1 General introduction

1.1 Plant Secondary metabolites

Since ancient times, plants have been regarded as a source for molecules that support healing injuries, skin protection, pain relief, and numerous other applications (Petrovska, 2012). For more than a century the mysteries of plants as pharmaceuticals is studied, whereby little by little new knowledge is being found every day. An interesting group of molecules, which has important medicinal, health, and other beneficial functions, are secondary plant metabolites. The term “secondary” refers to metabolites, which do not belong to “primary” metabolites and are not essential for plant life, i. e. not involved in growth, production, and reproduction processes (Hartmann, 2007). These include a large group of structurally diverse compounds, which are synthesized in plants to increase fitness during the interactions between the plant and its environment (Pagare *et al.*, 2015). Secondary plant metabolites contribute to defence mechanisms against pathogens and protect plants against biotic and abiotic stress factors (Khare *et al.*, 2020). The abundance of secondary metabolites differs between plant species, and organs in the plant, depending on environmental conditions, physiology, growth stage, and stage of development (Khare *et al.*, 2020). There are different groups of secondary plant metabolites, including phenolic compounds, alkaloids, and terpenoids (Chomel *et al.*, 2016). Phenolic compounds contain benzene rings, with one or more hydroxyl substituents (Lin *et al.*, 2016; Velderrain-Rodriguez *et al.*, 2014). Around 10,000 phenolic compounds are isolated (Chomel *et al.*, 2016). They are synthesized in the shikimic acid pathway of plants and pentose phosphate pathway through phenylpropanoid metabolization (Randhir *et al.*, 2004). Phenolic compounds have several ecological benefits for plants, such as defence responses, and for humans, such as dietary impacts, for example as antioxidant agents (Lin *et al.*, 2016). With 10,000 compounds being isolated (Chomel *et al.*, 2016), alkaloid compounds are found not only in plants but also in bacteria, fungi, and animals (Cushnie *et al.*, 2014). They are characterized by carrying a nitrogen atom in their structure and have physiochemical, pharmacological, and toxicological properties (Cushnie *et al.*, 2014). Terpenoids are the largest group of plant secondary metabolites (Y. Zhang *et al.*, 2017). They derive from terpenes, hydrocarbons with carbon skeletons derived from isoprene units. Terpenoids may contain oxygen and their carbon skeleton may differ from the strict additive structure of isoprene units (Y. Zhang *et al.*, 2017).

1.2 Terpenoids

Terpenoids (also referred to as isoprenoids) are found in all biological domains, with plants being their main producers (Pichersky & Raguso, 2018). It is assumed that they emerged alongside the formation of primitive membranes at the very origins of cellular life (Ourisson G. & Y., 1994). Ancient archaebacterial diphytanylglycerol ether membrane components, polyprenols, and derived steranes and sterols are assumed to represent early terpenoid predecessors (Ourisson G. & Y., 1994). With more than 80,000 compounds, terpenoids are regarded as the largest group of plant secondary metabolites (Yazaki *et al.*, 2017). All terpenoids include the common building block isoprene (C_5H_8) in their structure (Figure 1.2-1).

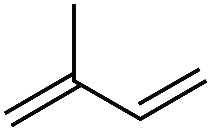

Figure 1.2-1 Structure of the isoprene unit (2-methylbuta-1,3-diene)

Based on the number of isoprene units (C_5) included, terpenoids can be divided in hemi (C_5)-, mono (C_{10})-, sesqui (C_{15})-, di (C_{20})-, sester (C_{25})-, tri (C_{30})-, tetra (C_{40})-, and poly-terpenoids, which contain one, two, three, four, five, six, eight, and more than eight isoprene units, respectively (Ghosh, 2016). They are structurally diverse and occur as cyclic, or linear compounds with different number of carbon atoms and versatile functional groups, which give them numerous natural functions. They play a major role in plant defence against pathogens (Cheng Ai-Xia *et al.*, 2007; Singh & Sharma, 2015). There are numerous examples showing the wide range of possible applications of terpenoids by humans. In the nutritional and food industry, terpenoids are used as additives and flavours. Some examples are the carotenoid bixin, an orange-red coloured pigment naturally found in the seeds of the achiote tree (*Bixa Orellana*) that is used as a natural dye in various foods, textiles, paintings, and cosmetic products (Rivera-Madrid *et al.*, 2016). Another example is the non-provitamin A carotenoid lycopene, that is responsible for the red colours seen in tomatoes, and pink grapefruit (Story *et al.*, 2010). Lycopene is used in the food industry (Pichersky & Raguso, 2018) and has been identified as an antioxidant agent with potential anticancer properties (P. Chen *et al.*, 2015). Numerous terpenoid compounds existing in essential oils have been applied in the fragrance and perfume industry. For instance, the monocyclic monoterpene D-limonene, occurring in citrus plants, is used as fragrance additive in perfume, and soap

production (Anandakumar *et al.*, 2021). Another example is geraniol, an acyclic monoterpenoid with a characteristic rose-like odour, a constituent of plant essential oils like rose, citronella, or palmarosa oil. Geraniol is widely used as a fragrance material in perfumes, cosmetics or household products (Gerke *et al.*, 2020). A huge number of terpenoids are present in vegetables and herbs and serve as well-known drugs in traditional medicine. Today, terpenoids are being intensively applied and studied for application as pharmaceuticals and for medical applications. The diterpene Taxol is one of the most well-known antitumoral drugs that has been used for over one million patients (Gallego-Jara *et al.*, 2020). Another important example is the sesquiterpene artemisinin, extracted from *Artemisia annua L.* (Qinghao) or recombinantly produced with yeast. Artemisinin was discovered from the Chinese herbal plant as an effective anti-malaria compound and has been used since the 1990s for treatment of malaria (Miller & Su, 2011).

In plants, terpenoids are synthesized from the precursor molecules isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). In higher plants, these molecules are generated in two different pathways: in plastids via the 2-*C*-methyl-*D*-erythritol-4-phosphate (MEP) pathway, and in the cytosol via the mevalonate (MVA) pathway (Cheng Ai-Xia *et al.*, 2007) (Figure 1.2-2).

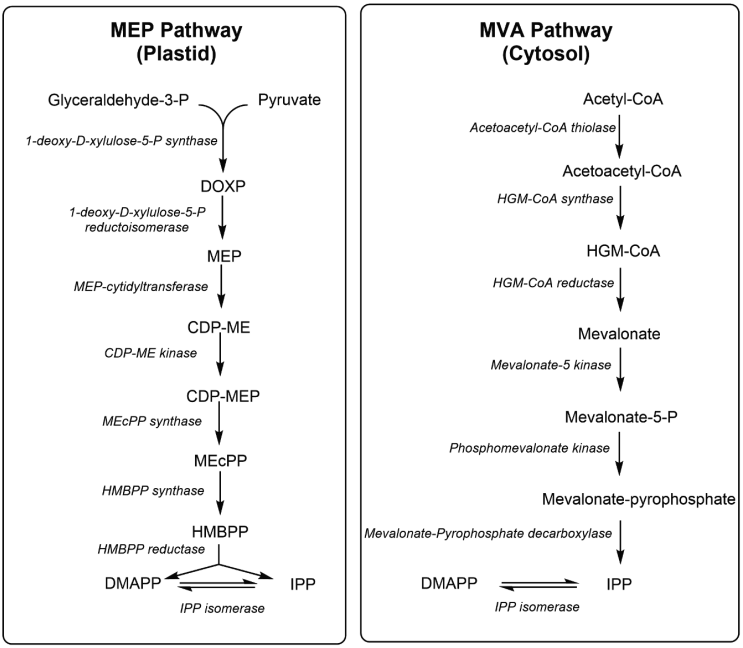

Figure 1.2-2 Biosynthesis pathway of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) from the 2-*C*-methyl-*D*-erythritol-4-phosphate (MEP) pathway in plastids and the mevalonate (MVA) pathway in the cytosol of plant cells. Abbreviations: DOXP: 1-deoxy-D-xylulose-5-P, MEP: 2-c-methyl-D-erythritol-4-P, CDP-ME: 4-diphosphocytidyl-2-c-methyl-D-erythritol, CDP-MEP: CDP-ME 2-phosphate, MEcPP: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, HMBPP: 1- hydroxy-2-methyl-2-butenyl 4-diphosphate. HGM-CoA: 3-hydroxy-3- methylglutaryl CoA. Figure adapted from (Rodriguez-Concepcion & Boronat, 2015)

IPP and DMAPP are converted by a class of prenyl transferase family proteins (F. Zhou & Pichersky, 2020) to the precursors of different terpenoid classes. These prenyl transferases are named according to the product they generate (Moses *et al.*, 2013). Through head-to-tail condensation of IPP and DMAPP, catalysed by geranyl diphosphate (GPP) synthase, GPP is synthetized, which is a precursor for of monoterpenoids (C_{10}). By fusing of GPP with an additional IPP catalysed by the enzyme farnesyl diphosphate synthase (Y. J. Zhao *et al.*, 2015) the sesquiterpenoid (C_{15}) precursor farnesyl diphosphate (FPP) is produced. Fusing FPP with IPP by geranylgeranyl diphosphate synthase (Fei Zhou *et al.*, 2017) generates geranylgeranyl diphosphate (GGPP) from which diterpenoids (C_{20}) are synthetized (Figure 1.2-3). Furthermore, condensation of two FPP or two GGPP molecules forms the central substrates of triterpenoid (C_{30}) and carotenoids (C_{40}), respectively (Karunanithi & Zerbe, 2019).

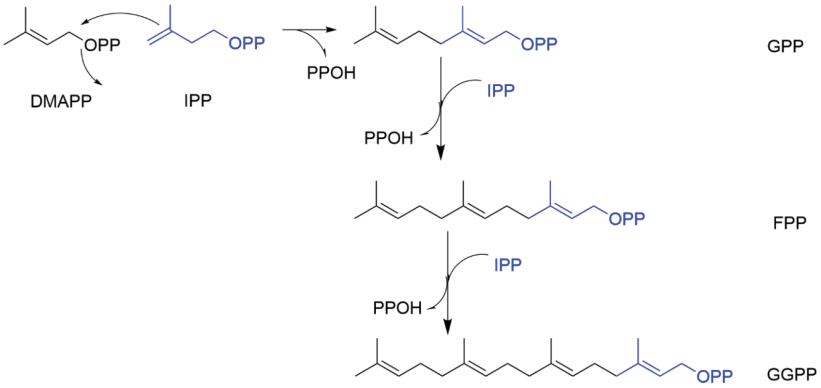

Figure 1.2-3 Head-to-tail condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) catalysed by the enzyme family prenyl transferases. Geranyl diphosphate catalyses the formation of GPP from IPP and DMAPP. Addition of one more IPP to GPP produces farnesyl diphosphate (FPP) catalysed by farnesyl diphosphate synthase. FPP is converted to geranylgeranyl diphosphate (GGPP) by geranylgeranyl diphosphate synthase.

Figure 1.2-4 shows an overview of the most relevant reaction steps in terpenoid synthesis in plants. The fact that MVA and MEP pathways are located in different compartments leads to a broader ability for a specialized terpenoid synthesis pathway and a better control of isoprenoid pools in different compartments (Karunanithi & Zerbe, 2019). MEP-derived mono- and diterpenoids, carotenoids, plastoquinones, and chlorophyll are synthetized in plastids, while MVA-derived sesquiterpenoids, sterols, and triterpenoids are located in the cytosol (Rodriguez-Concepcion & Boronat, 2015; Vranova *et al.*, 2013; Wille *et al.*, 2004). The physical separation of MEP- and MVA-pathways is tightly regulated by the cell. For instance, genome-wide co-expression studies in *Arabidopsis* showed minimal interaction between MVA and MEP genes (Rodriguez-Concepcion & Boronat, 2015; Vranova *et al.*, 2013; Wille *et al.*, 2004). On the other hand, active transfers of IPP, DMAPP, GPP, and FPP across the plastidial membrane shows that there is some cross-talk between the two pathways and compartments (Karunanithi & Zerbe, 2019).

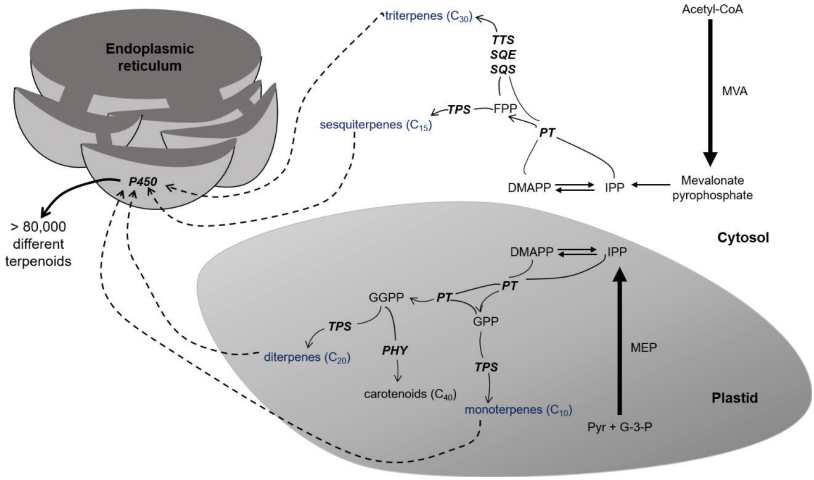

Figure 1.2-4 Overview of terpenoid biosynthesis in higher plants. Terpenoids are derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) that are synthetized via the cytosolic mevalonate pathway (MVA) from acetyl-CoA, and the plastidial 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway from pyruvate (Pyr) glyceraldehyde-3-phosphate (G-3-P). Prenyl transferases (PT) convert IPP and DMAPP to different intermediates geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). These intermediates are further converted to mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), and triterpenes (C_{30}). C_{10}-C_{20} terpenes are synthetized by terpene synthases (TPS). Synthesis of triterpenes starts with conversion of two FPP molecules to squalene by squalene synthase (SQS). Squalene epoxidase (SQE) converts squalene to 2,3-oxidosqualene, which is further converted to different triterpenes via triterpene synthase (TTS). The cytochrome P450 monooxygenase (P450) catalyses the oxygenation of these products. Further possible functional decorations lead to production of more than 80,000 distinct natural products. Figure adapted from (Karunanithi & Zerbe, 2019).

As shown in Figure 1.2-4, a large group of different terpenoids can be synthetized from a few substrate molecules. The terpenoid synthases, including trans-isoprenyl diphosphate synthases, squalene synthases, and terpene synthases, as well as triterpene cyclases, are regarded as drivers of terpenoid diversification. TSs are involved in construction of the basic backbone structure of terpenoids. While trans-isoprenyl diphosphate synthases and squalene synthases form basic linear chains, terpene synthases and triterpene cyclases cyclize and rearrange these, thus generating a huge diversity in terpenoid structures. Additionally, cytochrome P450 monooxygenases (CYPs) are further modifying the terpenoids (Boutanaev *et al.*, 2015). The mentioned enzymes enable the synthesis of terpenoids with complex structures and different functional groups, catering for a wide range of functions. Glycosyltransferases, not

depicted in Figure 1.2-4, further increase the biochemical space triterpenoids cover (Kurze *et al.*, 2022; Rahimi *et al.*, 2019). Due to their special importance in this thesis, triterpenoids are explained in more detail in the following paragraph.

1.1.1 Triterpenoids

The class of triterpenoids with 30 carbon units in their backbone comprises a vast group of different compounds, which are especially of great interest for applications in the pharmaceutical and cosmetic sectors. In combination with saccharide chains, triterpenoids form compounds called triterpenoid saponins, which gain huge interest for their beneficial properties. Saponins are non-volatile compounds composed of a polar (water-soluble) saccharide chain, and a non-polar (fat-soluble) aglycone (Singh *et al.*, 2017). Based on the aglycone moieties, one distinguishes triterpenoid saponins (dammaranes, ursanes, oleananes, lupanes, hopanes, etc.) and the sterol glycosides. Triterpenoid saponins are more diverse in their structure. These surface-active natural compounds have the ability to foam (hence the name saponin Latin "*sapo*", soap) (Podolak *et al.*, 2010). Due to their vast pharmacological benefits, such as anti-cancer, anti-inflammatory, anti-oxidant, cardioprotective, and many other activities, saponins are investigated intensively in for pharmaceutical purposes (Podolak *et al.*, 2010; Rao & Sung, 1995; Sparg *et al.*, 2004; W. Zhang *et al.*, 2013). Some famous examples are dammarene-type saponins from *Panax ginseng* (Chinese ginseng), a traditional medicine plant (Leung & Wong, 2010; Man *et al.*, 2010). Another example is glycyrrhizin from *Glycyrrhiza glabra*, which shows anti-viral, anti-bacterial, anti-fungal, and anti-inflammatory activities (Pastorino et al., 2018). Additionally, glycyrrhizin is discussed as a new potential therapeutic agent of natural origin for the potential treatment of COVID-19 infections caused by the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Al-Kamel & Grundmann, 2021).

More than 100 different triterpenoid carbon skeletons are found in plants (R. Xu *et al.*, 2004). Despite this huge diversity in their structure, all triterpenoids derive from the precursor 2,3-oxidosqualene (Shibuya *et al.*, 1999), which is synthetized from squalene by squalene epoxidase (SQE) (see Figure 1.2-5) (Basyuni *et al.*, 2007).

2,3-oxidosqualene, besides squalene, is the common precursor for phytosterols and triterpenoids in plants. The initial diversifying step in biosynthesis of triterpenoids is generated by an enzyme family of oxidosqualene cyclases (OSC), which convert 2,3-oxidosqualene to intermediate molecules of different triterpenoids (Y. Zhang *et al.*, 2017), such as tricyclic, tetracyclic, and pentacyclic triterpenoids (Figure 1.2-5). Pentacyclic triterpenoids comprise a large group of triterpenoids. For instance, oleanolic acid, which is abundant in plants of the *Oleaceae* family, is a pentacyclic

triterpenoid, which exerts pharmacological activities (Ayeleso *et al.*, 2017; X. Wang *et al.*, 2010). Due to its hepatoprotective effects, oleanolic acid has been used as a hepatic drug for over 20 years in China (H. Zhao *et al.*, 2013). Another example is ursolic acid, which naturally occurs in several plants such as in apples (Jager *et al.*, 2009) also showing tremendous health promoting factors (Seo *et al.*, 2018). This thesis mainly focusses on lupane-type pentacyclic triterpenoids, which occur in many plants, especially in birch trees (*Betula*). Extracts of the outer bark of different types of birch predominantly contain pentacyclic triterpenoids, with betulin being the main component, which causes the white colour of the birch bark (Demets *et al.*, 2022). In the outer part of the bark, betulin protects the plant from damaging environmental factors: radiation, bacteria, fungi, viruses, and insects (Kuznetsova *et al.*, 2014). All pentacyclic triterpenoids deriving from lupeol exhibit anti-viral, anti-HIV, wound-healing, and anti-cancer properties (Rios & Manez, 2018). Betulin shows wound-healing potentials (Metelmann *et al.*, 2013), and is used in the skin care and cosmetic industry. Its derivative betulinic acid is especially famous because of its strong anticancer activities. Betulinic acid has shown to be the selective inhibitor of the growth of human melanoma cells and other cancer cells (Surowiak *et al.*, 2009), exhibits cytotoxicity in several cancer cell lines and is capable of inducing apoptosis in cancer cells (Surowiak *et al.*, 2009).

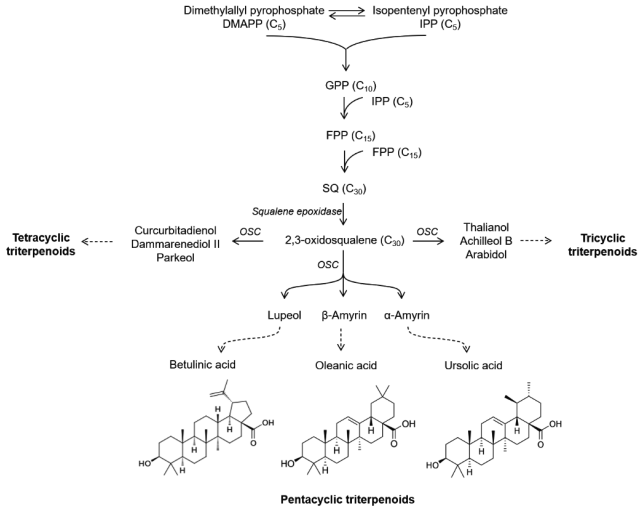

Figure 1.2-5 Simplified biosynthesis route of triterpenoids in plants. Squalene epoxidase converts squalene (SQ) to 2,3-oxidosqualene, the common precursor of triterpenoids. Oxidosqualene cyclases (OSC) catalyse the

conversion of 2,3-oxidosqualene to a different class of tricyclic, tetracyclic, and pentacyclic triterpenoids.

Figure 1.2-6 shows the reaction steps from 2,3-oxidosqualene to betulinic acid. Lupeol synthase (LUS) forms lupeol from oxidosqualene. Conversion of lupeol to betulinic acid is realized by a cytochrome P450 monooxygenase (CYP) and the respective reductase (CPR) with the precursor molecules betulin and betulin aldehyde.