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Feature Review

Phytocannabinoids: Origins and Biosynthesis

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Phytocannabinoids are bioactive natural products found in some flowering plants, liverworts, and fungi that can be beneficial for the treatment of human ailments such as pain, anxiety, and cachexia. Targeted biosynthesis of cannabinoids with desirable properties requires identification of the underlying genes and their expression in a suitable heterologous host. We provide an overview of the structural classification of phytocannabinoids based on their decorated resorcinol core and the bioactivities of naturally occurring cannabinoids, and we review current knowledge of phytocannabinoid biosynthesis in *Cannabis*, *Rhododendron*, and *Radula* species. We also highlight the potential *in planta* roles of phytocannabinoids and the opportunity for synthetic biology approaches based on combinatorial biochemistry and protein engineering to produce cannabinoid derivatives with improved properties.

Highlights

Phytocannabinoids are bioactive terpenoids that were thought to be exclusive to *Cannabis sativa*, but have now also been discovered in *Rhododendron* species, some legumes, the liverwort genus *Radula*, and some fungi.

Many cannabinoids display promising non-hallucinogenic bioactivities that are determined by the variable nature of the side chain and prenyl group defined by the enzymes involved in their synthesis.

The biosynthesis of cannabinoids in *C. sativa* is fully elucidated, whereas the pathways in *Rhododendron* and *Radula* have only recently gained research attention.

Cannabinoid biosynthesis is highly modular, enabling use of the modules identified in synthetic biology-based combinatorial approaches, as demonstrated by the generation of new-to-nature cannabinoids in *Saccharomyces cerevisiae*.

The ecological functions of cannabinoids include protection against UV light and desiccation, as well as in plant defense.

Phytocannabinoids

The term **phytocannabinoid** (see Glossary, also cannabinoid) defines **meroterpenoids** with a resorcinyl core typically decorated with a *para*-positioned isoprenyl, alkyl, or aralkyl side chain [1]. The alkyl side chain typically contains an odd number of carbon atoms, where orcinoids contain one carbon, varinoids three, and olivetoids five. Cannabinoids with an even number of carbon atoms in the side chain are known but rare. The term cannabinoid generally refers to molecules with a characteristic chemical structure; however, the term may also refer to pharmacological ligands of human endocannabinoid receptors [2]. In this review we use the chemical definition of cannabinoids.

Phytocannabinoids occur in flowering plants, **liverworts**, and fungi (Figure 1, Key Figure). They were first isolated from *Cannabis sativa* L. (Cannabaceae), a plant with a long and controversial history of use and abuse [3]. The mammalian brain has receptors that respond to compounds found in *C. sativa*. Accordingly, these receptors were named cannabinoid receptors (CB_x) and are the basis of the **endocannabinoid system**. Studies in human and animals demonstrated that the endocannabinoid system regulates a broad range of biological functions, including memory, mood, brain reward systems, and drug addiction, as well as metabolic processes such as lipolysis, glucose metabolism, and energy balance [2]. More than 113 different cannabinoids have been isolated from *C. sativa* and these are classified into distinct types: cannabigerols (CBGs), cannabichromenes (CBCs), cannabidiols (CBDs), (–)-Δ^9-*trans*-tetrahydrocannabinols (Δ^9-THCs), (–)-Δ^8-*trans*-tetrahydrocannabinols (Δ^8-THCs), cannabicyclols (CBLs), cannabielsoins (CBEs), cannabinols (CBNs), cannabinodiols (CBNDs), cannabitriols (CBTs), and the miscellaneous cannabinoids (Figure 2) [4]. *C. sativa* predominantly produces alkyl type cannabinoids that carry a monoterpene isoprenyl moiety (C10) and a pentyl side chain (C5) [1]. The most abundant constituents are *trans*-Δ^9-THC, CBD, CBC, and CBG, together with their respective acid forms (Δ^9-THCA, CBDA, CBCA, and CBGA) [5]. Cannabinoid biosynthetic pathways typically generate acidic cannabinoids (C22, 'pre-cannabinoids') as the final products [6]. Further modified cannabinoids are spontaneous breakdown or conversion products resulting from, for example, oxidation, decarboxylation and cyclization, or are formed during isolation [7]. These conversions take place because of the poor oxidative stability of alkylic cannabinoids, in particular Δ^9-THC [1]. *C. sativa*

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Key Figure

Illustration of the Structural Characteristics of Cannabinoids Found in Different Plant Species

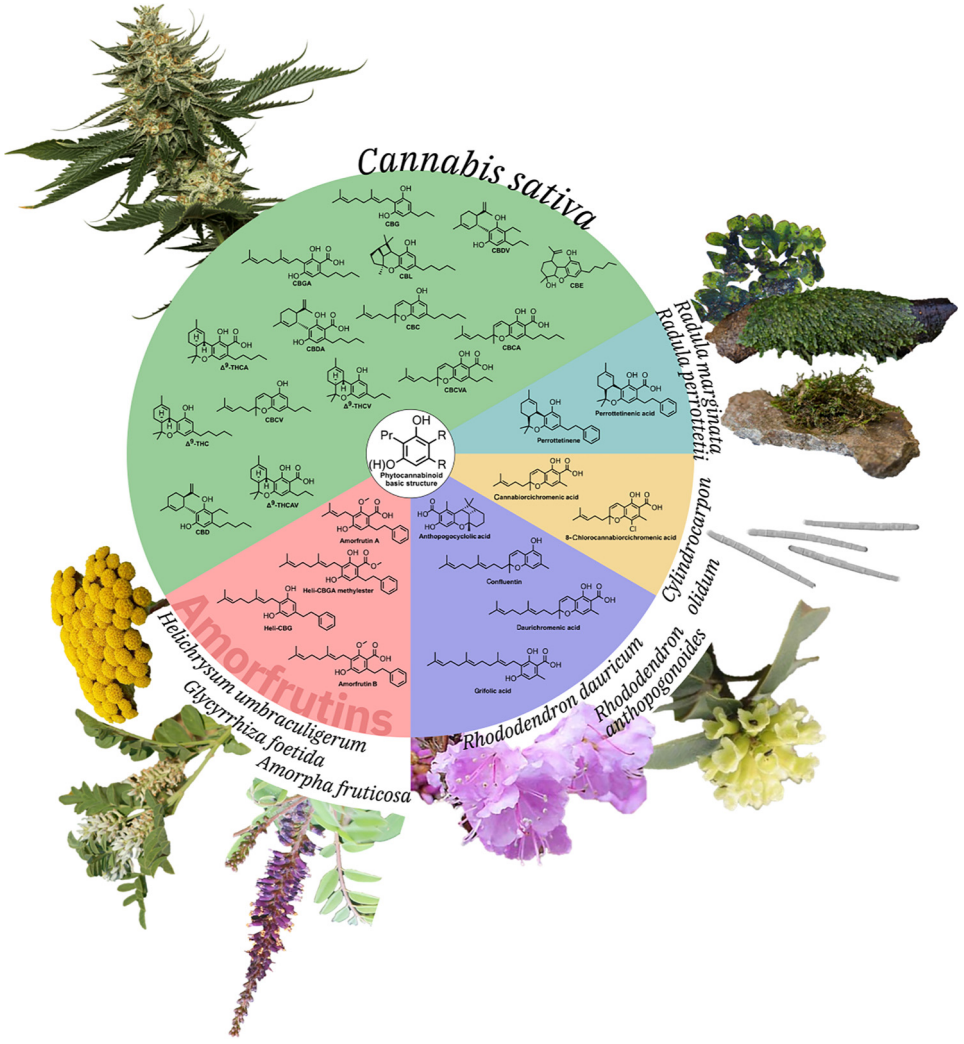


Figure 1. Abbreviations: CBC, cannabichromene; CBCA, cannabichromenic acid; CBCV, cannabichromevarine; CBCVA, cannabichromevarinic acid; CBD, cannabidiol; CBDA, cannabidiolic acid; CBDV, cannabidivarine; CBE, cannabielsoin; CBG, cannabigerol; CBGA, cannabigerolic acid; CBL, cannabicyclol; Δ^9-THC, Δ^9-tetrahydrocannabinol; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; Δ^9-THCAV, Δ^9-tetrahydrocannabivarinic acid; Δ^9-THCV, Δ^9-tetrahydrocannabivarine.

is the best-studied and most common and productive source of phytocannabinoids, but is not the only organism capable of synthesizing this group of bioactive natural products.

Several *Rhododendron* species (Ericaceae) produce bioactive meroterpenoids with a cannabinoid backbone [8]. *Rhododendron* cannabinoids typically belong to the CBC type decorated

Glossary

Behavioral tetrad: the four distinct behavioral changes that are associated with the consumption of Δ^9-tetrahydrocannabinol (Δ^9-THC): hypothermia, catalepsy, hypolocomotion, and analgesia.
Cytochrome P450 (CYP) enzymes: a superfamily of heme-containing enzymes that typically function as monooxygenases.
Elepidotes: *Rhododendron* species that lack glandular scales.
Endocannabinoid system: a biological system in mammals based on endocannabinoids that modulate vegetative functions including pain, sleep, mood, and appetite.
Gland cells: highly specialized cells for the efficient and safe production and storage of plant defense compounds such as cannabinoids and terpenoids. Based on their morphology, gland cells are classified as glandular trichomes or glandular scales.
Half-maximal effective concentration (EC_{50}): the concentration of a compound that is necessary to obtain its half-maximal effect.
Lepidotes: *Rhododendron* species possessing glandular scales.
Liverworts: a division of land plants that are closely related to mosses.
Meroterpenoids: terpenoids whose chemical structure also contains a phenolic moiety derived from phenylpropanoid metabolism.
Methylerythritol 4-phosphate (MEP) pathway: this pathway is located in plastids and generates the linear C5, C10, C20, and C40 isoprenoid precursors from which terpenoids are formed.
Natural deep eutectic solvents (NADES): these are composed of specific molar ratios of polar and ionic small molecules that via aqueous–aqueous phase partitioning may constitute a new phase in biological systems. NADES are excellent solvents for natural products and some enzymes.
Phytocannabinoids: a group of specialized metabolites that were first identified in *Cannabis sativa*. Extracts of *C. sativa* contain the psychoactive Δ^9-THC as well as cannabidiol (CBD) that offer beneficial health effects in humans.
Polyketide synthase (PKS): an enzyme that catalyzes the synthesis of polyketides by sequential elongation of different types of starter molecules with

with an orcinol side chain (i.e., a methyl group). *Rhododendron dauricum* L. is native to Northeastern Asia and produces grifolic acid (GFA), daurichromenic acid (DCA), and confluentin (decarboxylated DCA), as well as rhododaurichromenic acids A and B. All these cannabinoids carry a sesquiterpene moiety [9]. *Rhododendron adamsii* Rehder grows in the expanses of Eastern Siberia and Mongolia and is used in folk medicine for preparing a stimulating tonic and as an adaptogenic remedy [10]. It produces cannabigerorcynic acid, cannabigerorcynic acid methylester, DCA, and chromane/chromene meroterpenoids [10]. *Rhododendron anthopogonoides* Maxim. grows in Southern China and is used as expectorant and for the treatment of chronic bronchitis [11,12]. Phytochemical studies revealed that it contains the cannabinoid-like chromane and chromene derivatives anthopogocyclolic acid, anthopogochromenic acid, cannabiorcichromenic acid, and cannabiorcicyclolic acid [11,12]. Lastly, *Rhododendron rubiginosum* Franch. *var. rubiginosum*, a shrub endemic to Southwest China, was found to contain anthopogochromenes A and B, as well as rubiginosins A–G [8].

The flowering plant *Helichrysum umbraculigerum* Less. (Asteraceae) from South Africa, the edible roots of *Glycyrrhiza foetida* Desf. (licorice; Fabaceae), and *Amorpha fruticosa* L. (bastard indigo; Fabaceae) contain so-called amorfrutins, bioactive compounds with a cannabinoid backbone [13,14]. These cannabinoids carry an aralkyl side chain and are thus characterized as prenylated bibenzyls [1,14].

Liverworts (Marchantiophyta; also called 'hepatics') are prolific producers of compounds with a bibenzyl backbone, such as lunularic acid and vittatin [15]. Cannabinoids with a bibenzyl structure have been isolated from *Radula perrottetii* Gottsche ex Steph., *R. marginata* Taylor ex Gottsche, and *R. laxiramea* Steph. (Radulaceae), liverworts that are native to the northern island of New Zealand. These bibenzyl analogs of Δ^9-THC, (–)-*cis*-perrottetinene, and (–)-*cis*-perrottetinenic acid are noteworthy for their inverted stereoconfiguration compared to non-bryophyte cannabinoids [16–19].

Beyond plants, cannabinoids are present in a few fungal organisms. Mycorrhizal fungi of the genus *Albatrellus* (Albatrellaceae) produce GFA and the derivatives grifolin, neogrifolin, and confluentin [20,21]. Furthermore, *Cylindrocarpon olidum* (Nectriaceae) produces cannabiorcichromenic acid and the halogenated cannabinoid 8-chlorocannabiorcichromenic acid [22].

Sites of Phytocannabinoid Biosynthesis and Their Possible Functional Roles

C. sativa accumulates phytocannabinoids and terpenes in glandular trichomes located all over the aerial parts of the plant and in highest density on the female flowers [7,23]. No glandular trichomes are found on the root surfaces, and the root tissue therefore does not accumulate phytocannabinoids [24]. Glandular trichomes may be classified as sessile trichomes or stalked trichomes. Stalked glandular trichomes were recently shown to develop from sessile trichomes [25]. Glandular trichomes accumulate cannabinoids in a balloon-shaped cavity that is filled by secretory vesicles [25–28]. When a trichome ruptures, for example during high ambient temperatures or as a result of herbivory, its contents form a sticky coating on the plant surface that is orchestrated by the viscous, non-crystallizing properties of cannabinoids [29]. The noxious substance glues the mandibles and legs of potential herbivores and prevents desiccation, resembling the waxy glaze of cacti and other succulents from dry environments [30]. The amount of cannabinoids formed correlates positively with increased temperatures and imposed heat stress, as well as with low soil moisture and poor mineral nutrient content [31,32]. The latter was indicated by a negative correlation between mineral supply and cannabinoid production [33,34]. Cannabinoid production may also provide an evolutionary advantage by functioning as sun-

C2 carbon units derived from malonyl-CoA.

Terpenoids: the largest group of bioactive natural products of plants; terpenoids are produced from isoprenoid precursors of different chain lengths.

Therapeutic index (TI): a quantitative measurement of the relative safety of a drug.

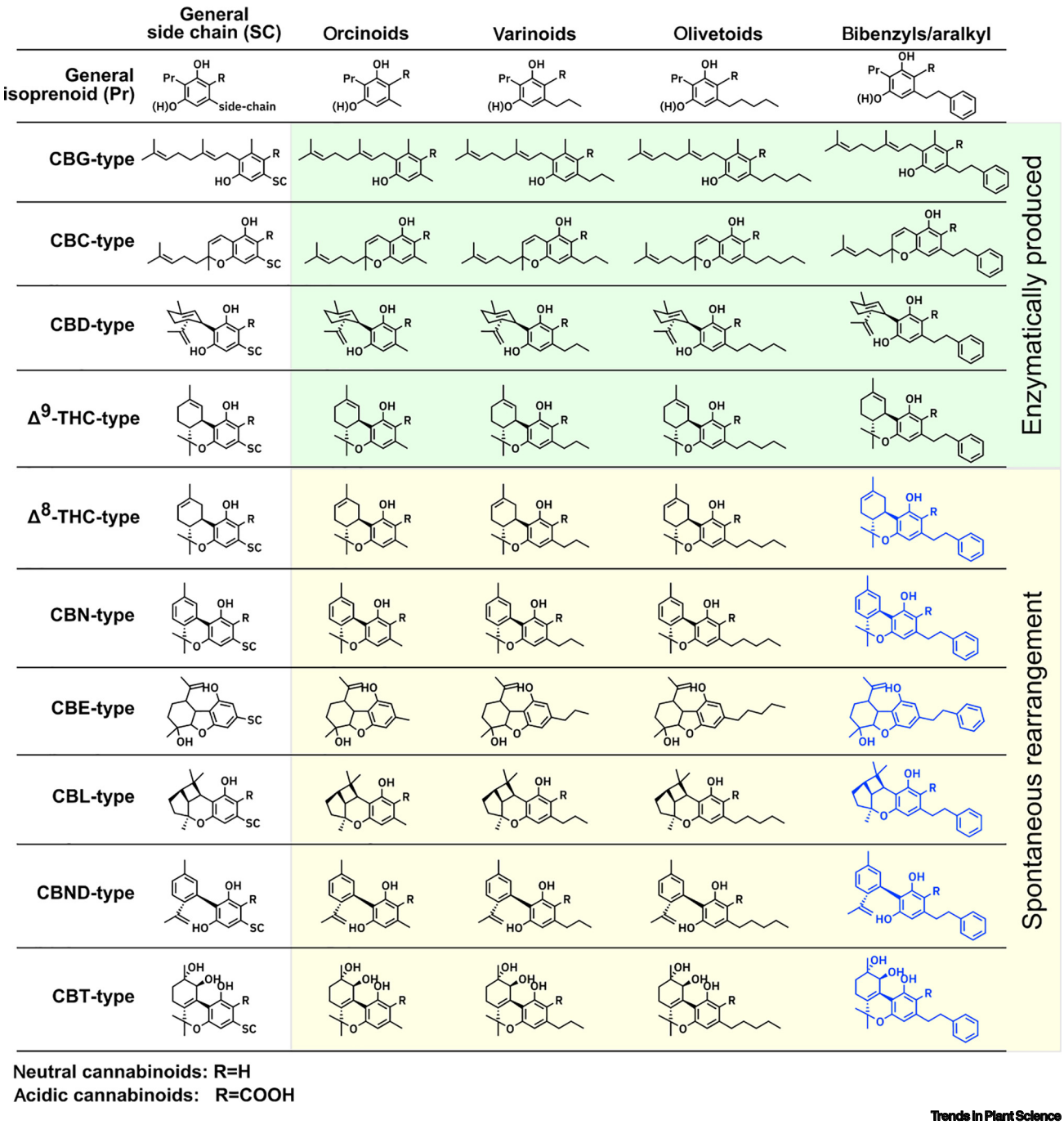


Figure 2. Overview of Cannabinoids Derived from an Orcinoid, Varinoid, Olivetoid, or Bibenzyl/Aralkyl Backbone. Black structures have been isolated from natural sources [1]. Blue structures have not yet been found *in planta*. Abbreviations: CBC, cannabichromene; CBD, cannabidiol; CBE, cannabielsoin; CBG, cannabigerol; CBL, cannabicyclol; CBN, cannabinol; CBND, cannabinodiol; CBT, cannabitriol; Pr, prenyl; SC, side chain; Δ^8-THC, Δ^8-tetrahydrocannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol.

screens that absorb biologically destructive UV-B radiation (280–315 nm) [35]. Significantly increased cannabinoid production was measured in *Cannabis* flowers after UV-B-induced stress [35]. Thus phytocannabinoids convey various biologically beneficial properties.

Compared with the *in planta* roles of cannabinoids in *C. sativa*, little is known about the function of cannabinoids in *Rhododendron* species. Rhododendrons are separated into two major groups, **elepidotes** and **lepidotes**. Elepidotes are large-leaved rhododendrons that are devoid of scales on their leaves. Lepidote rhododendrons have small leaves covered by glandular scales [36]. The scales are located predominantly on the abaxial leaf surface, but are also present on the adaxial side (ratio ca 20:1) [36]. These multicellular morphological features of epidermal origin consist of a stalk and a circular expanded cap [36]. The leaf scales contain lipophilic globules that contain specialized metabolites such as terpenes, and function in insect deterrence [37]. *R. dauricum* produces DCA that accumulates in the apoplast of the glandular scales, presumably serving as a chemical defense barrier based on its antimicrobial activities, together with its precursor grifolic acid (GA) and the reaction side-product H_2O_2 [38].

Liverworts (Marchantiophyta) possess oil bodies – membrane-bound cell organelles containing suspensions of **terpenoids** and aromatic oils in a carbohydrate- or protein-enriched matrix [39]. The morphology of the oil bodies is used to distinguish the different *Marchantiophyta* species, and oil bodies have been proposed to serve several ecological functions [40]. Liverworts are in general not damaged by fungi and bacteria, insect larvae and adults, or slugs, snails, and small mammals [39]. The compounds found in oil bodies bring forth characteristic pungent, odiferous, and/or bitter-tasting compounds that display a wide range of bioactivities [39]. It is noteworthy that most sesqui- and di-terpenoids produced by liverworts show *cis* configuration, in contrast to those found in most higher plants, which have *trans* configuration [41], although sesqui- and di-terpenoids in the *Eremophila* genus and *Solanum* spp. are exceptions [42–45]. In addition, the proposed ecological functions of oil bodies include resilience to cold temperatures, excessive light, UV radiation, and desiccation [40,46]. Liverworts are unable to biosynthesize abscisic acid. Instead, they produce the bibenzylic dihydrostilbenoid lunularic acid that has plant hormone activity [47]. The ecological function of perrottetinenic acid and perrottetinene is unclear, but might resemble those of cannabinoids in *C. sativa.* An extensive review on liverworts with a focus on *Radula marginata* taxonomy, genetics, cannabinoid phytochemistry, and pharmacology was recently published by Hussain *et al.* [19]. Mosses (Bryophyta) and hornworts (Anthocerotophyta) do not possess oil bodies [40,48].

Phytocannabinoids and Human Health

Cannabis preparations have been used as medicines since ancient times [3,49]. They have been investigated for their antimicrobial activity against bacteria and fungi, and were found to be effective against a range of infectious diseases in humans and to represent potent antibiotics. Many fungi can metabolize cannabinoids [50], and this may reflect why cannabinoids seem to be effective only against a few fungal pathogens such as *Phomopsis ganjae* [51]. Their distinct pharmacological potential is commercially relevant. An increasing number of countries are relaxing their legislation around *C. sativa* and its phytocannabinoids. As a result, the global industry around legal cannabis-derived products is growing rapidly, and is likely to represent a projected US$ 57 billion market by 2027 [52].

Antimicrobial Effects of Cannabinoids

Cannabis extracts possess antimicrobial activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, as well as against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, but exhibits no activity towards the pathogenic fungi *Candida albicans* and *Aspergillus niger* [53]. When tested individually, the major cannabinoids CBG, CBD, CBC, Δ^9-THC, and CBN display antibiotic activity against methicillin-resistant *Staphylococcus aureus* [54]. Δ^9-THC and CBD exhibit bactericidal effect against Gram-positive staphylococci and streptococci in the 1–5 μg/ml range, but not against Gram-negative bacteria [55]. This

indicates that components of the complex cannabis extracts other than Δ^9-THC and CBD, such as some of the terpenoids, are responsible for the bactericidal effects towards Gram-negative bacteria. DCA and GFA from *Albatrellus dispansus* also displayed antimicrobial activity against Gram-positive bacteria [21]. This activity is attributed to the presence of a prenyl group in DCA and GFA because the resorcinolic core alone shows no antimicrobial activity [54]. Interestingly, the prenyl moieties show structural similarities to bioactive monoterpenes.

Bioactivities of *Cannabis* Cannabinoids

Neutral Cannabinoids

In human, cannabinoids display a variety of bioactivities owing to their interaction with the G protein-coupled cannabinoid receptors (GPCRs) CB_1 and CB_2, transient receptor potential (TRP) ion channels, and peroxisome proliferator-activated receptor γ (PPARγ) [13,56–58]. CB_1 is the most abundant GPCR in the central nervous system [59]. CB_2 is located predominantly in cells and tissues of the immune system [59]. Some of the medical properties attributed to the different cannabinoids as specified later require validation by thorough scientific studies [60].

Δ^9-THC exerts pleiotropic effects in humans, including analgesic responses, relaxation, dysphoria, tolerance, and dependence [61]. This reflects agonistic effects of Δ^9-THC on CB_1 activation of β-arrestin 2 recruitment and signaling [61,62]. Δ^9-THC is the major psychoactive constituent of *Cannabis* [49]. The drug dronabinol (Marinol®) contains Δ^9-THC in sesame oil, and is marketed as an efficient antiemetic for patients receiving cancer chemotherapy and as an appetite stimulant for persons with acquired immunodeficiency syndrome (AIDS). Doses tailored for chronic administration positively affected weight and appetite [2]. Δ^9-THC is also used to improve sleep [63]. Δ^8-THC is an isomer of Δ^9-THC that exhibits a similar although slightly weaker activity profile on cannabis receptors [1,64]. Δ^8-THC can reduce intraocular pressure in humans and therefore displays antiglaucoma activity [64].

In human, CBD exerts its pharmacological effect on the central nervous system and peripheral regions through its high-potency antagonistic activity on CB_1 and CB_2 as well as by serving as an allosteric modulator of the μ-opioid receptor [65]. CBD has no toxic effects in humans at doses between 10 and 700 mg, and is administered in a highly purified form as Epidiolex® to patients with treatment-resistant epilepsy associated with CDKL5 deficiency disorder and several syndromes [66,67]. CBD has also been assigned anticonvulsive, antianxiety, antipsychotic, antinausea, and antirheumatoid arthritis properties [68]. CBD is not psychoactive and has low to no side effects when administered at recommended levels in pharmacotherapy [69]. Administration of CBD decreases Δ^9-THC-elicited psychotic symptoms and reduces the negative impact of Δ^9-THC on hippocampus-dependent memory [70].

CBG is an additional non-psychoactive phytocannabinoid that has low affinity for CB_1 and CB_2 (~500-fold lower than Δ^9-THC), but has significant activity towards several ligand-gated cation channels of the TRP superfamily. It acts as an agonist of TRPV1 (TRP type vanilloid 1) and TRPA1 (TRP type ankyrin 1), and as a potent inhibitor of TRPM8 (TRP type melastatin 8) [71–73].

CBC is non-psychotropic and does not interact with CB_1 and CB_2, but inhibits endocannabinoid inactivation and activates TRPA1, resulting in protective effects against intestinal inflammation in experimental model systems [74]. CBC resulted in dose-dependent anti-inflammatory activity in a lipopolysaccharide-induced paw edema model [75]. As of 2020, no human study with CBC is available.

Δ^9-THCV is a non-psychoactive CB_1 neutral antagonist with potential against obesity-associated glucose intolerance [76]. Δ^9-THCV and CBD are both suggested as possible therapeutic agents for the treatment of obesity- and metabolic syndrome-related hepatosteatosis [77–79]. In a double-blind placebo-controlled study, Δ^9-THCV significantly decreased fasting plasma glucose, improved pancreatic β cell function, adiponectin, and apolipoprotein A, whereas plasma high-density lipoprotein was unaffected [80].

Cannabinoid Acids

The cannabinoid acids produced by *C. sativa* include Δ^9-THCA, CBDA, CBGA, and CBCA. These compounds do not show any cannabimimetic (i.e., psychotropic) effects [81]. Most Δ^9-THCA samples contain low amounts of Δ^9-THC because of spontaneous decarboxylation. This lability hampers clinical applications. Upon chemical synthesis, Δ^9-THCA can be obtained in two isoforms, named Δ^9-THCA-A and Δ^9-THCA-B. The biosynthesis in *C. sativa* is regiospecific and only results in the formation of Δ^9-THCA-A. Δ^9-THCA binds to PPARγ with higher affinity than Δ^9-THC and displays low affinity at CB_1 and CB_2 [82,83]. In mice treated with 3-NPA, Δ^9-THCA acts as a neuroprotective via a PPARγ-dependent pathway [83]. Δ^9-THCA-enriched fractions of ethanol extracts from unheated *C. sativa* inhibit tumor necrosis factor α levels in culture supernatants from U937 macrophages and peripheral blood macrophages in a dose-dependent manner after stimulation with lipopolysaccharide [84]. Δ^9-THCA and Δ^9-THC also show distinct effects on phosphatidylcholine-specific phospholipase C activity, but via different pathways [84].

CBDA produces dose-dependent antihyperalgesia and anti-inflammatory effects in mice with carrageenan-induced acute inflammation [85]. In addition, CBDA (or Δ^9-THC) administered by oral gavage 60 minutes before carrageenan produced antihyperalgesia effects. CBDA (and CBD) induced a reduction in resting tissue tension of isolated intestinal segments of house musk shrews via a non-neuronally mediated pathway (i.e., independently of binding to CB_1 or CB_2) [86].

Bibenzylic Cannabinoids

Bibenzylic (aralkylic) analogs of CBG (bibenzyl-CBG) from *H. umbraculigerum* and some liverworts contain a phenethyl side chain instead of a pentyl side chain. Compared with CBG, bibenzyl-CBG displays decreased affinity for the metabotropic CB_1 and CB_2 receptors but retains affinity for the ionotropic receptors TRPV 1–4 and TRPA1, and exhibits increased affinity towards TRPM 8 [14]. These compounds belong to the amorfrutin class of compounds isolated from *Amorpha fruticosa* and *Glycyrrhiza foetida*. Amorfrutins are natural activators of PPARγ and exert potent anti-inflammatory effects [13,87].

Perrottetinene is a bibenzylic cannabinoid produced by the liverworts *Radula perrotteti* [18], *Radula marginata* [88], and *R. laxiramea* [89]. Structurally, it resembles Δ^9-THC, distinguishing itself with an aromatic instead of a pentyl side chain. Perrottetinene is a psychoactive CB_1 agonist that induces the **behavioral tetrad** of hypothermia, catalepsy, hypolocomotion, and analgesia. Interestingly, the cyclohexene ring of perrottetinene has a stereochemical *cis* configuration as opposed to the *trans* configuration of *trans*-Δ^9-THC. In contrast to the situation in higher plants, the production of enantiomers is a hallmark of liverwort specialized metabolism [39].

Rhododendron Cannabinoids

Prenylated orcinoids and their derivatives from *Rhododendron* spp. display various bioactivities, particularly in the immune system [90,91]. Most of these phytocannabinoids share a chromane/chromene scaffold and are grouped into CBC or CBL types. They are associated with anticancer,

antimicrobial, anti-inflammatory, antithrombotic, and antipsychotic activities and show very low toxicity in humans [92]. Daurichromenic acid (DCA) and rhododaurichromenic acids A and B exert the most effective anti-HIV activities, as monitored using acutely infected H9 cells. DCA has a **half-maximal effective concentration** (EC_{50}) of 15 nM [38], lower than the EC_{50} value for the positive control drug azidothymidine (44 nM) [93]. Furthermore, DCA displayed anti-HIV activity with an EC_{50} of 5.67 ng ml^{-1} and a **therapeutic index** (TI) of 3710 [94]. Rhododaurichromanic acid A also showed potent anti-HIV activity, with an EC_{50} of 370 ng ml^{-1} and a therapeutic index of 92 [94]. Anthopogocyclolic acid (CBL-type), anthopogochromenic acid (CBL-type), and cannabiorcichromenic acid (CBC-type) from *R. anthopogonoides* inhibit histamine release and thus exhibit antiallergic effects [11,12,91].

The focus on possible effects and synergies of Δ^9-THC and CBD in most pharmacological studies has resulted in under-representation of the minor cannabinoids in clinical trials. The low abundance of the minor cannabinoids in the plant renders their possible pharmacological effects non-obvious and their acquisition for initial trial experiments difficult and expensive. The other plant sources of cannabinoids do not accumulate cannabinoids at high levels and are often endangered species. This underpins the demand for biotechnology-based production of the rare cannabinoids to obtain an understanding of their possible contributions to phytocannabinoid-based medications. A prerequisite for such an approach is knowledge of the biosynthetic pathways that generate cannabinoids in *Cannabis sativa*, *Rhododenron dauricum*, and *Radula marginata*.

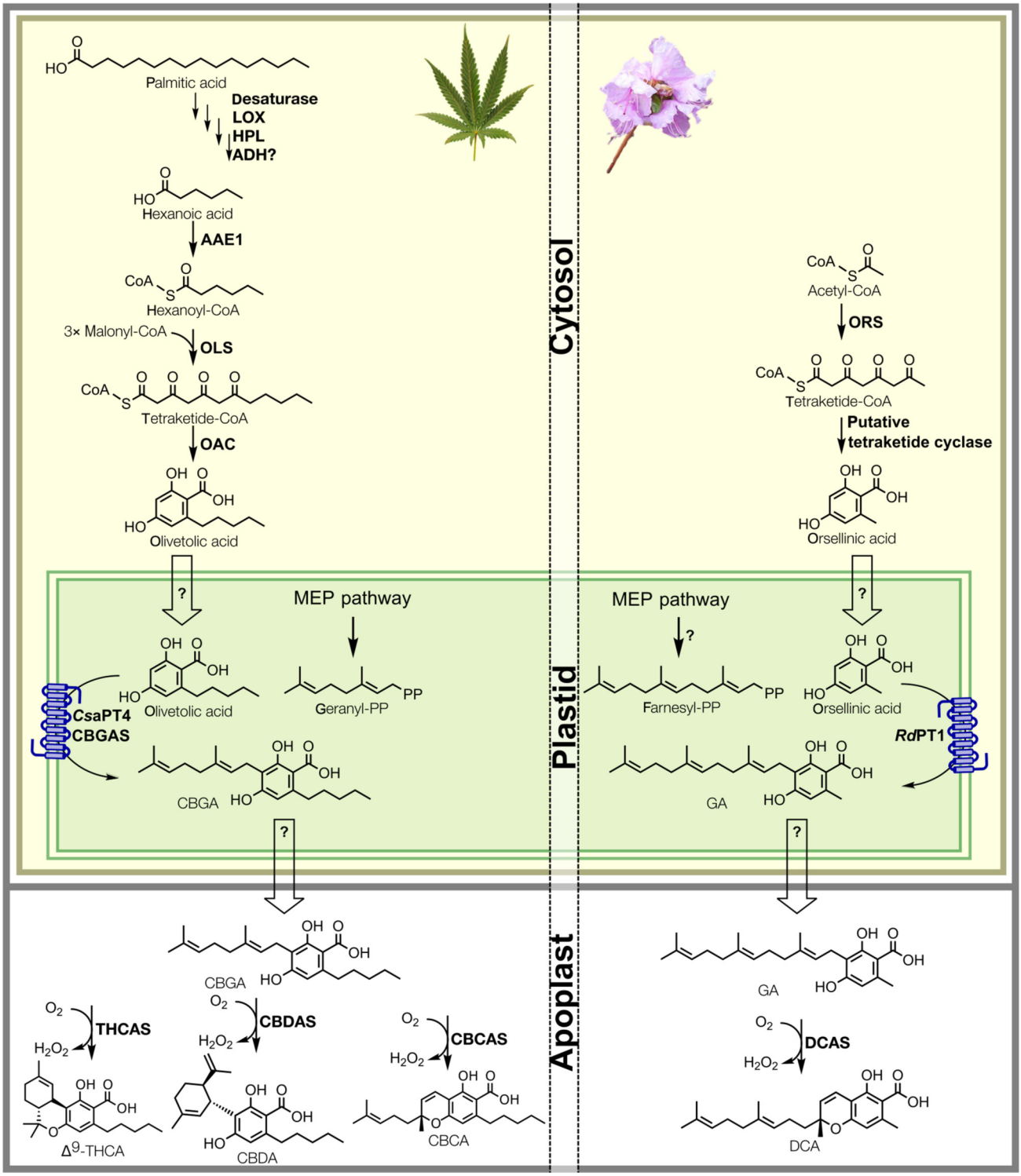
Biosynthesis and Biotechnological Production of Cannabinoids

Biotechnology-based production of cannabinoids requires a biological system that can provide a cellular supply of the precursor isoprenoid units, coordinated expression of all the genes encoding the enzymes catalyzing the entire biosynthetic pathway for the desired cannabinoid, and eventually enzyme engineering to utilize specific starter molecules. Currently known plant sources from which the biosynthetic genes can be obtained are *Cannabis sativa*, *Rhododendron dauricum*, and *Radula marginata.* A synthetic biology approach would likely involve combinatorial uses of the genes encoding biosynthetic enzymes with optimal catalytic properties independently of the plant species. Functional interaction to avoid autotoxicity from the accumulation of high levels of intermediates is also a selection parameter in such approaches.

Cannabinoid Biosynthesis in *Cannabis sativa*

The pathway for phytocannabinoid biosynthesis in *Cannabis* has recently been elucidated (Figure 3) [95, T. Gülck *et al.*, unpublished]. The pathway is split between different cell types and organelles: the cytosol of the **gland cells**, the plastids, and the extracellular storage cavity. Starting in the cytosol, the precursor molecule hexanoic acid is most likely made available through oxidative cleavage of fatty acids, such as palmitic acid. The geranyl diphosphate (GPP) used for prenylation of olivetolic acid (OA) originates from the **methylerythritol 4-phosphate (MEP) pathway**, which typically operates in plastidial organelles in eukaryotic cells (normally chloroplasts) [96]. Oxidative cyclization and storage of the final products take place outside the gland cells in the resin cavity [97,98]. It remains to be resolved how the pathway intermediates are transported between the different compartments. Most likely transport proteins and vesicle trafficking play key roles in mobilizing intermediates across the morphologically highly specialized interface between the gland cells and the storage cavity (Figure 3).

The biosynthesis of cannabinoids involves integration of key steps in polyketide and isoprenoid metabolism. Hexanoic acid is used as the polyketide starter molecule and is most likely generated from C18 fatty acids, that are sequentially desaturated, peroxygenated, and cleaved into a C6



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(the hexanoic acid) and a C12 product by the action of desaturases, lipoxygenases, and hydroperoxide lyases, respectively. This pathway to C6 alkyl compounds was suggested by Stout *et al.* [99], supported by Livingston *et al.* [25] who observed high desaturase, lipoxygenase (LOX), and hydroperoxide lyase (HPL) expression in trichome-specific transcriptomes. In a reaction catalyzed by acyl-activating enzyme 1 (AAE1), hexanoic acid is converted into the activated thioester hexanoyl-CoA [99] that is elongated with malonyl-CoA as a C2 donor in a reaction catalyzed by olivetol synthase (OLS) and cyclized by olivetolic acid cyclase (OAC) to produce olivetolic acid (OA) [100,101]. The production of OA from hexanoic acid takes place in the cytosol.

GPP (C10 isoprenoid) is synthesized by the plastidial non-mevalonate-dependent isoprenoid (MEP) pathway, as shown by incorporation studies with ^{13}C-labeled precursors [96]. Cannabigerolic acid synthase (CBGAS, *Csa*PT4) uses GPP to prenylate OA, forming the branch-point intermediate and the first *bona fide* cannabinoid compound CBGA. CBGA is the direct precursor for the common cannabinoids which are decorated with an alkylic pentyl side chain [95, T. Gülck *et al.*, unpublished,102]. CBGAS is a transmembrane aromatic prenyltransferase (aPT) and carries a plastid localization signal. It remains to be determined into which plastid membrane CBGAS is integrated and whether its active site faces the inner or the outer side of that membrane. The flavoproteins Δ^9-tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) are secreted into the extracellular space and convert CBGA to Δ^9-THCA and CBDA, respectively. These conversions proceed as oxidative cyclization reactions, via reduction of molecular oxygen (O_2), that generate hydrogen peroxide (H_2O_2) as a side-product [97,103]. The reaction mechanism for cannabichromenic acid synthase (CBCAS) is most likely also dependent on FAD and O_2 even though the enzyme was initially reported to be independent of flavin cofactors and O_2 [104]. At that time, CBCAS, CBDAS, and THCAS were all considered to be oxidoreductases that required neither cofactors nor coenzymes [104–106]. CBDAS and THCAS were subsequently characterized as flavoproteins that are strictly dependent on the presence of O_2 as an electron acceptor, as was daurichromenic acid synthase (DCAS; see later) [38,103]. The high sequence similarity between CBCAS and THCAS (96% at the nucleotide level) indicates that CBCAS is an O_2-dependent flavoprotein that catalyzes the oxidocyclization of CBGA to CBCA with H_2O_2 as side-product. All these oxidocyclases carry a secretion signal peptide and are exported to the extracellular resin space [6,103,107]. THCAS and CBDAS are catalytically active in the resin space, but it remains to be demonstrated whether their activity is exclusive to the extracellular space [97].

Δ^9-THCA, CBDA, and CBCA are the end-products of the enzymatic biosynthesis of cannabinoids with a pentyl side chain. When exposed to heat (pyrolysis during smoking or baking), radiation, or spontaneously during storage, the compounds undergo decarboxylation and 'spontaneous rearrangement' reactions (Figure 2) [1,108].

Cannabinoids with unusual alkyl side chains (C_1–C_4) are produced by the same enzymes, but from the respective short-chain fatty acyl-CoAs and with lower affinity (acetyl-CoA, propanoyl-CoA, butanoyl-CoA, or pentaoyl-CoA, respectively) [1,79,95].

Figure 3. Proposed Subcellular Distribution of Enzymes Catalyzing Phytocannabinoid Biosynthesis in *Cannabis sativa* (left) and *Rhododendron dauricum* (right). Enzymes are located in the cytosol (yellow), plastids (green), or apoplastic space (white). Question marks indicate unknown transport mechanisms. Polyketide formation takes place in the cytosol, prenylation in the plastid, and oxidocyclization and storage in the apoplast. Abbreviations: AAE1, acyl-activating enzyme 1; ADH, alcohol dehydrogenase; CBCA, cannabichromenic acid; CBCAS, cannabichromenic acid synthase; CBDA, cannabidiolic acid; CBDAS, cannabidiolic acid synthase; CBGA, cannabigerolic acid; CBGAS, cannabigerolic acid synthase; *Csa*PT4, *C. sativa* aromatic prenyltransferase 4; DCA, daurichromenic acid; DCAS, daurichromenic acid synthase; GA, grifolic acid; HPL, hydroperoxide lyase; LOX, lipoxygenase; MEP, methylerythritol 4-phosphate; OAC, olivetolic acid cyclase; OLS, olivetol synthase; ORS, orselinic acid synthase; *Rd*PT1, *R. dauricum* prenyltransferase 1; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; Δ^9-THCAS, Δ^9-tetrahydrocannabinolic acid synthase.

Biosynthesis of Daurichromenic Acid (DCA) and Derivatives Thereof

Rhododendron dauricum produces the phytocannabinoid DCA. All carbon atoms in DCA are derived from acetyl-CoA and farnesyl-CoA, with orsellinic acid (OA) and grifolic acid (GA) as key intermediates [38,90,109,110]. Specialized glandular scales on the surface of predominantly young leaves are the place of DCA biosynthesis and storage [38]. The morphology of the scales is different from that of the capitate stalked trichomes in *Cannabis sativa*, being defined by an expanded cap that comprises central cells and rim cells without an obvious secretory cavity [38]. As in *C. sativa*, the phytocannabinoid biosynthesis is split between processes taking place in the cytosol (polyketide pathway), the plastid (MEP pathway and *Rd*PT1 activity), and the apoplastic space, where DCA, confluentin and other hydrophobic metabolites such as essential oils accumulate [37]. GA and DCA are phytotoxic compounds that induce cell death in cell cultures of *R. dauricum* [9]. H_2O_2 formed as a side product in DCA biosynthesis also induces apoptosis-related reactions resulting in cell death, albeit at increased concentrations in comparison with GA and DCA. To avoid autotoxicity and cellular damage, the accumulation of DCA is therefore likely to take place in the apoplast. An increased concentration of H_2O_2 alongside DCA on the outer rim of the glandular scales may fortify the plant self-defense system [9]. For the DCAS-catalyzed reaction to take place, the phytotoxic GA must be exported from its intracellular site of synthesis before it can exert a detrimental effect. The subcellular transport mechanisms in *R. dauricum* are not yet understood and remain an interesting scientific challenge to address.

The DCA pathway begins with chain extension of acetyl-CoA by the type III **polyketide synthase** (PKS) orcinol synthase (ORS) in a reaction where three units of malonyl-CoA are the carbon donor (Figure 3). The predominant product of recombinant and purified ORS is orcinol; orsellinic acid (OSA), triacetic acid lactone, phloroacetophenone, and tetraacetic acid lactone are minor products. OSA is the substrate for the subsequent reaction, but not orcinol. Interestingly, it was found that addition of purified *C. sativa* olivetolic acid cyclase (OAC) to ORS led to an increase in OSA production and a concomitant decrease in orcinol production in an OAC dose-dependent manner [90]. Thus, the existence of a non-identified tetraketide cyclase in *R. dauricum* was suggested.

The regiospecific farnesylation step is catalyzed by the aromatic farnesyltransferase *Rd*PT1. Surprisingly, the farnesyl-CoA is derived from the plastidial MEP pathway, as shown by inhibitor studies. Inhibition of the MEP pathway with clomazone led to a decrease in OSA and DCA, whereas inhibition of the mevalonate-dependent pathway with mevastatin led to their increase, probably owing to compensatory upregulation of the MEP pathway [109]. *Rd*PT1 shows moderate sequence identity to known UbiA aromatic PTs and is localized within the plastid compartment (Figure 3) [109]. *Rd*PT1 may use geranyl-CoA and geranylgeranyl-CoA as alternative prenyl donors but at activity rates of only 13% and 2.5% of the activity obtained with farnesyl-CoA. Lastly, the cannabichromene (CBC) scaffold is formed in an oxidative cyclization reaction catalyzed by the flavoprotein DCA synthase (DCAS) [38]. Similarly to the oxidocyclases THCAS and CBDAS from *C. sativa*, DCAS is secreted and is enzymatically active outside in the apoplastic space. The reaction proceeds with concomitant release of H_2O_2 [38]. Confluentin is the decarboxylated form of DCA and has been isolated from *R. dauricum* [111]. It is possibly produced by spontaneous decarboxylation upon irradiation, heat, and storage, comparable with the slow decarboxylation of acidic cannabinoids to the neutral forms in *C. sativa* glandular trichomes [1].

In a synthetic biology-based approach, combinatorial biosynthesis of DCA as well as of a halogenated derivative of DCA was established in *Aspergillus oryzae* NSAR1, using genes from the fungus *Stachybotrytis bisbyi* PYH05-7 and *Fusarium* sp. in combination with *Rd*DCAS [112]. The type I PKS *Stb*A chain was found to elongate the starter molecule acetyl-CoA, using

malonyl-CoA as a carbon donor, and to cyclize the tetraketide product to establish the orcinoic core OSA. Using farnesyl-CoA as a donor and OSA as the acceptor, the UbiA prenyltransferase *Stb*C was used to biosynthesize GFA [112] which was converted into DCA using the *Rd*DCAS enzyme. The halogenase AscD from *Fusarium* was used to introduce a chlorine atom on the aromatic ring, forming 5-chloroDCA. Cyclization of the halogenated GA by *Rd*DCAS was not affected by the presence of the halogen atom [112]. Halogenated meroterpenoids derived from OSA display bioactivity [112].

Biosynthesis of Bibenzyl Cannabinoids in *Radula marginata*

Mining of a *de novo* assembled *Radula marginata* transcriptome published in 2018 afforded candidate genes for enzymes in GPP synthesis [113]. Stilbene acid or dihydrostilbene acid were indicated as the key precursor for synthesis of the bibenzyl CBGA analog [113]. Stilbene acids are rare in nature, but compounds of this type are present in *Hydrangea macrophylla* var. *thunbergii* (hydrangic acid, lunularic acid) [114], liverworts such as *Marchantia polymorpha* L. (prelunularic acid and lunularic acid) [115], and *Convolvulus hystrix* [116].

Stilbene acids are most likely biosynthesized from a type III PKS using common CoA-activated precursor molecules such as coumaroyl-CoA or dihydrocoumaroyl-CoA [117]. These starter molecules are extended with units of malonyl-CoA via decarboxylation involving condensation reactions, generating a polyketide intermediate that, depending on the enzyme systems present, may give rise to different core structures [117]. In the case of hydrangic acid, the starter molecule is thought to be coumaroyl-CoA, which is then extended using three units of malonyl-CoA as a C2 donor to produce a tetraketide intermediate in a reaction catalyzed by an unknown stilbene synthase (STS)-type PKS enzyme. Reduction of the polyketide chain by a ketoreductase (KR) is then thought to be followed by an STS-like C2-to-C7 intramolecular aldol condensation [118] in which unusual retention of the carboxylic group generates hydrangic acid. The possible involvement of a KR is based on the lack of the C5-hydroxyl group on the aromatic ring of the hydrangic acid compared with the structure of stilbene acid. The loss of the hydroxyl group can be explained by cyclization with a reduced C5 carbonyl group of the tetraketide [118].

Prelunularic acid, the precursor for lunularic acid, is most likely formed by a type III PKS, called bibenzyl synthase (BBS), that uses dihydrocoumaroyl-CoA as the starter molecule for extension with three units of malonyl-CoA as the carbon donor [117,118]. It is likely that the cyclization also takes place on a reduced polyketide given the observed lack of a hydroxyl group at position C5 of the A ring [114,115,118]. In both cases, the retention of the carboxylic group is unusual for an STS, leading to the proposition that the STS enzymes involved in the synthesis of bibenzyl cannabinoids represent a novel class of type III PKS enzymes that have an altered carboxylate-retaining reaction mechanism, and that the KR enzyme involved has acquired the additional ability to catalyze the cyclization or that a hypothetical cyclase might assist in the formation of the ring [114,118]. The discovery of the dimeric α + β barrel protein olivetolic acid cyclase (OAC) in *C. sativa* represented the first plant enzyme that catalyzes the proposed C2–C7 intramolecular aldol condensation with carboxylate retention [101]. Structural analyses of OAC disclosed a unique active-site cavity containing the pentyl-binding hydrophobic pocket and the polyketide binding site, as well as a lack of thioesterase and aromatase activities [119]. In summary, the stilbene acid or dihydrostilbene acid precursor in *R. marginata* is likely derived from coumaroyl-CoA or cinnamoyl-CoA, respectively, which is chain-elongated by a type III PKS and cyclized by a putative tetraketide cyclase (dihydrostilbene acid cyclase, DHAC).

R. marginata produces perrottetinenic acid. The transcriptome of this liverwort harbors an mRNA sequence encoding a type III PKS identified as a stilbene acid synthase (*Rm*SAS) and that

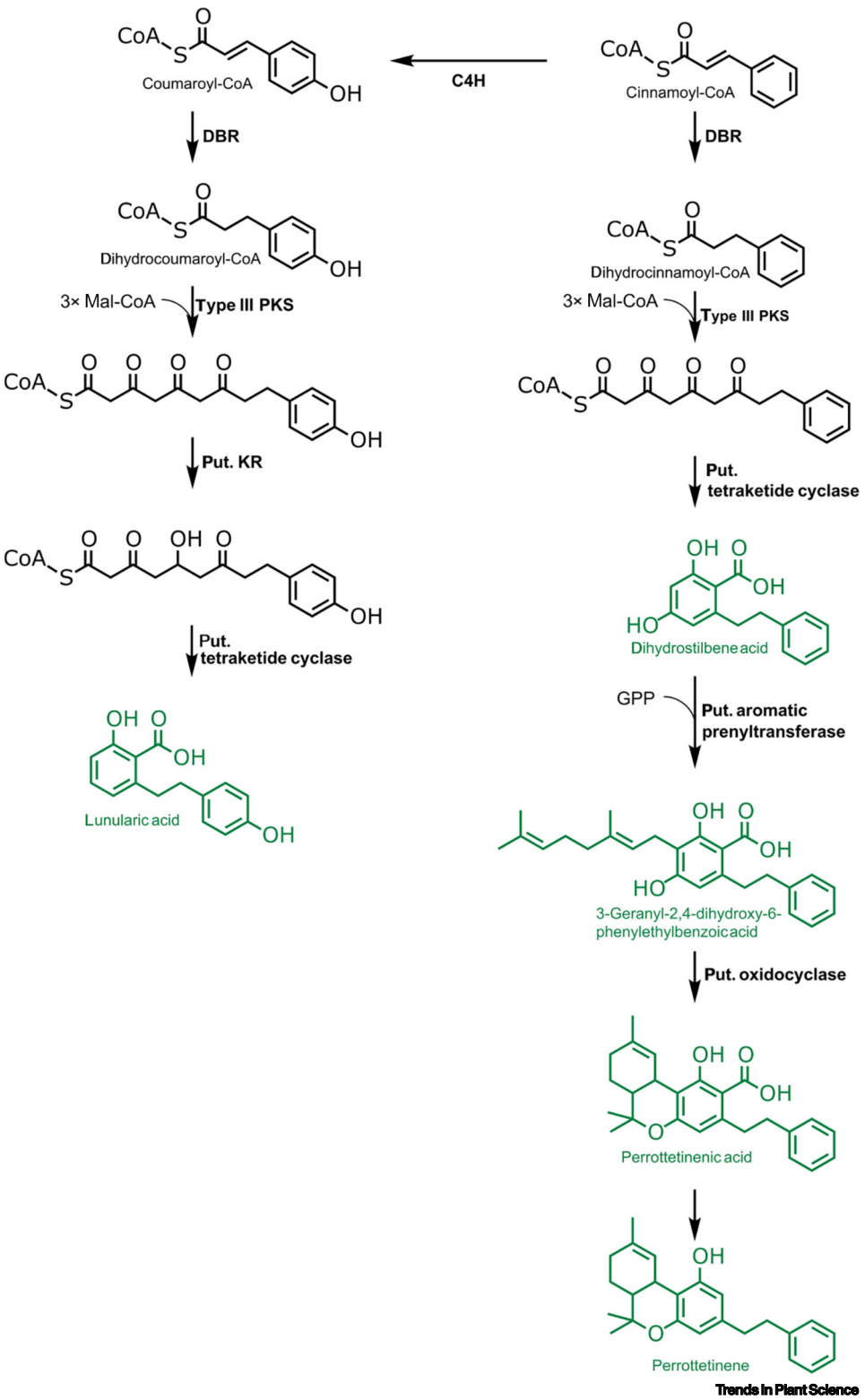
displays 60% amino acid sequence homology to a stilbene-carboxylate synthase from *Marchantia polymorpha* (PDB ID 2P0U) [113]. This PKS is most likely responsible for chain elongation of the aromatic precursor (Figure 4). Other putative enzymes involved in the biosynthesis of perrottetinenic acid are a double-bond reductase (DBR) that generates dihydrocinnamoyl-CoA from the phenylpropanoid precursor, an aromatic prenyltransferase (*Rma*PT), and an oxidocyclase (perrottetinenic acid synthase, PAS). Assuming that the biosynthesis of the bibenzyl cannabinoids is distributed across the liverwort cells in a similar manner as the biosynthetic pathways in *Cannabis* and *Rhododendron*, transcripts encoding the respective enzymes should be expressed accordingly. Hence, DBR and DHAC should localize to the cytosol, the *Rma*PT to the plastid, and the PAS to the oil body. Corresponding signal peptides might serve as indicators for selection of the encoding genes. Table 1 presents an overview of currently known pathway genes. The table also highlights the knowledge gaps in pathway discovery, and genes used for secondary modifications of cannabinoids.

Biotechnological Production of Phytocannabinoids in Heterologous Hosts

Cannabinoids can be produced using biotechnology approaches using different production platforms including plants, fungi, and bacteria. Comparison of these production platforms is beyond the scope of this review, but ample literature is available on the latest advances. *C. sativa*-based production of cannabinoids using explants and micropropagation has been reviewed by Lata *et al.* [120] and Schachtsiek *et al.* [121], and was recently expanded by Kodym and Leeb [122]. In addition to *C. sativa*, the high-biomass crop *Nicotiana benthamiana* was identified as a promising heterologous host to produce cannabinoids *in planta*. *N. benthamiana* is an established production platform for proteins and bioactive natural products [123–126], and possesses glandular trichomes that can be utilized to avoid autotoxicity resulting from cannabinoid pathway intermediates [121]. The use of *in vitro* plant cell cultures for cannabinoid production covering genetic transformation, cell suspension cultures, hairy/adventitious roots, and heterologous systems has been reviewed [127]. Yields of 1.04 μg Δ^9-THCA g^{-1} dry weight have been obtained in hairy roots [128] and 0.121 g Δ^9-THCA l^{-1} h^{-1} from direct precursor feeding [129]. Microbial production of cannabinoids remains limited [93, T. Gülck *et al.*, unpublished]. Production of cannabinoids from galactose was achieved in *Saccharomyces cerevisiae* [95], reaching titers of 1.4 mg l^{-1} CBGA, 2.3 mg l^{-1} Δ^9-THCA, and 8.0 mg l^{-1} CBDA. Their platform also led to the production of minor cannabinoids including tetrahydrocannabidivarinic acid (Δ^9-THCVA; 4.8 mg l^{-1}) and cannabidivarinic acid (CBDVA; 0.006 mg l^{-1}).

Synthetic Biology Approaches in Phytocannabinoid Production

As discussed earlier, phytocannabinoids are terpenophenolic compounds whose precursors are provided by the polyketide and MEP isoprenoid biosynthetic pathways. In heterologous production systems, optimizing and engineering these two pathways to provide adequate amounts of the initial precursors is an obvious challenge. In the biosynthesis of cannabinoids, these two pathways are linked by the action of aromatic prenyl transferases. Aromatic prenyltransferases are ubiquitous in animals [130], plants [131], fungi [132], and bacteria [133] and their different reaction spectra serve to diversify the production of aromatic metabolites such as the phenylpropanoids, flavonoids, and coumarins in plants [134]. In combination with modules involved in phytocannabinoid synthesis in different species of higher plants, liverworts, and fungi, the option arises to use an aromatic prenyltransferase-based approach for the production of new-to-nature phytocannabinoids in heterologous hosts based on combinatorial uses of the modules across species. Likewise, coexpression of specific pairs of aromatic prenyltransferases may result in increased phytocannabinoid production or altered product profiles if new catalytic patterns arise as a result of heterodimerization [T. Gülck *et al.*, unpublished]. The availability of aromatic prenyl transferases with different donor and acceptor preference specificities thus



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provides a plethora of opportunities to diversify phytocannabinoid production. Such combinatorial approaches in the diterpenoid area have given rise to new-to-nature core structures [135].

Like the hop plant (*Humulus lupulus*) [136], *C. sativa* also expresses chalcone isomerase-like proteins (CHILs) in trichomes. These are polyketide-binding proteins, and coexpression of these *CHIL* genes in heterologous cannabinoid-producing systems may serve to boost production levels [T. Gülck *et al.*, unpublished].

In addition to combinatorial biochemistry, enzyme promiscuity may be used successfully to incorporate non-natural precursors such as pentanoic acid, heptanoic acid, 4-methylhexanoic acid, 5-hexenoic acid, and 6-heptynoic acid into the cannabinoids produced. Functionalization of the side chain with an alkene or alkyne terminal group would enable post-fermentation click-chemistry-based modifications, as illustrated by copper-catalyzed azide–alkyne cycloaddition onto the 6-heptynoic acid analogs of CBGA and Δ^9-THCA with an azide conjugate [95].

The functionality of phytocannabinoids may be further diversified by other types of derivatization [T. Gülck *et al.*, unpublished]. Glycosylation may serve to avoid autotoxicity in the production host, increase solubility, and in a therapeutic context improve the absorption, distribution, metabolism, and excretion (ADME) characteristics of a drug candidate. Collections of regio- and substrate-specific glycosyltransferases to carry out such modification reactions are available [T. Gülck *et al.*, unpublished, 137]. Derivatization using acyltransferases or halogenation reactions offers additional opportunities. GA and DCA were successfully halogenated by coexpression of the halogenase AscD from *Fusarium* sp. [112]. Halogenation of GA did not hamper the conversion to DCA by DCAS. Halogenated natural products occur predominantly in marine sources and can display antibacterial, antifungal, antiparasitic, antiviral, antitumor, anti-inflammatory, and antioxidant activities [138]. With these options at hand, synthetic biology will have a major impact on the design of phytocannabinoids with optimal properties for specific uses.

Possible Storage of High Amounts of Cannabinoids in Natural Deep Eutectic Solvents to Maintain Cell Homeostasis

The resin inside glandular trichomes of *C. sativa*, the apoplast of glandular scales in *R. dauricum*, and oil bodies in *R. marginata* are the storage environments and sites of the oxycyclase-catalyzed last step in the synthesis of cannabinoids [5,37,40,97]. ^{1}H NMR-based metabolomics of *C. sativa* trichomes revealed co-storage of the cannabinoids with an abundance of sugars, amino acids, choline, organic acids, and terpenes [5]. Sugars, choline, and organic acids are typical constituents that, when present in appropriate stoichiometric ratios, form **natural deep eutectic solvents** (NADESs) [139]. NMR studies demonstrate that NADES-forming constituents exhibit proton mediated intermolecular interactions resulting in their aggregation into larger structures in the liquid phase [140]. NADESs are eminent solvents for natural products and retain this property when the water content is below 40% [141]. Based on these observations, NADESs have been proposed to embody a third membrane-less solvent phase in biological systems [139,142–146]. Δ^9-THCA and CBDA are virtually insoluble in water, thus supporting NADES-mediated solubilization in glandular trichomes and oil bodies [64,147]. The catalytic activity of the oxidocyclases THCAS, CBDAS, and DCAS within the trichome compartments of *C. sativa* and *R. dauricum* is accompanied by the release of stoichiometric amounts of H_2O_2 [97].

Figure 4. Proposed Biosynthetic Pathways for Lunularic Acid and Perrottetinene. Green highlighted structures represent compounds isolated from *Radula perrotteti* or *R. marginata*. Lunularic acid, a common liverwort phytohormone, contains a bibenzyl-core, but lacks the C5-hydroxy group. Abbreviations: DBR, double-bond reductase; GPP, geranyl diphosphate; KR, ketoreductase; Mal-CoA, malonyl-CoA; PKS, polyketide synthase; Put., putative.

Table 1. Enzymes Involved in Phytocannabinoid Biosynthesis or Cannabinoid Metabolism

Organism	Biosynthetic role	Enzyme	GenBank accession	Refs
C. sativa	Precursor supply	AAE1	JN717233.1	[99]
	Type III PKS	TKS	AB164375.1	[100]
	Tetraketide cyclase	OAC	JN679224.1	[101]
	C-Prenyltransferase	CBGAS	BK010648.1	[95]
	Oxidocyclase	THCAS	AB057805.1	[6]
		CBDAS	AB292682.1	[106]
		CBCAS	N/A[a]	[104]
R. dauricum	Precursor supply	Unknown		
	Type III PKS	ORS	LC133082.1	[90]
	Tetraketide cyclase	Unknown		
	C-prenyltransferase	*Rd*PT1	N/A	[109]
	Oxidocyclase	DCAS	LC184180.1	[38]
R. marginata	Precursor supply	Unknown		
	Type III PKS	Putative SAS	N/A	[113]
	Tetraketide cyclase	Unknown		
	C-prenyltransferase	Unknown		
	oxidocyclase	Unknown		
S. bisbyi PYH05-7	Type I PKS	StbA	LC125467.1	[112]
S. bisbyi PYH05-7	C-Prenyltransferase	StbC	LC125467.1	[112]
Secondary modifications				
S. bisbyi PYH05-7	Reductase	StbB	LC125467.1	[112]
Fusarium sp.	Halogenase	AscD	LC228576.1	[112]
H. sapiens	Monooxygenase	CYP3A4	NM_017460.6	[149]
Stevia rebaudiana	*O*-Glycosylation	UGT71E1	AY345976.1	[150]
Oryza sativa	*O*-Glycosylation	UGT5	XP_015622068.1	[151]

[a]N/A, not available.

Nevertheless, they retain their enzyme activity despite insignificant catalase activity in the *C. sativa* glandular trichomes. NADES embedment may stabilize the oxidocyclases. In parallel with the ability of a NADES to serve as a biological solvent in which biosynthetic enzymes can remain active, the storage of large amounts of natural products in a NADES-based separate liquid phase would enable the plant cell to maintain cell homeostasis [97,139,142]. The **cytochrome P450 (CYP) enzymes** CYP79A1 and CYP71E1 and the P450 oxidoreductase involved in synthesis of the cyanogenic glucoside dhurrin were recently shown to be stabilized in a NADES [148]. In efforts to engineer the production of high titers of cannabinoids in heterologous hosts, appropriate storage of the cannabinoids will need to be part of the overall engineering strategy to avoid autotoxicity issues.

Concluding Remarks and Future Outlook

The biosynthetic genes involved in phytocannabinoid biosynthesis across the plant species reviewed here can be used in a combinatorial fashion to construct desired new-to-nature phytocannabinoid structures based on combining unique prenyl moieties, side chains, and cyclized core structures (see Outstanding Questions). Potentially, it is possible to produce any type of cannabinoid once additional biosynthetic systems have been elucidated and a large collection of enzyme modules is established and available for additional engineering. The

Outstanding Questions

The biosynthetic pathways for amorfrutins in *Amorpha fruticosa*, *Helichrysum umbraculigerum*, and *Glycyrrhiza foetida* are unknown. Elucidation of their biosynthesis might provide cannabinoid specific *O*-methyltransferases and alternative aromatic precursors as new modules for use in combinatorial approaches to build new cannabinoid structures.

In cannabinoid biosynthesis, autotoxic intermediates and end-products such as CBGA, CBDA, GFA, and DCA are generated and safely accumulated in specialized storage organs such as capitate stalked trichomes, glandular scales, or extracellular storage cavities. How are the intermediates and end-products formed and transported without exerting toxicity? Does vesicle trafficking play a key role in enabling the compounds to cross the morphologically highly specialized interface between the gland cells and the storage cavity?

The oxidocyclases catalyzing the last steps in cannabinoid synthesis in *Cannabis sativa* are secreted into the non-aqueous storage space, where they retain their enzymatic activity. Does the oxidocyclization reaction take place exclusively in the apoplastic space to protect the gland cells, or does the reaction already take place during transport (e.g., inside transport vesicles)?

NADES have been proposed as a third phase in biological systems. Do NADES provide a specific reaction and/or storage space for cannabinoids in cannabis, rhododendron, and liverworts?

The biosynthetic pathways of cannabinoids are being investigated, but very little is known about their regulation. It is known that the stochiometric balance between for example OLS and OAC is crucial to avoid the accumulation of unwanted autocyclization products of the tetraketide intermediate. Which transcriptional, translational, and post-translational mechanisms are in operation to optimize the pathway fluxes? Which mechanisms respond to environmental changes?

membrane-bound aromatic prenyltransferases catalyze a key step in cannabinoid synthesis and are envisaged to pose the largest engineering challenges. The discovery of OAC orthologs for tetraketide cyclization with carboxylate retention, and of type III PKS enzymes specific for diverse aromatic starter molecules, are promising focus areas. Comparative studies between the type III PKS from *R. dauricum* (forming orcinoid cannabinoids) and *C. sativa* (forming predominantly olivetoids, but also varinoids) might give insight into their specificity for different starter molecule structures and allow protein engineering towards desired chain lengths. Secondary modifications of cannabinoids such as halogenation, oxygenation, and glycosylation may be used to modulate their physicochemical properties and thus positively affect their pharmacological properties by improving their bioavailability. Enzymes catalyzing some of these modifications could possibly be obtained from the *C. sativa* mycoflora or from the microbiome of humans heavily exposed to cannabinoids. Semi-synthetic approaches combining biosynthesis with chemical modification will no doubt offer further types of cannabinoids.

The availability of heterologously produced isolated cannabinoids provides an opportunity to scrutinize the contributions of individual cannabinoids to the reported therapeutic and psychoactive effects, including specific combinatorial effects. Lastly, quantitative chemical profiling of glandular scales from *Rhododendron dauricum* and oil bodies from *Radula marginata* is expected to provide insights into the occurrence and importance of NADES in offering storage possibilities in heterologous hosts and thereby avoiding autotoxicity.

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