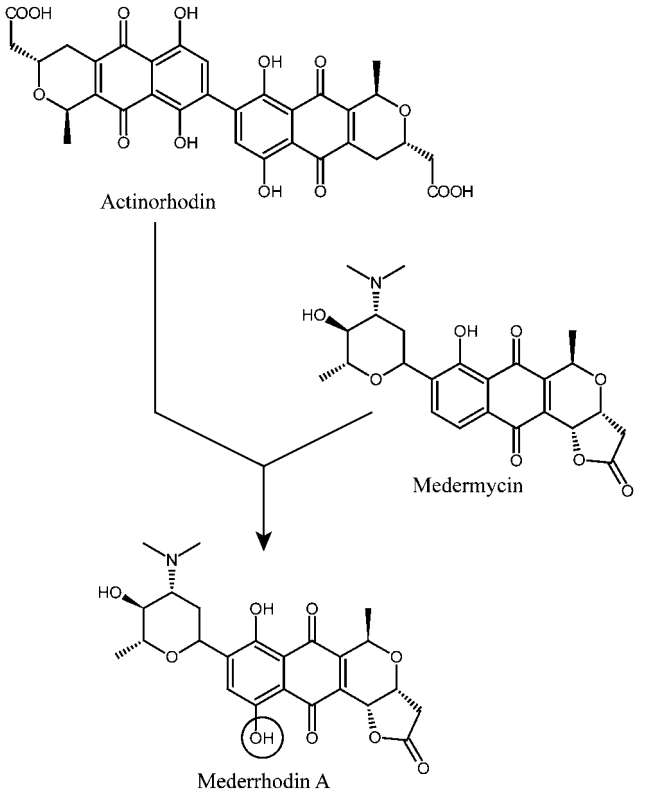


**Fig. 1** Mederrhodin A, produced by cloning of actinorhodin biosynthesis genes into a medermycin-producing *Streptomyces* strain.[19]

method, referred to as 'Lego-ization of polyketide biosynthesis', to assemble synthetic polyketide synthases that catalyzed the production of new polyketides.[30,31]

Combinatorial biosynthesis in microorganisms has been successfully applied to other classes of microbial natural products as well, such as the indolocarbazoles, a class of compounds with potential applications in cancer therapy.[32] Characterization of the rebeccamycin biosynthetic gene cluster paved the way for its combinatorial stacking with selected genes from other indolocarbazole biosynthetic pathways, resulting in the production of over 24 novel indolocarbazole derivatives in the microbial host.[33,34]

Recent, groundbreaking, technological advances in molecular biology, functional genomics and biotechnology will substantially increase the capacity of combinatorial biosynthesis.[22] For instance, with the emergence of next-generation sequencing techniques, entire microbial genomes can be sequenced, facilitating the discovery of biosynthetic gene clusters, which can be applied in combinatorial biosynthesis platforms. Obviously, because these generic technologies are also applicable to plants, they will undoubtedly allow the exploitation of the unrivalled complex metabolic arsenal of plants (see below) and open a new era for plant-based drug discovery in the near future.

## 3 The metabolic power of plants

### 3.1 Broad substrate specificity

Although plants produce a vast array of metabolites with very diverse structures and biological activities, most of the plant

metabolites are derived from only a limited number of chemical scaffolds. For instance, in *Catharanthus roseus* (Madagascar periwinkle), over 120 different terpenoid indole alkaloids (TIAs) are biosynthesized from one precursor molecule, *i.e.* strictosidine. Starting from this precursor, the structural variation is obtained *via* a highly branched pathway, containing tens of specific enzymatic conversions and spontaneous chemical reactions.[35] Similar cases have been reported in multiple plant species and various metabolite classes. For example, over 60 different ginsenosides derived from the ubiquitous precursor 2,3-oxidosqualene have been isolated from *Panax quinquefolius* (American ginseng), and novel structures continue to be discovered.[36,37]

Several factors collectively contribute to the enormous structural diversity of plant metabolites. Plants contain a large number of proteins capable of catalyzing regio- and stereoselective reactions. Cells from any plant species or organ express over 10 000 genes, of which a substantial fraction encode enzymes with a role in metabolism. For instance, in the model plant *Arabidopsis thaliana*, approximately 20% (5506 out of 27 416) of the annotated protein-encoding genes in the genome are currently predicted to catalyze enzymatic reactions (TAIR10 Genome release, http://www.arabidopsis.org/ and AraCyc 7.0 Release, http://www.plantcyc.org/). Furthermore, alternative reading frames, gene fusions and alternative splicing of pre-mRNA can lead to structurally and functionally different proteins originating from a single gene.[38,39] Also at the protein level, structural variation is further enlarged. Multifunctional enzymes can accept a range of substrates, catalyze distinct reactions, or form different products.[39] The multi-amyrin synthase from *Pisum sativum* (pea) produces eight different triterpene scaffolds from the 2,3-oxidosqualene precursor. Consequently, only one enzyme can already account for nearly 10% of the natural variation in triterpene skeletons.[40] Perhaps the most impressive cases reported to date are represented by the sesquiterpene synthases, δ-selinene synthase and γ-humulene synthase, from *Abies grandis* (Grand fir), which can produce 34 and 52 different sesquiterpene scaffolds, respectively.[41] Interestingly, directed evolution strategies based on the knowledge of the active sites of these promiscuous enzymes allowed the creation of enzymes with altered (new or narrower) specificity and activity,[42] illustrating the enormous potential of plant enzymes for metabolic engineering toward the production of new-to-nature plant-derived products.

In future combinatorial biosynthesis programs, the availability of substrates for the substrate-flexible enzymes will become key. For instance, the recombinant alcohol acyltransferase of *Fragaria* × *ananassa* (strawberry) has been shown to accept a broad range of acyl-CoAs as precursors to form the volatile esters of strawberry fruit. However, the substrate tolerance of the recombinant enzyme and the volatile esters occurring in strawberries did not correspond, indicating that the availability of the precursors rather than the substrate specificity of the enzymes governs the volatile ester formation.[43]

### 3.2 Biotransformation

Biotransformation is defined as the process in which one chemical is transformed into another by means of a biocatalyst. Whole cells (either from microorganisms, animals or plants), cell extracts, or (partially) purified enzymes can be used as biocatalysts.[44] As purified enzymes are expensive, difficult to isolate, and need to be externally supplied with cofactors during the reaction, whole cells are considered as the preferred biocatalysts, because they are relatively cheap, easy to obtain, provide a protective environment for the enzymes from shear forces, and supply essential cofactors for the biotransformation. However, in whole cells it might be more difficult to avoid alongside side reactions and to control or reproduce the cells.[45]

Because of the dazzlingly high number of enzymes present in whole plant cells (see above), biotransformation can be an excellent tool to obtain novel compounds. Typically, biotransformation substrates are cheap and readily available products, which can be transformed into more rare and, thus, more expensive compounds.[46,47] Over the last few decades, numerous studies have reported on the biotransformation of exogenous substrates by plant cells (for an excellent review from 1978 to 2003, see Ishihara *et al.*[46]). The advances in this field after 2003 are summarized in Table 1.

The applications of biotransformations in pharmacognosy are very broad and are illustrated below with some of the most representative examples. First, biotransformation can generate novel, potentially bioactive molecules that may serve as candidate drugs. For instance, paeonol supplied to hairy roots of *Panax ginseng* was converted into glycosylated derivatives. Paeonol glycosides have radical-scavenging effects and, after biotransformation, two of the five detected paeonol glycosides were novel.[48] Second, biotransformation can alter the physical properties of a compound. For instance, the natural antioxidants genistein and quercetin, which have antiallergic and anti-inflammatory activities, cannot be applied as drugs because of their water-insolubility and low absorbability after oral administration. Glycosylation of flavonoids increases their water solubility and, in turn, possibly enhanced bioavailability of the compound. Indeed, feeding genistein and quercetin to cultured cells of *Ipomoea batatas* (sweet potato) and *Marchantia polymorpha* (liverwort) resulted in the glycosylation of these compounds and, consequently, increased their pharmaceutical applicability.[49] Third, the production of enantiopure compounds or molecules of one chirality is essential in the synthesis of organic molecules of biological interest.[50] As enzymes are stereoselective biocatalysts, biotransformation can be applied to produce enantiopure compounds. For instance, in hairy root cultures of *Daucus carota* (carrot), the supplemented prochiral ketone acetophenone was reduced to 1-phenylethanol, consisting of more than 98% of the *S*-isomer.[51]

### 3.3 Mutasynthesis

A major limitation of biotransformation is its inability to generate targeted modifications on the applied substrates. Knowledge of metabolic pathways gives the opportunity to introduce particular desired modifications on the supplied precursor molecules. This is the principle behind Precursor-Directed Biosynthesis (PDB), in which a compound analogous to the precursor is added to a secondary metabolite-producing organism. This precursor analog is used by the wild-type organism in competition with the naturally occurring precursor,

 

**Table 1** Overview of biotransformations by plant biocatalysts from 2003 onward. For earlier literature on plant-based biotransformation, see ref. 46

| Substrate | Species | Type | Product | Ref. |
|---|---|---|---|---|
| **1.Production of novel compounds** | | | | |
| Ingenol-3-angelate | *Hordeum vulgare*<br>*Oryza sativa*<br>*Panax quinquefolium*<br>*Nicotiana tabacum* | Cell suspension cultures | 16-Hydroxy-ingenol-3-angelate | 52 |
| Taxadienes | *Ginkgo biloba* | Cell suspension culture | Polyoxygenated taxadienes (6 novel) | 53 |
| Chinensiolide B | *Catharanthus roseus*<br>*Platycodon grandiflorum* | Cell suspension cultures | Guaianolides (2 novel) | 54 |
| Cinnamic acid, *p*-coumaric acid, Caffeic acid, Ferulic acid | *Eucalyptus perriniana* | Cell suspension culture | Several glycosylated and hydroxylated phenylpropanoids (2 novel) | 55 |
| Naringin and naringenin | *Eucalyptus perriniana* | Cell suspension culture | Naringin and naringenin glycosides (2 novel) | 56 |
| Sesamol | *Nicotiana tabacum*<br>*Eucalyptus perriniana* | Cell suspension cultures | Sesamol glycosides (2 novel) | 57 |
| Fluorophenols | *Nicotiana tabacum* | Cell suspension culture | Fluorophenol glycosides (2 novel) | 58 |
| Farnesol<br>Geraniol<br>(*S*)-perillyl alcohol | *Eucalyptus perriniana*<br>*Strophanthus gratus*<br>*Phytolacca americana* | Cell suspension cultures | β-Glucosides (3 novel) | 59 |
| Hesperetin | *Ipomoea batatas*<br>*Eucalyptus perriniana* | Cell suspension cultures | Hesperitin glycosides (3 novel) | 60 |
| Hydroxybenzoic acids | *Panax ginseng* | Hairy roots | Hydroxybenzoic acids glycosides (1 novel) | 61 |
| Capsaicin,<br>8-Nordihydrocapsaicin | *Catharanthus roseus* | Cell suspension culture | Capsaicin and 8-nordihydrocapsaicin glycosides (4 novel) | 62 |
| Raspberry ketone,<br>Zingerone | *Phytolacca americana* | Cell suspension culture | β-Glucosides (4 novel) | 63 |
| Tocopherols | *Eucalyptus perriniana* | Cell suspension culture | Tocopherol glycosides (4 novel) | 64 |
| Thymol, Carvacrol, Eugenol | *Eucalyptus perriniana* | Cell suspension culture | Glycosides (3 novel) | 65 |
| Vitamin E + homologs<br>Vitamin A | *Phytolacca americana*<br>*Catharanthus roseus* | Cell suspension cultures | Glycosides (4 novel) | 66 |
| Vitamin E homolog | *Phytolacca americana*<br>*Catharanthus roseus*<br>*Eucalyptus perriniana* | Cell suspension cultures | Glycosides (1 novel) | 67 |
| Paeonol | *Panax ginseng* | Hairy roots | Paeonol glycosides (2 novel) | 48 |
| Curcumin | *Catharanthus roseus* | Cell suspension culture | Curcumin glycosides (5 novel) | 68 |
| Zerumbone | *Caragana chamlagu* | Cell suspension culture | Zerumbone derivatives (2 novel) | 69 |
| 4(20),11(12)-taxadienes | *Asparagus officinalis*<br>*Platycodon grandiflorum*<br>*Catharanthus roseus*<br>*Phytolacca acinosa* | Cell suspension cultures | Taxanes (12 novel) | 70 |
| 4(20),11(12)-taxadienes | *Ginkgo biloba* | Cell suspension culture | Taxanes (2 novel) | 71 |
| α-Santonin | *Asparagus officinalis* | Cell suspension culture | Hydroxy-α-santonin | 72 |
| α-Santonin | *Catharanthus roseus*<br>*Ginkgo biloba*<br>*Platycodon grandiflorum*<br>*Taxus cuspidate*<br>*Phytolacca asinosa* | Cell suspension cultures | α-Santonin derivatives (4 novel) | 73 |
| 14-Deacetoxyl sinenxan A | *Ginkgo biloba* | Cell suspension culture | Taxadienes (2 novel) | 74 |
| **2.Alteration of physical properties** | | | | |
| Capsaicin | *Panax ginseng* | Cell suspension culture | Capsaicin β-glycosides | 75 |
| Genistein<br>Quercetin | *Ipomoea batatas*<br>*Marchantia polymorpha* | Cell suspension cultures | β-Glucosides | 49 |
| Daidzein | *Eucalyptus perriniana* | Cell suspension culture | Daidzein glycosides | 76 |
| β-Thujaplicin | *Nicotiana tabacum* | Immobilized cells | β-Thujaplicin glycosides | 77 |
| **3.Production of enantiopure compounds** | | | | |
| L-Tyrosine | *Portulaca grandiflora* | Cell suspension culture | 3,4-Dihydroxy L-phenylalanine (L-DOPA) | 78 |
| Benzofuran-2-yl-methyl ketone | *Daucus carota* | Root pieces | (*S*)-1-Benzofuran-2-yl-ethanol | 79 |
| Racemic 1-arylethanols | *Ocimum basilicum* | Cell suspension culture | (*R*)-Arylethanols + ketones | 80 |
| α-Pinene | *Psychotria brachyceras*<br>*Rauvolfia sellowii* | Cell suspension cultures | *trans*-Verbenol + verbenone | 81 |
| α-Pinene | *Picea abies* | Cell suspension culture | *trans*-Verbenol + verbenone | 82 |
| Enol acetates | *Marchantia polymorpha* | Purified proteins | α-Alkylated ketones | 83 |
| α-Ionone<br>β-Ionone | *Caragana chamlagu* | Cell suspension culture | 3-Oxo-α-ionone, 5,6-epoxy-β-ionone | 84 |
| Aliphatic and aromatic aldehydes and ketones | *Cocos nucifera* | Coconut water | Acids, amines and azoxyderivative | 85 |
| Cyclic 3-oxo-amines | *Daucus carota* | Root pieces | Cyclic amino-alcohols | 86 |
| Substituted fluorenones | Various plant species | Ground fruits and vegetables | Substituted fluorenols | 87 |
| Aromatic ketones | *Phaseolus angularis* | Crude enzyme extract | Aromatic alcohols | 88 |
| Ketones | *Raphanus sativus* | Germinated plants | Alcohols | 89 |

**Table 1** (*Contd.*)

| Substrate | Species | Type | Product | Ref. |
|---|---|---|---|---|
| Ketones | Various plant species | Cell suspension cultures<br>Differentiated tissues | Alcohols | 90 |
| Ketones | Various plant species | Fruit and vegetable pieces | Alcohols | 91 |
| Ketones | *Cyanidioschyzon merolae*<br>*Cyanidium caldarium* | Algal cultures | Alcohols | 92 |
| Ketones | Various plant species | Fruit and vegetable pieces | Alcohols | 93 |
| Substituted acenaphthenequinones | Various plant species | Ground fruits and vegetables | Hydroxyacenaphthenones | 94 |
| Aliphatic and aromatic aldehydes and ketones | *Saccharum officinarum* | Sugarcane juice | Acids, amines | 95 |
| Dydrogesterone | *Azadirachta indica* | Cell suspension culture | 20*R*-Hydroxy-9β,10α-pregna-4,6-diene-3-one | 96 |
| Substituted α-tetralones | *Daucus carota* | Root pieces | Homochiral α-tetralols | 97 |
| Racemic secondary alcohols | *Manihot esculenta* | Immobilized enzyme preparation | Acylation of one racemate | 98 |
| Aromatic aldehydes and ketones | *Passiflora edulis* | Fruit peel | Alcohols | 99 |
| Aliphatic and aromatic aldehydes and ketones | *Manihot esculenta*<br>*Manihot dulcis* | Root pieces | Alcohols | 100 |
| Aliphatic and aromatic acids | Various plant species | Cell suspension cultures<br>Differentiated tissues | Alcohols | 101 |
| 2-Substituted tetrahydropyran-4-ones | *Daucus carota* | Root pieces | 2-Substituted tetrahydropyranols | 102 |
| Prochiral ketones | *Daucus carota* | Root pieces | (*S*)-Alcohols | 103 |
| Prochiral ketones | *Daucus carota* | Root pieces | Secondary alcohols | 104 |
| Acetophenone | *Daucus carota* | Hairy roots | (*S*)-Phenylethanol | 51 |
| Phenolic 1-Benzyl-*N*-methyltetrahydroisoquinolines | *Corydalis spp.*<br>*Macleaya spp.* | Cell suspension cultures | Oxygenated protoberberines | 105 |
| Ketones | Various plant species | Cell suspension cultures<br>Differentiated tissues | Homochiral secondary alcohols | 106 |
| Prochiral diketones | *Brassica napus* | Hairy roots | Homochiral alcohols | 107 |
| Aromatic γ-nitroketones | *Daucus carota* | Root pieces | (*S*)-Alcohols | 108 |
| Aromatic nitro compounds | *Vitis vinifera*<br>Various plant species | Fruit and vegetable pieces | Hydroxylamines | 109 |
| 3-Acetylisoxazoles | *Caragana chamlagu* | Cell suspension culture | (*S*)-Alcohols | 110 |
| Bromo- and methoxy-acetophenone derivatives | *Daucus carota*<br>*Apium graveolens* | Ground roots | (*S*)-Alcohols | 111 |
| Progesterone | *Marchantia polymorpha* | Cell suspension culture | 5α-Pregnane-3,20-dione | 112 |
| Carbonyl compounds | *Taxus brevifolia*<br>*Taxus globosa* | Cell suspension cultures | Reduced and cyclized compounds | 113 |
| Bromo- and methoxy-substituted 1-phenylethanol acetates | *Daucus carota*<br>*Apium graveolens* | Ground roots | Alcohols | 114 |
| Acetophenone derivatives | Various plant species | Fruit and vegetable pieces | Chiral (*R*)- and (*S*)-alcohols, Ketones | 115 |
| (*R*,*S*)-1-phenylethanol<br>α-Santonin<br>Lancerodiol p-hydroxybenzoate<br>8,9-Dehydronootkatone<br>Nootkatone | *Marchantia polymorpha* | Cell suspension culture | 1,2-Dihydro-α-santonin, 3,4-dihydrolancerodiol *p*-hydroxybenzoate, 13-hydroxy-8,9-dehydronootkatone, 9-hydroxynootkatone | 116 |
| 1-Arylethanols | *Cyanidioschyzon merolae* | Algal culture | Homochiral secondary alcohols | 117 |
| **4.Other biotransformations** | | | | |
| Prenyl alcohols | *Cucurbita maxima* | Cell suspension culture | Prenylcarboxylic acids | 118 |
| Valencene | *Gynostemma pentaphyllum*<br>*Caragana chamlagu*<br>*Hibiscus cannabinus* | Cell suspension cultures | Nootkatone + nootkatol | 119 |
| Thujopsene | *Hibiscus cannabinus*<br>*Nicotiana tabacum*<br>*Catharanthus roseus* | Cell suspension cultures | 3β-Hydroxy-4-thujopsene + mayurone + 3β-epoxythujopsan-5β-ol | 120 |
| Nitro-aromatic compounds | *Arracacia xanthorrhiza*<br>*Beta vulgaris* | Root pieces | Amines and acetamides | 121 |
| Dihydroartemisinic acid and artemisinic acid | *Catharanthus roseus*<br>*Panax quinquefolium* | Cell suspension cultures | Hydroxylated derivatives | 122 |
| Maleimides | *Nicotiana tabacum*<br>*Catharanthus roseus*<br>*Marchantia polymorpha*<br>*Parthenocissus tricuspidata*<br>*Gossypium hirsutum* | Cell suspension cultures | Succinimides | 123 |
| *p*-Hydroxybenzyl alcohol | *Datura tatula* | Hairy roots | Gastrodin | 124 |
| Cholesterol | *Dioscorea bulbifera* | Cell suspension culture | Diosgenin | 125 |

**Table 1** (*Contd.*)

| Substrate | Species | Type | Product | Ref. |
|---|---|---|---|---|
| Podophyllotoxin | *Hordeum vulgare* | Cell suspension culture | Isopicropodophyllone | 126 |
| 4-Hydroxybenzene derivatives | *Polygonum multiflorum* | Hairy roots | Corresponding glucosides | 127 |
| Nitrosobenzene + pyruvate | *Spinacia oleracea* | Leaf homogenate | *N*-Phenylglycolohydroxamic acid | 128 |
| Deoxypodophyllotoxin | *Linum flavum* | Cell suspension culture | 6-Methoxypodophyllotoxin 7-*O*-glucoside | 129 |
| Paeonol<br>Emodin | *Panax quinquefolium* | Cell suspension culture | Glycosylated, hydroxylated, and methylated compounds | 130 |
| Tryptophan | *Achillea millefolium* | Chopped plant | Melatonin | 131 |
| Valdecoxib | *Catharanthus roseus*<br>*Azadirachta indica*<br>*Capsicum annuum*<br>*Ervatamia heyneana*<br>*Nicotiana tabacum* | Cell suspension cultures | Hydroxylated, methylated and demethylated compounds | 132 |
| α-Bromo alkanone<br>α,α′-Dibromo alkanone | Five algal species | Algal cultures | α-Hydroxyketone, α-diketone | 133 |
| Hesperedin | *Daucus carota*<br>*Nicotiana tabacum* | Cell suspension cultures | Neohesperidin | 134 |
| Various substrates | *Apium graveolens*<br>*Daucus carota*<br>*Armoracia lapathifolia* | Ground roots | Various products | 135 |
| 2′,4′-Dihydroxychalcone | *Morus nigra* | Cell suspension culture | Isocordoin | 136 |
| Substituted indoles<br>Benzofuran + derivatives<br>Benzoxazole + derivatives | *Caragana chamlagu*<br>*Wasabia japonica* | Cell suspension cultures | Various products | 137 |
| Alkylcycloalkanediones | *Caragana chamlagu* | Cell suspension culture | Oxo carboxylic acids | 138 |



finally resulting in a mixture of the desired and the wild-type compounds.[139–141] Although PDB had originally been developed to generate structural diversity in secondary metabolites of microorganisms,[140] it proved to be applicable in plant secondary metabolism as well. Feeding unnatural tryptamine analogs to *C. roseus* seedlings or hairy roots led to the production of a whole range of novel alkaloids.[142] A major disadvantage of PDB, however, is the substrate specificities of the biosynthetic enzymes involved that limit the range of unnatural precursors that can be applied. Furthermore, the internal competition of the analogous precursor with the naturally occurring endogenous precursor(s) may lead to low yields of the novel compound or to complex product mixtures from which the desired molecule is difficult to isolate.

To overcome these limitations, mutational biosynthesis or mutasynthesis can be used. Mutasynthesis is a technique in which the analogous compound or mutasynthon is supplemented to a 'biosynthetic block mutant'. In this mutant, the biosynthetic pathway of the natural compound is blocked, so that only the analogous compound is produced.[139,140] In the first study published on mutasynthesis in plants, RNA silencing of tryptophan decarboxylase (Fig. 2) effectively blocked the biosynthesis of tryptamine in *C. roseus* hairy roots. As a consequence, TIAs did not accumulate in these cultures. Subsequent feeding of these 'mutant' hairy root cultures with an unnatural tryptamine analog yielded several novel TIAs. Importantly, the new compounds were not 'contaminated' by the TIAs naturally present in the 'wild-type' *C. roseus*.[143] Another way to generate novel molecules *via* mutasynthesis is to reorient the plant biosynthetic pathways by means of mutated enzymes. Engineering enzymes *via* deliberate mutations can alter their substrate selectivity. For instance, by engineering the binding pocket of the strictosidine synthase of *C. roseus*, mutant enzymes were created that accepted strictosidine analogs not recognized by the native enzyme. Ultimately, novel unnatural alkaloids were produced both in *in vitro* assays[144–146] and in transgenic plant cell cultures.[147,148] Mutasynthesis has also been applied to generate novel plant polyketides. By mutation of three amino acids in the sequence of the pentaketide chromone synthase (PCS) from the medicinal plant *Aloe arborescens* (Krantz aloe), an enzyme was created that could condense nine molecules of malonyl-CoA to form a novel nonaketide naphthopyrone.[149]

### 3.4 Combinatorial metabolism in hybrids

Hybridization is a frequently occurring phenomenon in plants that can alter their resistance against biotic or abiotic stresses, facilitate introgression of plant traits, or ultimately lead to the formation of new plant species. Often, quantitative and qualitative variations are found in the secondary chemistry of hybrid plants.[150] Hybrids may contain either all the secondary chemicals of both parental taxa, not certain parental chemicals, or novel chemicals that are absent in either of the parental taxa.[150,151] This latter form of hybrid metabolism is a "naturally" occurring form of combinatorial biochemistry in plants.

A classical example of natural hybridization leading to novel compounds is found in the *Helianthus* (sunflower) species that produce sesquiterpene lactones (STLs) in their flowers. *H. annuus*, a species that synthesizes niveusin- and argophyllin-type STLs in its foliar glandular trichomes, hybridizes naturally with *H. petiolaris*, a species without foliar glandular trichomes that produces budlein-type STLs. Natural hybridization between these two species resulted in three hybrid-derived species in which the major STLs differ from those produced by either of the parental taxa.[150] Furthermore, the F1 progeny of crosses between *H. annuus* and *H. debilis* contained novel STLs that were biosynthetic chimeras bearing the basic skeleton of one parent and the side chain of the other parent.[152]

In an extensive literature review[150] that underscored the potential to achieve combinatorial biochemistry in plants *via*

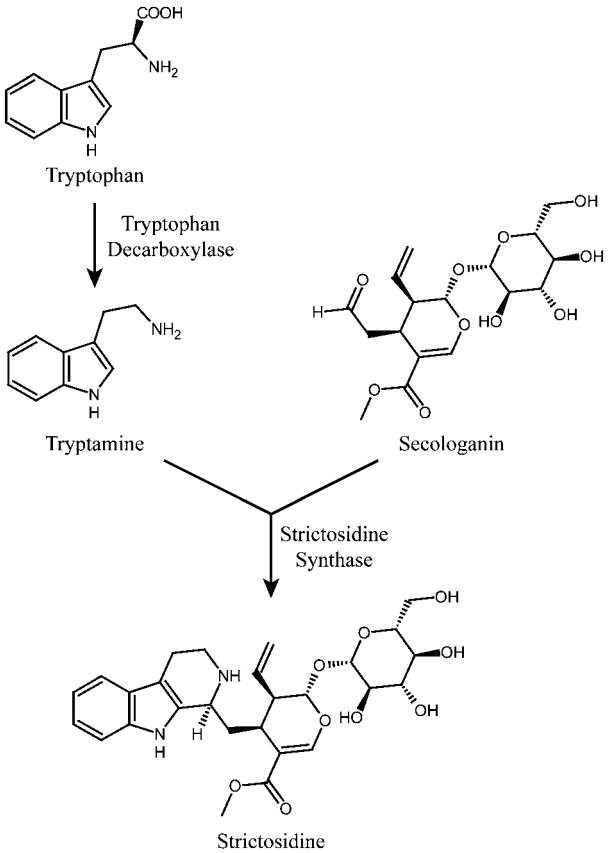


**Fig. 2** Condensation of tryptophan-derived tryptamine and secologanin to form strictosidine by the enzyme strictosidine synthase.

hybridization, 32 hybrid crosses were cited that led to the accumulation of novel compounds.[150] Several recent reports confirm this remarkable potential, a few of which are highlighted here. $F_1$ poplar hybrids (*Populus deltoides* cv 'S9-2′ × *P. trichocarpa* cv 'V24′) have been shown to synthesize an unknown flavanone compound not detectable in either of the parents. Furthermore, a metabolite quantitative trait locus had been identified that was postulated to control the accumulation level of the unknown flavanone compound.[153] Novel flavonoid compounds have also been discovered in hybrids of *Ziziphus mauritiana* (jujube)[154] and *Zea mays* (maize).[155] In addition, three novel terpenoids were found in hybrids of *Artemisia tridentata* (sagebrush)[156] and two new odor compounds in *Ophrys* (orchid) hybrids.[157]

Even crossings within different accessions, cultivars, or genotypes of a single plant species can result in combinatorial biochemistry, as demonstrated by a striking metabolite study of a collection of *A. thaliana* L*er*/Cvi Recombinant Inbred Lines (RILs). Untargeted liquid chromatography–time of flight mass spectrometry-based metabolomics revealed that more than one third of the masses detected in the RILs were not detectable in either of the parental accessions. This qualitative variety was attributed to the recombination of the parental genomes.[158]

Nonetheless, in the above 'classical' breeding cases, interspecific crossing barriers represent a major pitfall, which can partially be overcome in laboratory practice by somatic hybridization methods. In symmetric somatic hybridizations, hybrids with the complete genomes of both parents are obtained through protoplast fusions, whereas in asymmetric somatic hybridizations, the donor genome is fragmented prior to protoplast fusion, yielding hybrids with the complete recipient genome, but a partial donor genome. An elegant example of a somatic hybridization experiment was aimed at overcoming crossing barriers between wild and cultivated species of potatoes, more specifically of the wild-type, non-tuberous *Solanum brevidens* and the commonly cultivated, tuberous species, *S. tuberosum*.[159] The major glycoalkaloids normally found in the parental strains are tomatine (aglycone tomatidine) in *S. brevidens* and solanidine in *S. tuberosum*. The somatic hybrids, however, possessed large quantities of a novel glycoalkaloid, demissidine, not found in any of the parents and four other unidentified compounds, along with all of the parental glycoalkaloids (Fig. 3).[159] In another example, the interspecific crossing barrier of *Dendranthema morifolium* (chrysanthemum) and *Artemisia vulgaris* (mugwort) was overcome *via* embryo rescue. The resulting intergeneric hybrids produced a small number of hybrid-specific terpenes, besides those present in one or both of the parental plants.[160]

### 3.5 Activation of silent metabolism

Recent advances in genomics indicate that the full metabolic capacity of organisms is far from comprehensively exploited. Bioinformatics has been used as a tool in microorganisms to identify a number of 'orphan' biosynthetic pathways, *i.e.* pathways of which the encoded natural products are unknown.[162,163] For example, in the actinomycete *Streptomyces coelicolor*, only three antibiotics and a spore pigment had been described before its complete genome had been sequenced. Predictions based on the genome sequence revealed 18 more gene clusters potentially encoding the biosynthesis of various secondary metabolites.[164] Such observations illustrate that the currently used natural product compendia do not represent the full potential of the producing organisms.[163,165]

More than microorganisms, plant cells are capable of simultaneously accumulating several thousands of secondary metabolites, of which the exact biochemical role remains mostly unknown to date. Despite the already dazzling number of accumulating compounds, it is becoming clear that in plants, like in microorganisms, many biosynthetic pathways are not fully active, or at least not detectable at the resolution of the present metabolite profiling technologies. Accordingly, many enzymes that have been annotated exist without any apparent endogenous substrate, pointing towards the occurrence of 'occult' metabolic capabilities in plants. This phenomenon is also defined as silent metabolism[151] and can be 'awakened' by either providing the appropriate substrate to the existing enzymes or, alternatively, by triggering the inactive parts of the biosynthetic pathways. As such, ectopic onset of silent metabolism might generate unprecedented opportunities to obtain new enzyme-substrate combinations and serve as a tool to obtain novel plant-derived compounds.

In literature, several silent metabolism cases in plants have been described. Only recently, *Arabidopsis* has been found capable of producing volatile terpenes in its flowers.[166] Before this discovery, the terpene-producing capacity of the plant was only suspected, based on the 32 predicted terpene synthase genes in its genome. Scanning of the genomic regions of predicted

 

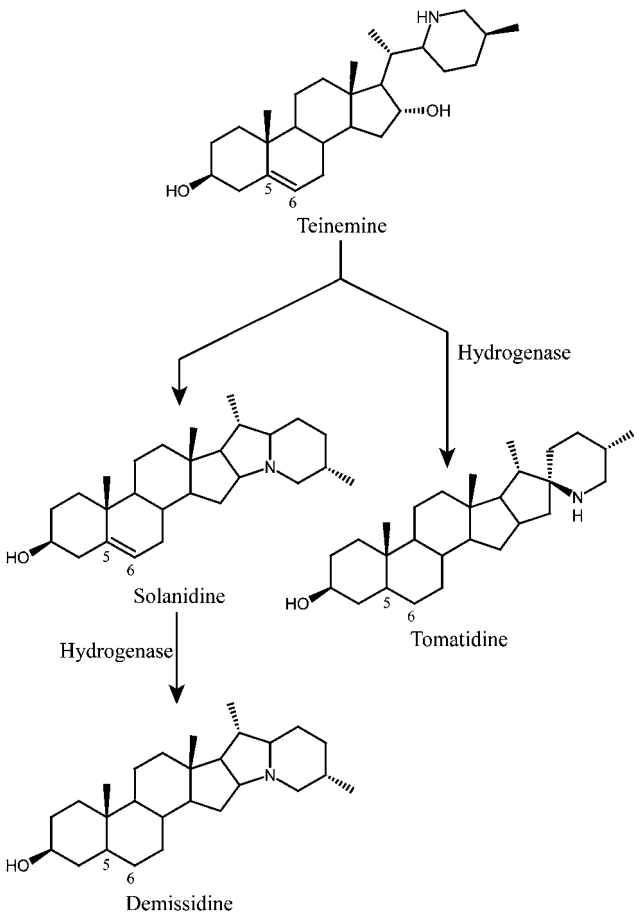


**Fig. 3** Biosynthesis of a novel alkaloid, demissidine, in somatic hybrid plants of *Solanum brevidens* and *Solanum tuberosum*. Both parental plants produce teinemine, whereas solanidine and tomatidine are only produced in *S. tuberosum* and *S. brevidens*, respectively.[159,161]

oxidosqualene cyclases revealed a gene operon that was exclusively expressed in *Arabidopsis* roots and potentially involved in plant secondary metabolism. Functional analysis of the oxidosqualene cyclase gene in yeast revealed that it catalyzed the formation of thalianol, a compound never reported in plants before. Subsequently, the occurrence of low amounts of the triterpene thalianol has been demonstrated in *Arabidopsis* roots.[167] Similarly, although no complex alkaloids have been detected in *Arabidopsis* yet, numerous homologs of enzymes involved in alkaloid biosynthesis in medicinal plants, including the tropane, pyrrolidine and indole alkaloid types, have been detected by genome scanning.[168] Thus, a well-studied plant, such as *Arabidopsis*, might also be capable of producing still undiscovered alkaloids, a hypothesis still needing to be substantiated.[169] More examples of silent metabolism have been recently described elsewhere.[151]

In a broader sense, tissue-, cell-, or even organelle-specific expression of genes in plants can be seen as silent metabolism as well. Activation of this alternative form of silent metabolism *via* ectopic expression can give rise to novel enzyme-substrate combinations, generating novel compounds. For instance, ectopic overexpression of the *Vitis vinifera* (grapevine) fruit-specific VvMybPA1 and VvMybPA2 MYB transcription factors activated protoanthocyanin biosynthesis in hairy roots. The MYB-overexpressing grapevine hairy roots accumulated one specific class of protoanthocyanins, the B-ring trihydroxylated units that are absent in control hairy roots.[170]

Finally, even (re)directing a metabolic pathway to a specific cellular compartment may activate silent metabolism and subsequent production of novel compounds. Targeting of a strawberry linalool/nerolidol synthase to the mitochondria instead of to the cytosol, its natural site, in transgenic *Arabidopsis*, produced small amounts of 4,8-dimethyl-1,3(*E*),7-nonatriene (*E*-DMNT), a new compound for *Arabidopsis*.[171] Hence, *Arabidopsis* contains the necessary enzymes to recognize and convert the product of the linalool/nerolidol synthase, (3*S*)-(*E*)-nerolidol, to *E*-DMNT, a signal molecule in plant defense that attracts enemies of herbivores.[171,172] Similarly, reallocating patchoulol synthase from its natural cytosolic location to the chloroplast of *Nicotiana tabacum* (tobacco) produced high amounts of patchoulol and an entire suite of novel sesquiterpenes.[173]

### 3.6 Combinatorial biosynthesis in plants

Ultimately, the successful development of a genuine combinatorial biosynthesis platform in plants will depend on the availability of an array of biosynthetic enzymes to ensure a broad substrate tolerance and a wide range of possible (un)natural substrates for the substrate-flexible enzymes. Meanwhile, in pioneering work on combinatorial biosynthesis in plants, new-to-nature TIA molecules have been generated successfully in transgenic *C. roseus* hairy roots. As mentioned above, strictosidine synthase, the enzyme that catalyzes the first committed step in the TIA biosynthesis, namely the condensation of tryptamine and secologanin to form strictosidine (Fig. 2), has been demonstrated to tolerate various modifications on the indole ring of tryptamine and to incorporate analogs containing benzofuran and benzothiophene heterocycles. Furthermore, the resulting unnatural condensation products are accepted by the downstream glucosidase, producing unnatural analogs of alkaloid intermediates as a consequence.[142,174] By introducing prokaryotic halogenases that chlorinate the indole ring of tryptophan into *C. roseus* hairy roots, chlorinated tryptophan was produced and used by the downstream TIA biosynthesis enzymes to form halogenated alkaloids *in planta*.[175] This finding is the first and, to the best of our knowledge, unique example of true combinatorial biosynthesis in plants reported to date, but it clearly underscores its huge potential.

## 4 Synthetic biology in 'model' plant systems

### 4.1 Pathway reconstitution

One of the major challenges in the development of plant-derived drugs is obtaining sufficient amounts of the compounds. In many, if not most, cases, the complex bioactive molecules are available only at very low concentrations *in planta*. Furthermore, either plant growth is very slow or the molecules are found in rare plant species, almost extinct, or recalcitrant to genetic transformation. As such, the establishment of combinatorial biosynthesis programs *in planta* might be compromised, at least in some plant species.

As an alternative approach, sets of plant biosynthesis enzymes can be produced in heterologous hosts, such as *Escherichia coli* or *Saccharomyces cerevisiae* (yeast), a technique referred to as heterologous biosynthesis or, more fashionably, 'synthetic biology'.[176] In addition, the production of the desired compounds can be increased in the heterologous host by combining heterologous biosynthesis with metabolic engineering of the pathways leading to the precursor molecules.[177–180] One of the most highly performing examples is the construction of a *S. cerevisiae* strain that was able to produce up to 100 mg $L^{-1}$ of artemisinic acid, a precursor molecule of the powerful anti-malarial drug artemisinin (Fig. 4). This endeavor was achieved by the co-expression of three genes of the plant *Artemisia annua* (sweet wormwood) in a yeast strain engineered for increased farnesyl pyrophosphate (FPP) production and decreased use of FPP for sterol biosynthesis.[181] The heterologous production of plant-derived natural products with significant medicinal potential in microorganisms have been reviewed recently,[10,176,182–185] therefore, we will not expand on this topic.

Plants with a fast, photosynthesis-driven accumulation of biomass and a large biosynthetic potential may serve as attractive hosts for synthetic biology programs as an alternative to the large-scale microbial production systems that often require expensive feedstocks.[173,186,187] Tobacco species are widely used not only as bioreactors for the production of pharmaceutically valuable recombinant proteins,[188] but also as a production vehicle for small molecules. Indeed, currently, the recreation of the biosynthesis pathway of several pharmacologically important natural products in tobacco is being explored, as illustrated by the reconstitution of the artemisinin biosynthetic pathway. In a pioneering study, the enzyme catalyzing the first committed step of the artemisinin biosynthesis, amorpha-4,11-diene synthase (ADS) (Fig. 4), of *A. annua* was introduced into *N. tabacum*, resulting in the accumulation of minute amounts (0.2–1.7 μg $kg^{-1}$ fresh weight) of amorpha-4,11-diene in the transgenic plants.[189] Co-expression of FFP synthase (FPS) and ADS in the plastids instead of the cytosol improved amorpha-4,11-diene accumulation in tobacco more than 1000-fold, up to 25 mg $kg^{-1}$ fresh weight.[173] The discovery of additional genes in the artemisinin biosynthesis pathway (Fig. 4)[181,190,191] will further help reconstruct the pathway in tobacco. In one study, CYP71AV1 and mitochondria-targeted FPS and ADS were transiently synthesized in *N. benthamiana* leaves by agro-infiltration. Surprisingly, no oxidation products of amorpha-4,11-diene were detected in the infiltrated leaves, but artemisinic acid-12-β-diglucoside was detected instead, indicating that the produced artemisinic acid was metabolized by glycosyl transferases endogenous to *N. benthamiana*.[186] In another study, heterologous production of *A. annua* ADS and CYP71AV1 in *N. tabacum* led to the accumulation of amorpha-4,11-diene and artemisinic alcohol, but not of artemisinic acid. When artemisinic aldehyde Δ11(13) double-bond reductase (DBR2) was co-transformed in combination or not with aldehyde dehydrogenase 1 (ALDH1), additional accumulation of dihydroartemisinic alcohol was observed, but neither artemisinic acid nor dihydroartemisinic acid were found.[192] The absence of the sesquiterpenoid acids was attributed to the cellular environment that might favor reduction of the produced sesquiterpenoid aldehydes, but no possible

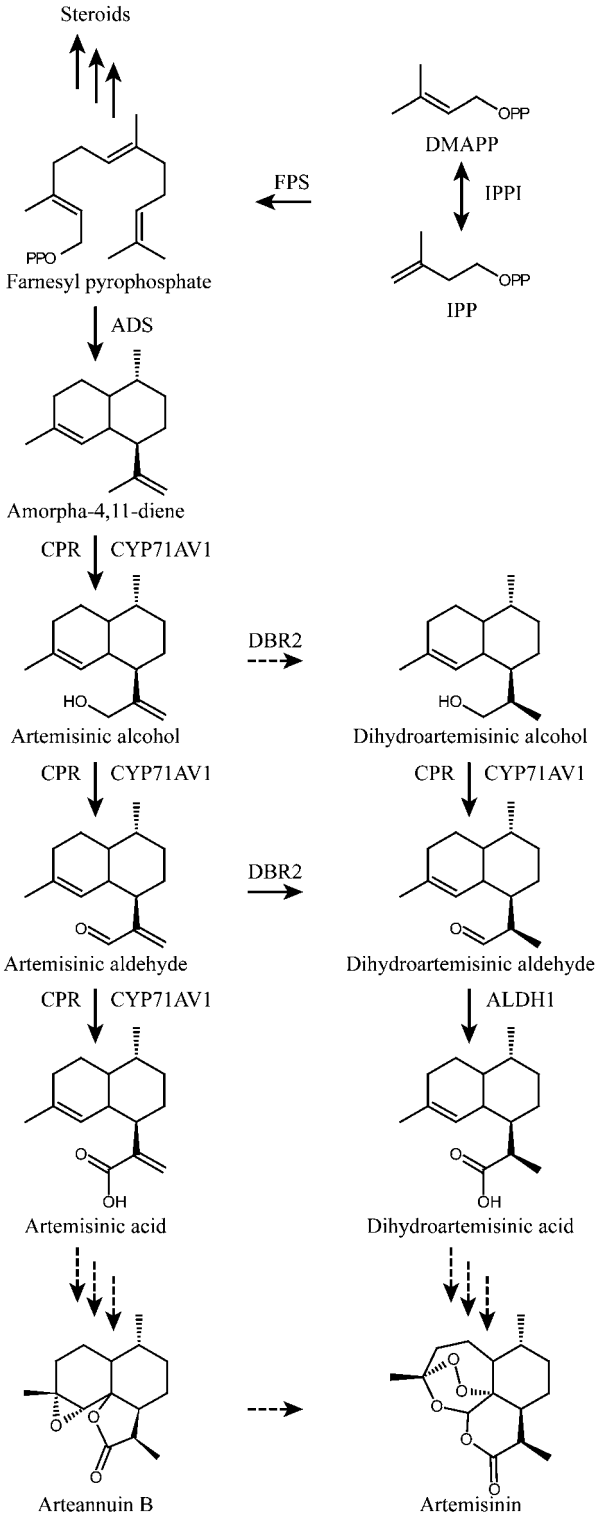


**Fig. 4** The artemisinin biosynthetic pathway. ADS: amorpha-4,11-diene synthase; ALDH1: aldehyde dehydrogenase 1; CPR: cytochrome P450 reductase; CYP71AV1: amorpha-4,11-diene monooxygenase; DBR2: artemisinic aldehyde Δ11(13) double-bond reductase; DMAPP: dimethylallyl pyrophosphate; FPS: farnesyl pyrophosphate synthase; IPP: isopentenyl pyrophosphate; IPPI: IPP isomerase. Dashed arrows indicate steps for which no enzymes have been identified.

accumulation of sesquiterpenoid acid glycosides was reported, in contrast to the study in *N. benthamiana*.[186,192]

Heterologous biosynthesis of several other types of natural products has been tried in tobacco as well. By the simultaneous expression of up to 13 genes from the *Arabidopsis* glucosinolate biosynthesis pathway, several types of glucosinolates were produced in *N. benthamiana*, a species that does not naturally produce them.[193–195] Several attempts have been undertaken to engineer monoterpene biosynthesis in *N. tabacum*. Limonene synthase of *Perilla frutescens* (green shiso) synthesized limonene, of which the amounts varied depending on the subcellular targeting of the transgene product, with the highest in plastids.[196] Similarly, tobacco plants transformed with three different monoterpene synthases from *Citrus limon* (lemon) emitted β-pinene, limonene, γ-terpinene and a number of side products in addition to the terpenoids emitted by wild-type plants.[197] Supertransformation of this tobacco line with limonene-3-hydroxylase from *Mentha spicata* (spearmint) resulted in the emission of (+)-*trans*-isopiperitenol and derivatives, such as 1,3,8-*p*-menthatriene, 1,5,8-*p*-menthatriene, *p*-cymene and isopiperitenone.[198]

Other plant species can serve as heterologous hosts as well. In one of the most famous examples, β-carotene (provitamin A) was biosynthesized in the endosperm of *Oryza sativa* (rice) grains by heterologous expression of several bacterial and plant-derived carotenoid biosynthesis genes. The resulting β-carotene-producing 'Golden Rice' could help combat vitamin A deficiency,[199,200] a common problem that yearly claims the lives of 0.6 million children less than 5 years old in developing countries.[201]

### 4.2 Co-expression of multiple genes

Most natural product biosynthesis pathways consist of an assembly of numerous enzymatic steps. Therefore, co-expression of multiple genes is an absolute prerequisite for synthetic biology in any (plant) host, but gene stacking remains one of the major technical challenges.[202] Conventional iterative gene stacking can be obtained by crossing homozygous transgenic lines or by sequential transformation of transgenic lines with additional transgenes. However, in the latter approaches, the different transgenes are usually all driven by their own promoter and are inserted randomly into the genome, leading to uncoordinated gene expression and potential transgene segregation in subsequent generations.[203] Furthermore, the need for multiple breeding generations converts these methods into extremely time-consuming enterprises.[204]

Alternatively, co-transformation allows the simultaneous introduction of two or more transgenes into the plant host in one single transformation event and is faster than iterative gene stacking. The co-introduced transgenes tend to be integrated on the same chromosomal locus, thus inherently avoiding segregation and large variations in transgene expression.[202,203] Traditionally, co-transformation makes use of different plasmids, each bearing a single gene (unlinked co-transformation). In one single transformation event, multiple T-DNAs are introduced into the host and integrated on the same chromosomal locus. However, unlinked co-transformation could lead to very complex loci consisting of multiple tandem or inverted T-DNA repeats, thereby increasing the risk of gene silencing.[202] To overcome these drawbacks, multiple genes can be included within a single T-DNA (linked co-transformation) with each gene driven by its own promoter. To avoid homology-dependent gene silencing, this method requires a battery of different promoters.[205] Moreover, the method becomes inefficient with numerous genes, because large (≥50 kb) binary vectors become unstable and undergo spontaneous deletions.[204]

Ultimately, co-transformation could be carried out with multicistronic expression systems. In plastids, which evolved from bacteria, the operon system for gene regulation is preserved and can be targeted for the coordinated expression of several genes as a single, polycistronic mRNA. Despite the potential of the technique, its application is limited, mainly due to the technological, species-bound bottlenecks in plastid engineering.[204,206,207] Alternatively, viral mechanisms could be employed to achieve multicistronic expression in plants. Internal ribosome entry sites (IRES) are nucleotide sequences that allow translation initiation in the middle of an mRNA sequence.[208] As such, two or more genes from a single T-DNA can be expressed coordinately. IRES are large sequences (500 bp) and translation of the second open reading frame (ORF) is much lower than that of the first ORF.[203] An alternative to IRES for the co-expression of multiple proteins in one single transformation step is the short viral 2A peptide (16–20 amino acids). In this system, a single gene encoding multiple proteins, linked by 2A sequences, is transcribed from a single promoter. During translation, the polyprotein self-processes, releasing each protein as a discrete translation product C-terminally fused to 2A.[203,209,210] Recently, constructs have been created to express two carotenoid biosynthesis genes in rice, linked either *via* the 2A or IRES systems. Analysis of the endosperm of the transgenic rice revealed that the carotenoid levels were ninefold higher in the 2A system, indicating its potential for multigene stacking.[205] The 2A system has also been successfully applied for the one-step transfer of the last three steps of the benzylglucosinolate pathway from *Arabidopsis* to tobacco.[211]

The above-mentioned methods, however, are still insufficient for the simultaneous transfer of complete biosynthesis pathways and suffer from random integration into the genome and from gene silencing.[212] A breakthrough technology in this field could be the use of plant artificial chromosomes or minichromosomes, which would allow the simultaneous introduction and expression of many genes from a synthetic chromosome.[212–215] Minichromosomes are obtained by trimming the arms from endogenous plant chromosomes. By creating additional restriction sites, large inserts can be introduced into the chromosomes. Although proof of concept of the technology has been achieved in maize,[215,216] further research is required to apply this promising, but technically challenging technology in other plant systems.

## 5 Bioactive terpenoid indole alkaloids

To highlight the relevance of structural variety for bioactivity in terms of drug potential, two classes of plant natural products, the TIAs and the saponins will be discussed. Alkaloids are nitrogen-containing molecules with a low molecular weight and are mostly derived from the amino acids Phe, Tyr, Trp, Lys and Arg. Approximately 20% of all plant species produce alkaloids. Traditionally, humans utilize the biological potency of alkaloids

for hunting and treatment of diseases, but also for less noble purposes, such as executions and warfare.[217]

An important set of alkaloids are the TIAs, a class of structurally diverse molecules of which many have interesting pharmaceutical activities. TIAs are mainly found in plants belonging to the families Apocynaceae, Icacinaceae, Loganiaceae, Nyssaceae and Rubiaceae and are derived from the common precursor molecule strictosidine, a condensation product of the terpenoid moiety secologanin and the tryptophan-derived indole moiety tryptamine (Fig. 2). The structural variability of TIAs in the different TIA-producing plant species is due to species-specific conversions of the common strictosidine precursor molecule that are either enzymatically catalyzed or occur through spontaneous chemical reactions.[174,218] These species-specific decorations have profound effects, not only on the final complexity of the structure itself, but also on its bioactivity, setting an example of the enormous resources available for combinatorial biochemistry, but still hardly explored within the plant kingdom. The structure and bioactivity of the most potent TIA drugs currently known are briefly described below.

The predominantly investigated TIA-producing plant species is *C. roseus* that in its various organs can produce over 120 different TIAs,[35,218] of which some with therapeutic activities used in pharmaceuticals. Vinblastine and its oxidized form vincristine (Fig. 5), two minor constituents of the complex *C. roseus* TIA mixture, are commercially used as anti-cancer drugs. These antineoplastic compounds bind tubulin, thereby inhibiting the assembly of microtubule structures and disrupting that of the mitotic spindle and the kinetochore, ultimately leading to an arrest in the metaphase of rapidly dividing cells, including cancer cells.[35,219] Vinblastine (brand names, *e.g.* Velban® and Velbe®) is mainly administered to treat lymphoma, but is also applied to treat bladder cancer, testicular cancer or Kaposi's sarcoma, whereas vincristine (brand names, *e.g.* Oncovin® and Vincasar Pfs®) is used to treat lymphoma, leukemia, and other types of cancer. *C. roseus* also accumulates the antihypertensive and antiarrhythmic TIA compounds ajmalicine (or raubasine; brand names, *e.g.* Circolene®, Hydrosarpan®, Isoarteril® and Lamuran®) and serpentine (Fig. 5).[35,174,220]

Another important genus of TIA-producing plants is *Rauwolfia*, in which the most pharmacologically important TIAs are reserpine, ajmaline, yohimbine and ajmalicine (Fig. 5). Reserpine (brand name Harmonyl®) is administered as an antihypertensive drug to control high blood pressure and as an antipsychotic to treat, for instance, schizophrenia, but is nowadays rarely used because of its numerous side-effects and the development of drugs with better efficacy. Ajmaline is an antiarrhythmic drug that, due to its short half-life, is very useful for acute intravenous treatment of abnormal heart rhythms, such as ventricular tachycardia and atrial fibrillations in patients with the Wolff–Parkinson–White syndrome.[221] Furthermore, it is also the drug of choice to search for ST elevations in electrocardiograms of patients suspected of suffering from the Brugada syndrome,[222] a genetic disease causing unanticipated ventricular fibrillation, which can lead to cardiac arrest and death.[223] Yohimbine (brand name Procomil®) is a psychoactive drug with stimulant and aphrodisiac effects. Yohimbine hydrochloride has potential clinical applications for the treatment of erectile dysfunction,[224,225] and can also be used as a mydriatic drug to aid eye examinations.[226] *Rauwolfia* species produce yohimbine only in limited amounts, hence the main natural source of the molecule is the African tree *Pausinystalia yohimbe* (yohimbe).[224]

The Chinese 'happy tree' *Camptotheca acuminata* was the first source of another important TIA, the antineoplastic compound camptothecin,[227] which was found later in a few other plant species as well.[228–235] Camptothecin (Fig. 5) binds to mammalian DNA topoisomerase I, thereby interfering with the breakage–reunion reaction of topoisomerase I *via* the stabilization of a reaction intermediate, namely the cleavable complex. The S-phase-specific toxicity of camptothecin is caused by the interaction between the stabilized topoisomerase I cleavable complexes and the moving replication forks, provoking an irreversible fork arrest and ultimately highly toxic double-strand DNA breaks, with cell death as a consequence.[236–241] Nowadays, camptothecin and its more water-soluble derivatives, topotecan and irinotecan, are common anticancer agents.[241] Topotecan hydrochloride (Hycamtin®) is mainly used for treatment of ovarian and lung cancer and CPT-11 or irinotecan hydrochloride (Camptosar® and Campto®) for colon cancer, frequently in combination with other types of chemotherapeutical drugs.

Other important TIAs include strychnine and quinine (Fig. 5). Strychnine is a poison produced by *Strychnos nux-vomica* (poison nut) that provokes muscular convulsions, eventually leading to death through asphyxia or sheer exhaustion and is used as a pesticide against small vertebrates, such as birds and rodents. Quinine is a bitter-tasting TIA with antipyretic, antimalarial, analgesic and anti-inflammatory activities. It is extracted from the bark of the *Cinchona* tree and has long been the drug-of-choice to treat malaria. Addition of quinine gives the distinctive bitter taste of tonic water.

To date, most advances in molecular characterization of the complex and strictly regulated TIA biosynthesis pathway have been made in *C. roseus*. The first step in the biosynthesis of all TIAs is the condensation of the precursor molecules tryptamine and secologanin by the enzyme strictosidine synthase. The indole moiety tryptamine is derived from the primary metabolite tryptophan *via* a single enzymatic step, the decarboxylation of the amino acid by the enzyme tryptophan decarboxylase (Fig. 2). The biosynthesis of the terpenoid (iridoid) moiety secologanin consists of several enzymatic steps, of which only a few are characterized, and starts with the hydroxylation of the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway-derived monoterpenoid geraniol.[242,243]

After the condensation of secologanin and tryptamine to strictosidine, the various TIA structures are generated through species-specific enzymatic reactions and spontaneous chemical modifications.[218] Because of the complexity of the pathway, most of the enzymes involved in the biosynthesis of the various TIA backbones are still unknown. Nonetheless, several subpathways, such as the six-step enzymatic conversion of tabersonine to vindoline in *C. roseus*[244] or the 10-step ajmaline biosynthesis in *R. serpentina*[245] have already been elucidated.

## 6 Bioactive triterpene saponins

Saponins constitute a structurally diverse group of amphipathic glycosides with a steroid, steroidal alkaloid, or triterpenoid aglycone backbone. The aglycone or sapogenin can be covalently

 

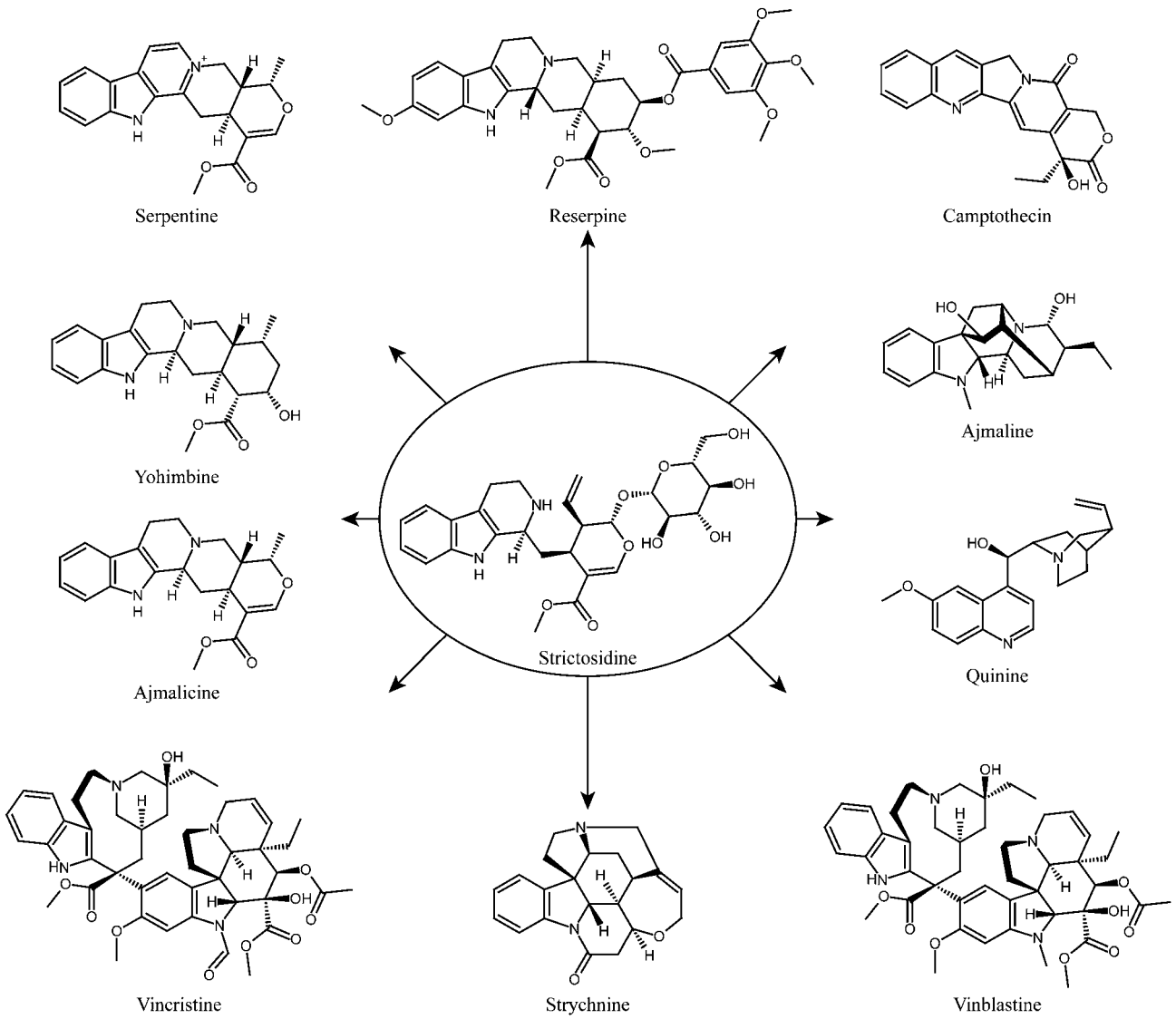


**Fig. 5** Overview of the molecular structures of pharmacologically important TIAs derived from strictosidine.

linked to one (monodesmosidic) or more (di- or tridesmosidic) sugar chains *via* a glycosidic bond. Saponins are surface-active agents that, when dissolved in water, form a colloidal solution that foams upon shaking. Accordingly, they are used as emulsifier and foaming agents in the food and beverage industries. Importantly, many saponins also possess pharmacological properties and are, therefore, commonly used in phytotherapy and cosmetics.[246]

Here, we will focus on the structure–activity relationships of only one type of saponins, the triterpene saponins. All terpenoids are derived by repetitive fusions of a branched five-carbon unit, isopentenyl diphosphate (IPP). The number of fused IPP units determines the classification of the terpenoids. Triterpenoids, to which also the steroids belong, contain 30 carbon atoms. The precursor $C_{30}$ unit, called squalene, is generated by the junction of two $C_{15}$ units, or farnesyl diphosphate, each of which consists of three IPP units[247] and is usually oxidized to its 2,3-epoxide, 2,3-oxidosqualene. The first committed step in the biosynthesis of triterpene saponins is the cyclization of 2,3-oxidosqualene. This step forms the branch point between the primary sterol and the secondary saponin metabolism (Fig. 6). Predating the emergence of plants, cycloartenol synthase (CAS), involved in primary steroid metabolism, is the ancestor enzyme of all plant oxidosqualene cyclases (OSCs) involved in secondary metabolism (Fig. 6).[248] Most plants possess OSCs that catalyze the formation of different triterpene skeletons. For instance, the genome of *Arabidopsis* contains up to 13 predicted OSC-encoding genes, producing various triterpenes of which the function *in planta* remains mostly unknown.[167] As such, nearly 100 distinct non-steroidal triterpene skeletons have been described in plants.[248] Usually, two distinct types of additional modifications of the cyclization products complete the saponin biosynthesis and dramatically increase the structural diversity. Firstly, cytochrome P450-mediated oxidations of the cyclization products give rise to different sapogenins. For instance, in the model plant *Medicago truncatula* (barrel medic), oxidation of the oleanane-type cyclization product β-amyrin leads to at least 10 different sapogenins[249] and in the medicinal plant *Panax ginseng* (Asian ginseng), two different saponin backbones, protopanaxadiol (PPD) and protopanaxatriol (PPT) are formed by oxidation of

the dammarane-type cyclization product dammarenediol[250] (Fig. 6). Subsequently (and/or concomitantly), the different sapogenins can be glycosylated at different positions. The attached sugar chains can be linear or branched and can contain different monosaccharides, such as glucose, galactose, glucuronic acid, xylose, or rhamnose. Other, less common, modifications, such as acylations, can further increase the structural variability of the saponins. Even within a single plant species, a very rich mixture of saponins can occur. In *P. ginseng* alone over 40 ginsenosides have been reported already, some of which have been discovered only recently.[36,251]

One of the most promising pharmacological activities of triterpenoid saponins is their ability to activate the mammalian immune system, making them candidate vaccine adjuvants.[252,253] QS-21, a saponin isolated from the bark of *Quillaja saponaria* (soapbark), has been evaluated in several clinical trials as a candidate adjuvant[253] and is far more effective than the currently accepted adjuvants for human vaccines, calcium and aluminium salts,[254] but toxicity, hemolytic side-effects and instability in the aqueous phase limit its current use.[252,253,255,256] Some of these undesired effects might be overcome by insights into the structure–activity relationship of saponins. A lot of adjuvant compounds have an amphipathic structure. Saponins also, with their lipophilic aglycone backbone and hydrophilic sugar chains have amphipathic properties. A study on the correlation between adjuvant activity and the amphipathic structure of saponins has revealed a clearly increasing adjuvanticity with an increasing amphipathicity of the molecules,[257]



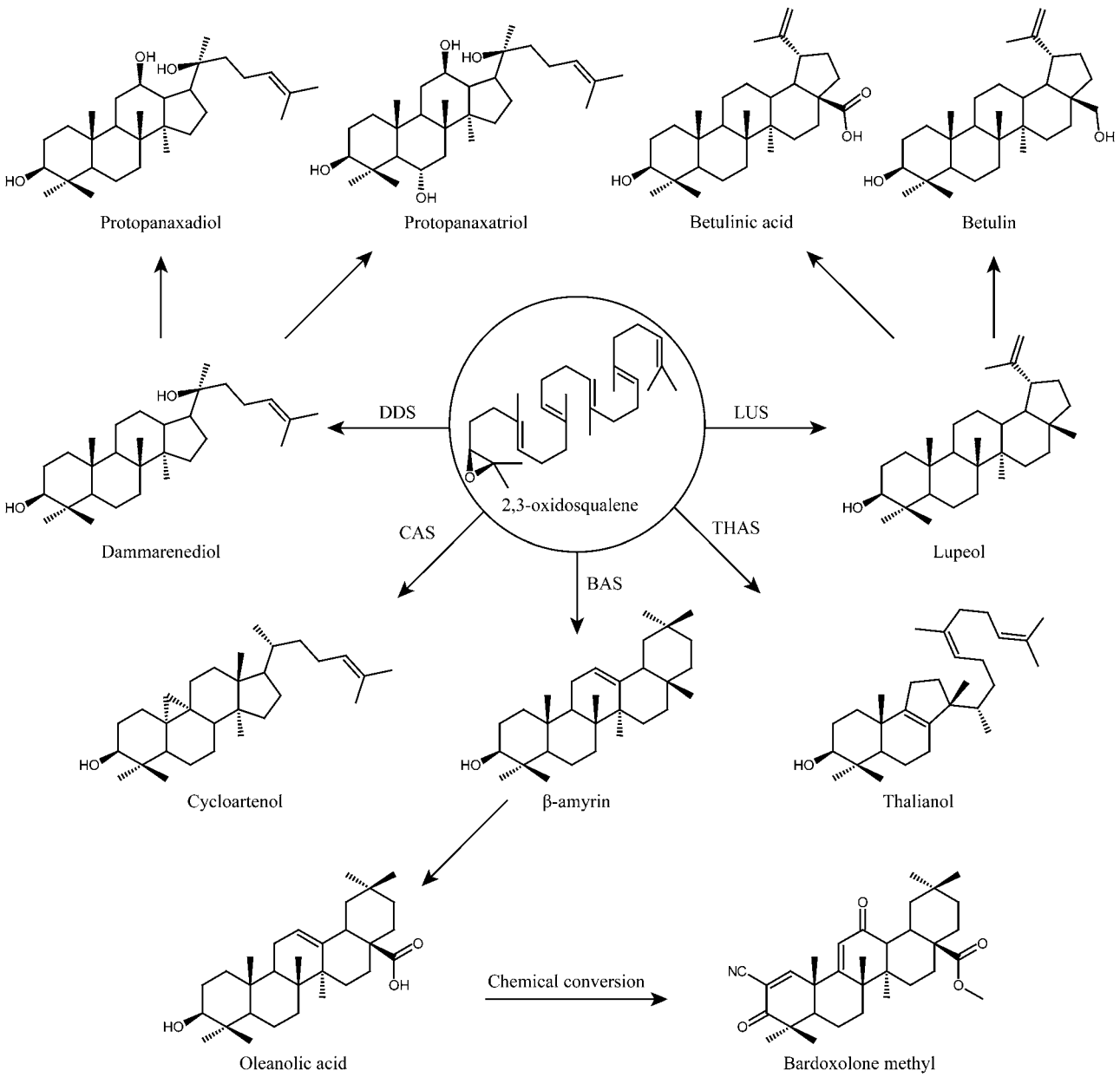


**Fig. 6** Biosynthesis of triterpene saponin aglycones from the common precursor 2,3-oxidosqualene. Following cyclization of 2,3-oxidosqualene by specific oxidosqualene cyclases, the cyclization products are oxidized at various positions. BAS, β-amyrin synthase; CAS, cycloartenol synthase; DDS, dammarenediol synthase; LUS, lupeol synthase; THAS, thalianol synthase.

 

whereas hemolytic effects are considered to be influenced by the affinity of the lipophilic aglycone to cholesterol in the cell membranes. The degree of affinity depends on the type of aglycone and side chains occurring on the aglycones.[253]

The immunostimulating complex (ISCOM®)-based vaccines also make use of the adjuvant properties of saponins, while overcoming their hemolytic effects.[258–260] Iscoms are spherical, cage-like structures, typically 40 nm in diameter, and form spontaneously when saponin, cholesterol and phospholipid are mixed under controlled conditions. Traditionally, the ISCOM®-based vaccines also use 'Quil A' saponin that is a crude saponin extract from the bark of *Q. saponaria*.[258] However, because the Quil A extract is a heterogeneous mixture of dozens of closely related saponins with different chemical or biological activities, variations in the composition of the mixture may lead to unpredictable *in vivo* effects. Hence, the current tendency is to use more purified fractions of the Quil A saponin extract, such as QS-21.[252,259,261] Several other medicinal plants contain structurally different saponins with adjuvant activities[253,262] and novel immunostimulatory nanoparticles could be developed by incorporation of these saponins in iscom-like nanostructures. Ginsomes, for instance, are nanoparticles containing saponins from the medicinal plant *P. ginseng* and enhance the IgG responses in mice, when they are administered together with the antigen, compared to administration of the antigen alone.[263]

Triterpene saponins can also serve in cancer treatments. One of the most studied triterpene molecules in this context is a compound isolated from the bark of *Betula alba* (white birch), betulinic acid (Fig. 6), which was shown to be a melanoma-specific cytotoxic agent able to inhibit tumor growth *in vitro* and *in vivo*, without systemic toxicity.[264–266] Betulinic acid affects the mitochondrial membrane potential of cancer cells, releasing mitochondrial proteins, such as cytochrome c, in the cytosol and triggering apoptosis in the cancer cells.[265,267] In contrast to other antineoplastic drugs, betulinic acid has no cytotoxic effects on nonmalignant cells, but the exact mechanism for the selectivity of betulinic acid toward malignant cells remains to be discovered.[266] Betulin (Fig. 6), which differs from betulinic acid only on position 28, can also induce apoptosis in cancer cells,[268–270] but its mode of action is different, despite its almost identical structure and similar effects on cancer cells. Unlike betulinic acid, betulin fails to trigger the release of cytochrome c from isolated mitochondria, suggesting another mechanism activating the intrinsic apoptotic pathway.[270]

In addition to adjuvant and anti-cancer properties, a plethora of other bioactivities have been attributed to triterpenoid saponins.[253,271–274] For instance, ginsenosides from *P. ginseng* alone are pharmacologically significant as anti-cancer, anti-diabetic, neuroprotective, cardioprotective, radioprotective, hypolipidemic, anti-amnesic and anti-aging agents.[37,250,275–277]

All of the pharmacologically potent triterpenes described above are naturally occurring compounds. Chemical modification of the triterpene scaffolds has led to the synthesis of semi-synthetic triterpenoids with improved pharmacological properties, when compared to the parent compounds. The most promising candidate drug of this sort is bardoxolone methyl, a chemical derivative of oleanolic acid (Fig. 6). Recently, bardoxolone methyl has been positively evaluated in a Phase-IIB clinical trial, in which its oral administration reduced the stage of disease and improved measures of kidney function in patients with chronic kidney disease (CKD) and Type 2 diabetes (see http://www.abbott.com/). Bardoxolone methyl activates the transcription factor Nrf2, inducing the antioxidant and detoxification enzymes of the phase 2 response.[278,279] This increased cellular antioxidant content prevents the activation of inflammatory signaling pathways, such as NF-κB and chronic "metabolic inflammation" that promotes Type 2 diabetes and CKD. Besides bardoxolone methyl, several other synthetic triterpenoids trigger Nrf2. Essential features required for the activation of Nrf2 are the presence of Michael acceptor groups (enones) on both the A and C rings of the molecules and a nitrile group at the $C_2$ position.[279] Nrf2 activation may also be useful for treatment of several types of cancer. Tumor-associated immune suppression is mediated by reactive oxygen species, contributes to tumor growth and limits the activity of cancer immunotherapy. Accordingly, blocking the tumor-associated immune suppression by treatment with bardoxolone methyl improved the immune response in tumor-bearing mice and cancer patients.[280]

## 7 Outlook: gene discovery for combinatorial biosynthesis of plant natural products

Plant-derived natural products are and will undoubtedly remain very important for various drug discovery programs. The structural diversity of these complex molecules may be enhanced with various combinatorial biosynthesis techniques. The low production amounts *in planta* may be tackled with the heterologous expression of plant genes in microorganisms, such as *E. coli* or yeast. Ultimately, plants themselves might be metabolically engineered. To exploit the full potential of plant natural products, knowledge of the genes involved in the biosynthesis of the metabolites is essential, but, until now, most, if not all, of the biosynthesis pathways remain incompletely understood.

Over the past five years, the commercialization of several next-generation sequencing techniques has revolutionized functional genomics, such as pyrosequencing in the 454 system,[281] bridge PCR in the Solexa technology,[282] sequencing by ligation in the AB SOLiD and Polonator platforms,[283,284] single molecule DNA sequencing in the HeliScope platform,[285] or, more recently, the application of single-molecule real-time DNA sequencing in the PacBio RS system.[286,287] This tremendous progress now brings the whole genome or transcriptome of any plant species within reach and will undoubtedly greatly accelerate gene discovery for secondary metabolite pathways. Simultaneously, the enormous amount of data generated by such next-generation sequencing platforms demands efficient strategies to identify and select the relevant biosynthetic genes from the pools of thousands of sequenced genes or, at least, requires efficient tools to exploit them. One manner to solve this problem would be the use of elicitors, such as the phytohormone jasmonate. Elicitors induce the production of a compound of interest in plants or plant cell cultures that, most often, is caused by the transcriptional activation of the genes involved. Hence, comparison of transcriptomes at various stages before and after elicitor treatment might pinpoint the desired subsets of the total gene pools as candidate genes responsible for the biosynthesis of the secondary metabolites.[161,288] Several studies have already demonstrated the feasibility of this approach. For instance, treatment of tobacco

Bright Yellow 2 (BY-2) cell cultures with jasmonate induces nicotine biosynthesis in the cells. Genome-wide transcript profiling of the jasmonate-elicited cell cultures established a tobacco gene compendium[289] from which several new proteins, including enzymes, regulators and transporters involved in nicotine biosynthesis were identified in subsequent functional screens.[290–294] In a similar series of studies in the triterpene saponin-producing model legume, *M. truncatula*, the response was investigated of the metabolome and transcriptome to different elicitors.[295] By analysis of unknown genes co-regulated with known triterpene saponin biosynthesis genes, glucosyltransferases involved in triterpene saponin biosynthesis were identified and characterized.[296,297] The identification of such sets of candidate genes involved in the biosynthesis of plant natural products will allow a high-throughput approach for combinatorial biosynthesis in plants or heterologous microbial systems. Alternatively, 'metagenomics' approaches may be applied to screen for plant enzymes that can catalyze the synthesis of new-to-nature compounds. Indeed, capturing the genetic resources of complex microbial communities in metagenome libraries already allowed the discovery of a wealth of new enzymatic diversities, of entirely new sequence classes and novel functionalities.[298] Hence, we can conclude that, thanks to the recent technical advances in gene discovery and functional genomics, the large-scale implementation of different combinatorial biosynthesis techniques in plants will offer a bright green future for drug discovery in plant-derived natural products.

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