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Biosynthesis and Emission of Isoprene, Methylbutanol and Other Volatile Plant Isoprenoids

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2.1 Introduction

Various phytogenic volatile organic compounds (VOCs) emitted by plants are found in the atmosphere, such as alkanes, alkenes, alcohols (e.g. methanol),[1] aldehydes, ethers, esters, carboxylic acids[2] and various types of isoprenoids.[3,4] A major part of these phytogenic VOCs is of isoprenoid origin being emitted from herbaceous plants and to a very high extent from green leaves of a great number of trees.[4–6] The largest proportion of volatile isoprenoids emitted from green vegetation consists of the volatile hemiterpene isoprene (2-methyl-1,3-butadiene, **1**), several monoterpenes and, yet much less important, certain sesquiterpenes (i.e. C_{15} isoprenoid compounds). Plants without isoprene emission or without formation of other isoprenoid volatiles can accumulate particular diterpenes, isoprenoid C_{20} products, such as different ginkgolides in the green leaves of ginkgo trees; however, such diterpenes are mostly accumulated within the plants since they are not very or no longer volatile.

Today a lot of emphasis is placed on the emission of volatile phytogenic isoprene and monoterpenes, due to their high impact on atmospheric chemistry and ozone formation.[3,7,8] Isoprene contributes to photochemical smog and enhances the formation of ozone. In order to better predict and control this biogenic isoprenoid emission and ozone formation, it is

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essential to understand the biological and physiological background of isoprene, monoterpene and sesquiterpene emission. Why and under which environmental conditions do plants emit these volatile isoprenoids? What types of change in the cell metabolism do and must occur in such plants to substantially reduce their amount of photosynthetically fixed carbon by the emission of isoprenoid VOCs, which essentially cuts down their productivity?

2.2 Plant Isoprenoids

Plants, animals and micro-organisms contain various primary isoprenoid compounds and partially also 'secondary' isoprenoid compounds (or natural products) that are composed of the C_5 units of 'active isoprene', which is known as isopentenyl diphosphate (IPP, **2**). These isoprenoid compounds are also termed terpenoids with hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes as C_5, C_{10}, C_{15}, C_{20}, C_{30} and C_{40} isoprenoids, respectively. Depending on the plant there exist: (*a*) hemiterpenes [C_5, e.g. isoprene (**1**)], (*b*) monoterpenes [C_{10}, e.g. geraniol (**3**), linalool (**4**), menthol (**5**), 1,4-cineol (**6**)], (*c*) sesquiterpenes [C_{15}, e.g. farnesol (**7**), bisabolol (**8**)], (*d*) diterpenes [C_{20}, e.g. phytol (**9**), camphorene (**10**), taxol (**11**), ginkgolides (e.g. ginkgolide A, **12**)], as shown in Figure 2.1, and (*e*) triterpenes [C_{30}, e.g. *Avena* saponins, oleanolic acid (**13**), cycloartenol (**14**)], (*f*) tetraterpenes [C_{40} compounds, such as the primary carotenoids β-carotene (**15**) and zeaxanthin (**16**), secondary carotenoids such as astaxanthin (**17**) and canthaxanthin (**18**) and the C_{20} apocarotenoid crocetin (**19**)], as shown in Figure 2.2. In several plant families very long-chain polyterpenes $(C_5H_8)_n$ are found (e.g. in rubber latex, gutta percha or in the chyle latex of several plant families, such as Euphorbiaceae or Asteraceae) whereby the number n for C_5 units ranges from about several thousands up to one million. Several plant compounds (i.e. the mixed prenyllipids), which are essential for the primary plant metabolism, obtain their lipophilic character by the possession of an isoprenoid side chain, such as the plastidic chlorophylls (phytyl chain), plastoquinone-9 (**20**, nona-prenyl side chain, Figure 2.2), phylloquinone K_1 (**21**, phytyl chain) or the mitochondrial ubiquinones Q-9 (**22**) and Q-10 (**23**) with nona-prenyl and deca-prenyl side chains, respectively.[9] In pure isoprenoid lipids (isoprene, phytol, menthol, cycloartenol, β-carotene) the complete carbon skeleton is composed of isoprenoid C_5 units, whereas in mixed prenyllipids (plastoquinone-9, phylloquinone K_1) the prenyl side chain is bound to a non-isoprenoid nucleus, such as a benzoquinone or naphthoquinone ring.

The 'biogenetic isoprene rule' (i.e. the composition of such terpenoid natural products from C_5 building blocks) was first detected in 1885 by Wallach,[10] and the head to tail addition of 'active C_5 units' was pointed out by Ruzicka *et al.*[11] This composition from a branched isoprenic C_5 unit is shown for several plant terpenoids in Figures 2.1 and 2.2. With respect to the biosynthesis of the isoprenoid chains, acetate[12] and acetyl-coenzyme A (acetyl-CoA)[13] were detected as biosynthetic precursors for cholesterol (triterpenoid derivatives) of mammals and fungi. Later mevalonic acid (**24**, MVA) was found as an intermediate[14] and IPP (**2**) as the active cellular biosynthetic C_5 unit (Figure 2.3).[15] Since ^{14}C-labelled acetate and acetyl-CoA were readily incorporated also into plant sterols and, in the case of the photosynthetic organism *Euglena*, also into carotenoids, it was generally accepted that all other isoprenoids of living cells and the large variety of different plant terpenoids including isoprene, monoterpernes, diterpenes, carotenoids

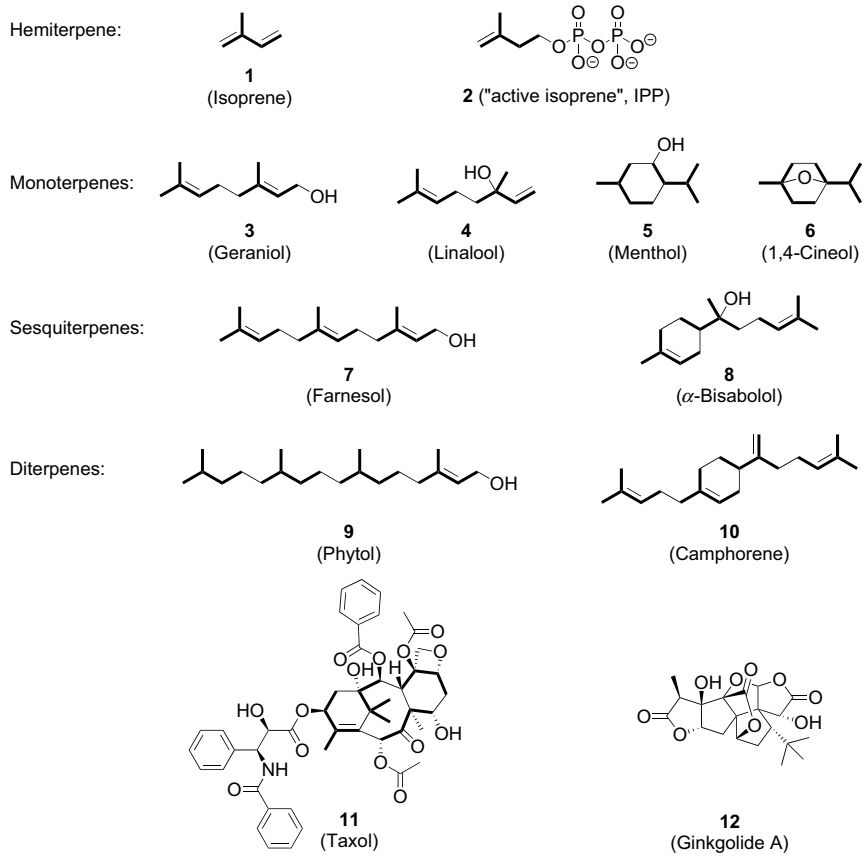


Figure 2.1 *Chemical structures of isoprene (**1**) and several other plant isoprenoids and terpenes (mono-, sesqui-, diterpenes) with indication of the isoprenoid C_5 units (printed in bold face) that make up the carbon skeleton of the final product*

and polyterpenes are synthesized via this isoprenoid pathway known as cytosolic acetate/mevalonate pathway.

For more than three decades it had been accepted that all plant terpenoids were synthesized from acetyl-CoA via this classical acetate/MVA pathway that proceeds in the cytosol of the plant cell catalysed by microsomes. This pathway is present in Plants, Fungi, Animals and Archaea, but generally not in Eubacteria.[16] An exception among the Eubacteria are only a few gram-positive cocci, such as *Streptococcus pneumonia*,[17] which obtained the MVA pathway apparently by lateral gene transfer. The acetate/MVA pathway starts from 3-acetyl-CoA, proceeds via hydroxyl-methylglutaryl-CoA (HMG-CoA) and mevalonate (MVA) to finally yield IPP.[18,19] This pathway can specifically be blocked by mevinolin (**25**) and other statins (e.g. cerivastatin, compactin, lovostatin), which inhibit the plant's microsomal enzyme HMG-CoA reductase, as first shown for mevinolin by Bach and Lichtenthaler.[20,21] Mevinolin (**25**) did, however, not block the bioynthesis and accumulation

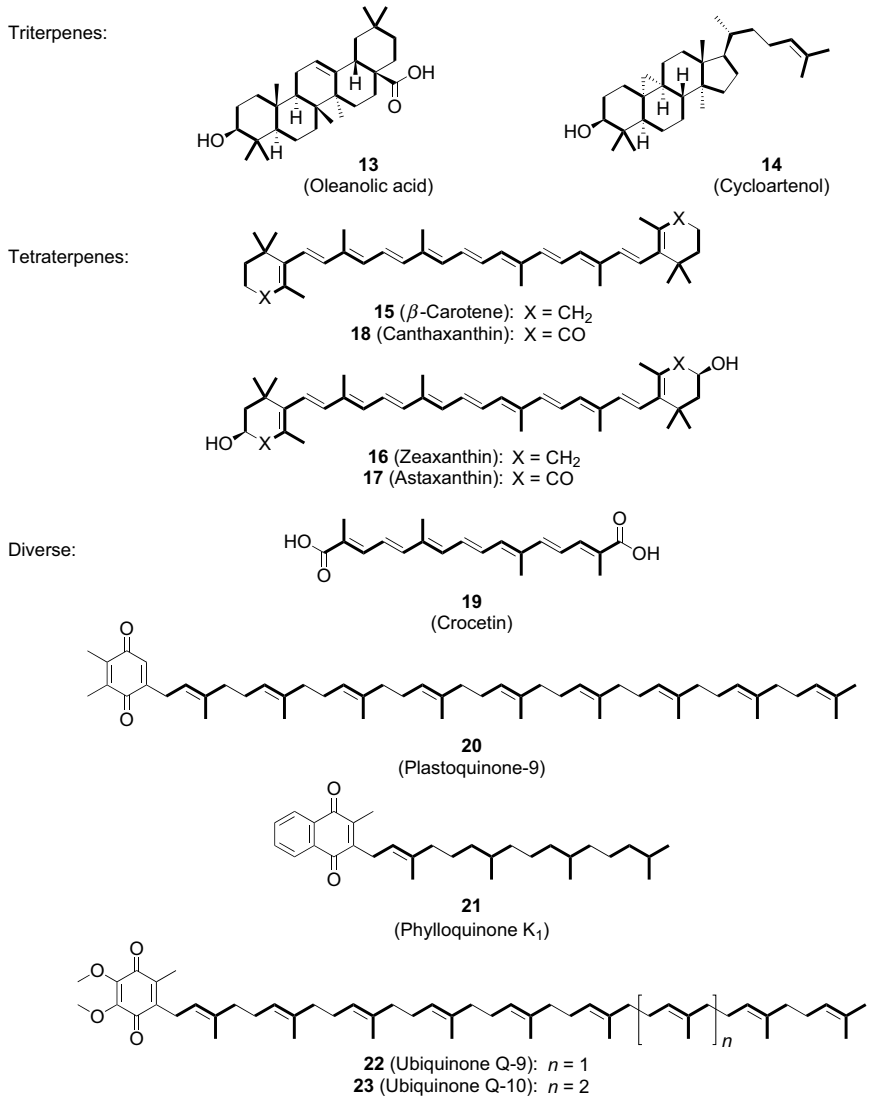


Figure 2.2 *Chemical structures of several plant isoprenoids and terpenes (tri- and tetraterpenes, some other structures) with indication of the isoprenoid C_5 units (printed in bold face) that make up the carbon skeleton of the final product*

of the isoprenoids in chloroplasts, such as carotenoids and chlorophylls (phytyl side chain).[22] This and other inconsistencies showing up in the labelling of plastidic isoprenoids (carotenoids, phytol chain of chlorophylls, prenyl side chain of plastoquinone-9), such as little or no labelling by ^{14}C-acetate or ^{14}C-mevalonate, finally led to the conclusion that chloroplasts might possess a separate biosynthetic pathway for IPP formation, as reviewed by Lichtenthaler *et al.*[19,23,24]

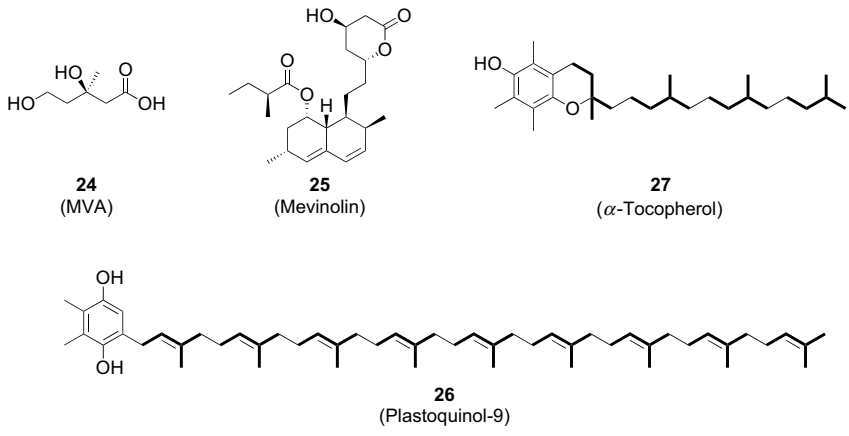


Figure 2.3 *Chemical structures of mevalonate (MVA, **24**), mevinolin (**25**), α-tocopherol (**27**) and plastoquinol-9 (**26**) with indication of the isoprenoid C_5 units (printed in bold face)*

2.3 Two IPP-Yielding Pathways in Plants

Today it is well known that plants possess two biochemically fully independent cellular pathways for the biosynthesis of isoprenoids:

- The *acetate/mevalonate* pathway (*acetate/MVA* pathway) in the cytosol that yields IPP for the biosynthesis of sterols, sesquiterpenes and ubiquinones.
- The *deoxyxylulose phosphate/methylerythritol phosphate* (*DOXP/MEP*) pathway in the chloroplasts that is responsible for the biosynthesis of carotenoids, chlorophylls (phytyl chain), plastoquinol-9 (**26**, nona-prenyl side chain), phylloquinone K_1 (**21**, phytyl side chain) and α-tocopherol (**27**, prenyl side chain) as well as isoprene, monoterpenes and diterpenes.

The DOXP/MEP pathway of chloroplasts was only detected in the early 1990s by the joint co-operation of the working groups of Lichtenthaler (Karlsruhe) and Rohmer (Mulhouse/Strasbourg) upon applying new labelling techniques.[23,25–27] When using (*a*) labelling with ^{13}C-glucose combined with high-resolution NMR spectroscopy and (*b*) deuterium-labelling complemented by mass spectroscopy, a new labelling pattern of plastidic isoprenoids was detected that did not at all correspond to a labelling via the cytosolic acetate/MVA pathway. This DOXP/MEP pathway of isoprenoid biosynthesis was shown to occur in all chloroplast-containing, photosynthetic oxygen-evolving organisms, such as green algae[28] and higher plants (first reported by Lichtenthaler *et al.*[23] and Zeidler *et al.*[29,30]) as well as in the free living prokaryotic cyanobacteria[25] which are supposed to have the same evolutionary ancestors as the endosymbiotic chloroplasts. Later, these findings were confirmed by several other research groups, as reviewed by Lichtenthaler[19,25,26] and Rohmer.[27] The only exception of a photosynthetic organism producing carotenoids from IPP synthesized via the acetate/mevalonate pathway is *Euglena* which, in the course of evolution, has lost its plastidic DOXP/MEP pathway of IPP biosynthesis.[16,25,31] Besides photosynthetic organisms, the DOXP/MEP pathway also occurs in the

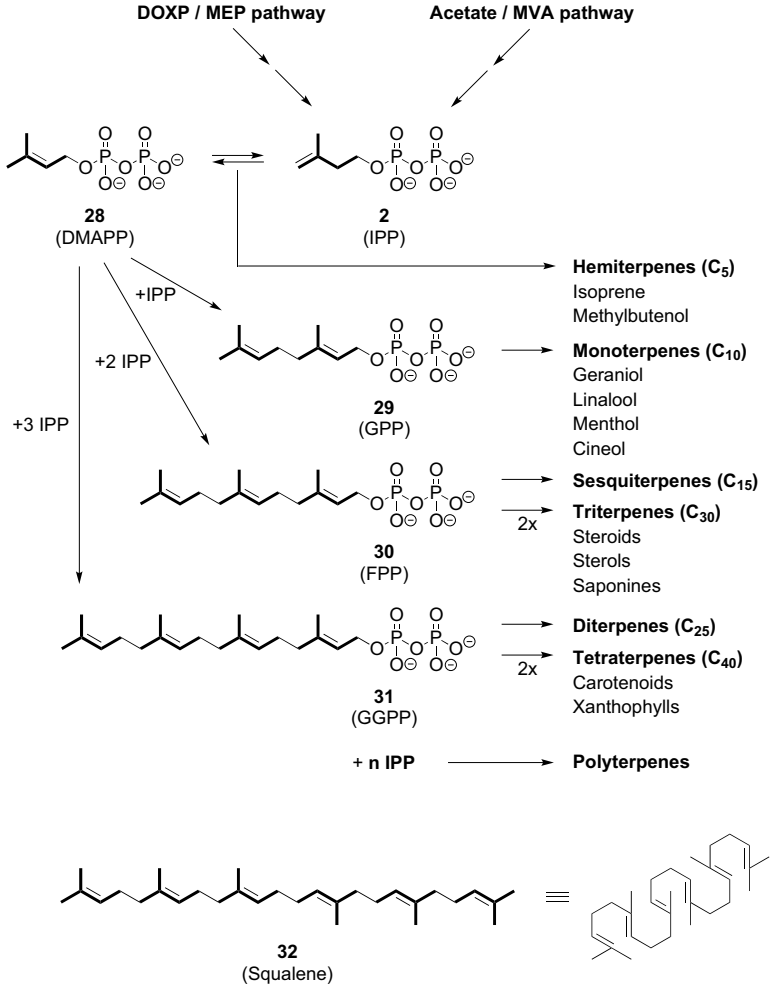
heterotrophic, mostly pathogenic eubacteria, such as *Escherichia coli*, *Haemophilus influenzae*, *Helicobacter pylori*, *Chlamydia pneumoniae*, *Mycobacterium tuberculosis* and *Vibrio cholerae*,[16,26] organisms which might have evolved from originally photosynthetic bacteria after loss of their photosynthetic competence.

2.4 Prenyl Chain Formation and Elongation

Both cellular isoprenoid pathways of photosynthetic organisms provide IPP which, via an isomerase, can be transferred to its isomer 3,3-dimethylallyl diphosphate (DMAPP, **28**, Scheme 2.1). By the action of the enzyme isoprene synthase, isoprene is directly formed from DMAPP. When the C_5 unit of IPP is added to the starter molecule DMAPP in a head to tail condensation, geranyl diphosphate (GPP, **29**), the C_{10} prenyl diphosphate, is formed. By head to tail condensation of one or two more IPP units to GPP, the C_{15} and C_{20} prenyl diphosphates are formed [i.e. farnesyl diphosphate (FPP, **30**) and geranylgeranyl diphosphate (GGPP, **31**); Scheme 2.1]. Sterols, being found in several cytosolic cell membranes, are synthesized via a tail to tail condensation of the C_{15} carbon skeletons of two FPP (dimerization), yielding first squalene (**32**) and then the triterpene cycloartenol (**14**, see Figure 2.2). The C_{30} isoprenoid structure is further modified, for example by the removal of three methyl groups, to form the final sterol ring structure. Carotenoids as tetraterpenoids are composed of eight isoprenic C_5 units. They are formed by a tail to tail condensation of two GGPP molecules followed by a successive desaturation of their C_{40} prenyl chain. This biogenetic relationship is summarized in Scheme 2.1. Higher prenyl chain homologues are synthesized via the addition of the C_5 carbon skeletons of several hundred or thousands of IPP molecules, a process which is the basis for the biosynthesis of polyterpenes, as found in the white latex of Euphorbiaceae and Asteraceae, for example in the latex of the natural rubber tree *Hevea brasiliensis* that primarily contains long-chain *cis*-1,4-polyisoprene molecules but also the plastidic isoprenoids plastoquinol-9 (**26**) and α-tocopherol (**27**).

2.5 Compartmentation of Plant Isoprenoid Biosynthesis

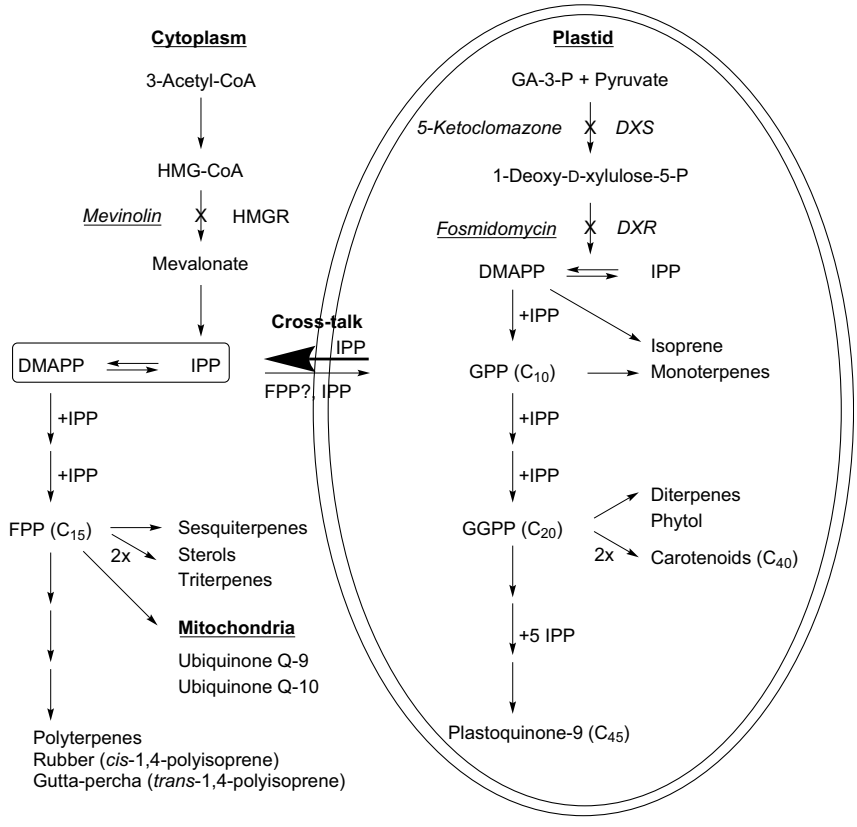
The compartmentation of isoprenoid biosynthesis in the plant cell is shown in Scheme 2.2. The *plastidic DOXP/MEP* pathway starts from glyceraldehyde-3-phosphate (GA-3-P, **33**, Figure 2.4) and pyruvate (**34**) with 1-deoxy-D-xylulose 5-phosphate (DOXP, **35**) as the first product that is transferred to DMAPP and IPP in six further enzymatic steps. By head to tail condensation the latter form GPP, to which more C_5 carbon skeletons of IPP are condensed to eventually form the isoprenoid chains of the different carotenoids and plastidic prenyl lipids, mono- and diterpenes, etc. In contrast, the *cytosolic acetate/mevalonate* pathway starts from 3-acetyl-CoA and provides IPP and DMAPP for cytosolic sterol biosynthesis, sesquiterpenes and polyterpernes as well as the prenyl side chains of ubiquinones Q-9 (**22**) and Q-10 (**23**). Both cellular isoprenoid pathways can specifically be inhibited: the acetate/mevalonate pathway by mevinolin (**25**) and other statins (target: HMG-CoA reductase),[20–22,32] whereas the DOXP/MEP pathway is specifically blocked by fosmidomycin (**36**; target: DOXP reductoisomerase, DXR)[26,30] and by 5-ketoclomazone (**37**; target: DOXP synthase, DXS),[26,33,34] as indicated in Scheme 2.2.



Scheme 2.1 *Biogenetic relationship of plant isoprenoids and terpenes being formed from the starter molecule dimethylallyl diphosphate (DMAPP) by head to tail condensation of one or more isopentenyl diphosphates (IPP). Depending on the type and cellular localization of the plant isoprenoid, the IPP molecules are derived either from the cytosolic acetate/mevalonate pathway or the plastidic DOXP/MEP pathway. Triterpenes and tetraterpenes are synthesized by dimerization (tail to tail condensation) of two farnesyl and geranylgeranyl diphosphates, respectively*[4,19]

2.6 The Enzyme Steps of the Plastidic DOXP/MEP Pathway of IPP Formation

The nonmevalonate DOXP/MEP pathway in plastids starting from GA-3-P (**33**) and pyruvate (**34**) requires seven enzymes, three ATP equivalents [adenosine-5′-triphosphate (ATP) or cytidine-5′-triphosphate (CTP)] and three nicotinamide adenine dinucleotide



Scheme 2.2 *Compartmentation of the two isoprenoid biosynthesis pathways in the plant cell. (1) The chloroplastidic DOXP/MEP pathway for the biosynthesis of the active C_5 units (IPP) for chlorophylls (phytyl side chain), carotenoids, prenylquinones (isoprenoid side chains), mono- and diterpenes. (2) The cytosolic acetate/mevalonate pathway of IPP biosynthesis for the formation of sterols, sesquiterpenes, triterpenes, polyterpenes and the prenyl side chain of the mitochondrial ubiquinones Q-9 and Q-10. Specific inhibition is indicated for the acetate/mevalonate pathway by mevinolin (target: HMG-CoA reductase = HMGR), the DOXP/MEP pathway by 5-ketoclomazone (target: DOXP-synthase = DXS) and fosmidomycin (target: DOXP-reductase = DXR). Possible cross-talk between the two cellular biosynthetic isoprenoid pathways, which primarily consists of an export of active C_5 units from chloroplasts to the cytosol,[42] is accentuated*[23,25,30,33,34,42]

phosphates (NADPH), as shown in Scheme 2.3. The enzymes and their genes are well defined today. The DOXP synthase (DXS), a thiamine-dependent transketolase-type enzyme, is the *first enzyme* of this IPP-producing pathway. It condenses pyruvate with GA-3-P, yielding DOXP, the first C_5 chain of this pathway. 5-Ketoclomazone (**37**), an oxidation product of the herbicide clomazone, specifically inhibits the DOXP synthase.[33,34] *Enzyme 2* is the DOXP reductoisomerase (DXR) and reduces DOXP to 2-*C*-methylerythritol-4-phosphate (MEP, **38**).[33,35] This enzymic reaction comprises, besides the NADPH catalysed reduction step, an intramolecular rearrangement of the carbon

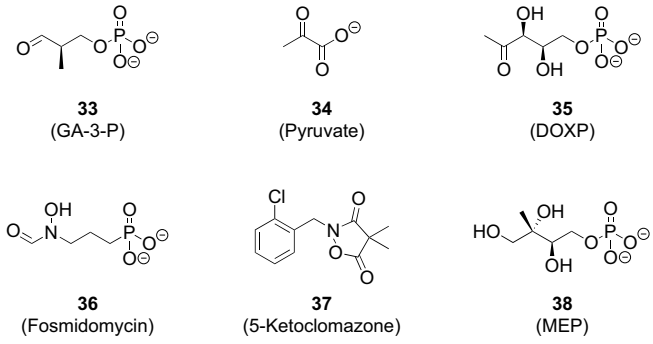


Figure 2.4 *Chemical structures of glyceraldehyde-3-phosphate (GA-3-P,* ***33****), pyruvate (****34****), deoxyxylulose phosphate (DOXP,* ***35****), 2-C-methylerythritol-4-phosphate (MEP,* ***38****) and enzyme inhibitors fosmidomycin (****36****) and 5-ketoclomazone (****37****)*

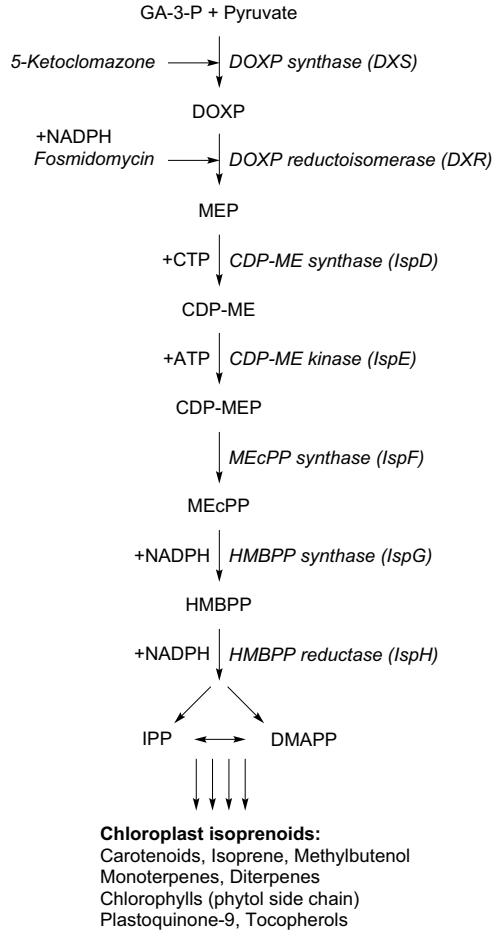
skeleton. The DOXP reductoisomerase is apparently the major regulatory step of the whole DOXP/MEP pathway. It is specifically blocked by fosmidomycin (**36**), as independently shown for plants[30,35] and for Eubacteria[36] that also possess the DOXP/MEP pathway. Fosmidomycin is a structural analogue of MEP, the intermediate in the enzymic reaction of DXR.

Enzyme 3, the CDP-ME synthase, catalyses the activation of MEP by CTP to form CDP-methyl-D-erythritol (CDP-ME, **39**, Figure 2.5).[37] *Enzyme 4*, CDP-ME kinase, phosphorylates CDP-ME to CDP-ME2P, which is transformed by *enzyme 5*, MEcPP synthase, to 2-*C*-methyl-D-erythritol-2,4-cyclo-diphosphate (MEcPP, **40**). *Enzyme 6*, HMBPP synthase, reduces MEcPP to 4-hydroxy-3-methyl-2-(*E*)-butenyl-diphosphate (HMBPP, **41**), as was independently verified by several groups.[38] *Enzyme 7*, the HMBPP reductase, was detected by several groups.[39] The HMBPP reductase (gene: *IspH*, formerly *lytB*) in one step yields two isoprenoid C_5 diphosphate products: IPP and its isomer DMAPP, usually at a ratio of 5:1 or 3:1. Depending on the cellular metabolic demand, IPP and DMAPP can be interconverted by a plastidic IPP isomerase.

All seven enzymes of the DOXP/MEP pathway have been isolated and their genes have been cloned in plants and eubacteria (as reviewed for the first five enzymes by Lichtenthaler[16,26] and for all seven enzymes by Rodríguez-Concepción and Boronat and by Eisenreich *et al.*[40]). These are *DXS*, *IspC* (*DXR*), *IspD* (*ygbP*), *IspE* (*ychB*), *IspF* (*ygbB*), *IspG* (*cpE*) and *IspH* (*lytB*), with the old names given in parentheses. The gene (*Idi*) for the plastidic IPP isomerase is also known. As in the case of many other chloroplast enzymes, the genes of the seven DOXP/MEP pathway enzymes are encoded by the nuclear genome, yet the final enzyme proteins operate in chloroplasts and also in non-green plastid forms. For this purpose the enzyme proteins of the DOXP/MEP pathway, being synthesized in the cytosol, possess a transit peptide sequence that directs them to their proper organelle, the chloroplast.

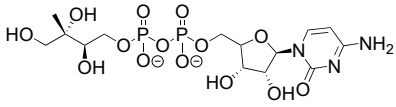
2.7 Cross-Talk Between the Two IPP Biosynthesis Pathways

There exists some cross-talk between the two cellular isoprenoid-forming pathways (see Scheme 2.2) that was first demonstrated by using cross-labelling of the plastidic isoprenoid

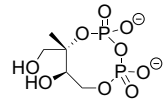


Scheme 2.3 *Biochemical sequence of the DOXP/MEP pathway of plastidic isoprenoid biosynthesis in plants yielding the active isoprenic C_5 units IPP and DMAPP used for biosynthesis of plastidic isoprenoids (chlorophylls, carotenoids) as well as isoprene, monoterpernes and diterpenes. The pathway, starting from pyruvate and GA-3-P, consists of seven enzymes and consumes three NADPH and three ATP equivalents to yield one active C_5 diphosphate (IPP or DMAPP). The first two enzymatic steps can specifically be inhibited by 5-ketoclomazone and fosmidomycin, respectively.*[26,30,33] *The genes of the seven enzymes have been cloned, their names are shown in italics in parentheses following the enzyme names. Via the enzyme IPP isomerase, whose gene (Idi) is also known, IPP and DMAPP can be converted into each other*

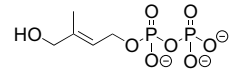
phytol (**9**, side chain of chlorophylls) and the cytosolic sterols from precursors of the plastidic DOXP/MEP pathway and the cytosolic mevalonate pathway in two algae and a higher plant (*Lemna gibba*) under photosynthetic conditions. When ^{14}C-labelled deoxyxylulose (^{14}C-DOX, ^{14}C-**42**, Figure 2.6) and tritium-labelled mevalonolactone (^{3}H-MVL, ^{3}H-**43**) were applied as tracers, the degree of radioactivity incorporation in phytol and sterols was determined. Phytol and sterols were labelled by their proper precursor



39
(CDP-ME)



40
(MEcPP)



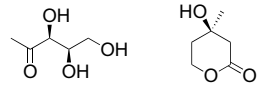
41
(HMBPP)

Figure 2.5 *Chemical structures of CDP-methyl-D-erythritol (CDP-ME,* ***39****), 2-C-methyl-D-erythritol-2,4-cyclo-diphosphate (MEcPP,* ***40****) and 4-hydroxy-3-methyl-2-(E)-butenyl diphosphate (HMBPP,* ***41****)*

substances ^{14}C-DOX and ^{3}H-MVL.[41] The rather good labelling of the cytosolic sterols from ^{14}C-DOX indicated an export of isoprenoid units from the chloroplast to the cytosol. A labelling of phytol from ^{3}H-MVL occurred only at very low rates[42] indicating that under photosynthetic conditions the cross-talk worked primarily in a chloroplast to cytosol direction. Other approaches were performed by several authors using specific inhibitors of the two cellular IPP-producing pathways (e.g. mevinolin (**25**) on the one hand and fosmidomycin (**36**) or 5-ketoclomazone (**37**) on the other hand), as reviewed by Lichtenthaler.[43] However, an adequate compensation for the inhibited activity of one cellular isoprenoid pathway by the second isoprenoid pathway of the other cell compartment apparently did not and does not occur under physiological standard conditions of growth.

It is evident from the currently available data of several laboratories that, under photosynthetic conditions, there occurs a unidirectional, relatively high flow of plastidic C_5 isoprenoid units into cytosolic sterol and sesquiterpene or polyterpene biosynthesis, as shown and reviewed by Lichtenthaler[42] and Nagegowda *et al.*[44] This export of isoprenoid C_5 units into cytosolic isoprenoid biosynthesis is presumably mediated by the plastidial unidirectional proton symport system[45] that can transport IPP and geranyl diphosphate (GPP, **29**) in a plastid to cytosol direction. Thus, the biomembranes of the chloroplast envelope play an essential role in mediating the cross-talk between both IPP-producing pathways. In addition, it is well known that the chloroplast envelope biomembranes also possess many other biosynthetic capacities and mechanisms[46] to transport various compounds, for example triose phosphates and phosphates, pyruvate and organic acids (malate, oxalacetate, glutamate) via special transporter/antiporter systems (triosephosphate/phosphate translocator, dicarboxylate transporter, phosphoenolpyruvate/phosphate transporter) from the chloroplast to the cytosol and vice versa.

In illuminated green leaves chloroplasts yield the starter substances GA-3-P and pyruvate in the course of the photosynthetic CO_2 fixation process (Calvin cycle) and in the



42
(DOX)

43
(MVL)

Figure 2.6 *Chemical structures of 1-deoxy-D-xylulose (DOX,* ***42****) and mevalonolactone (MVL,* ***43****)*

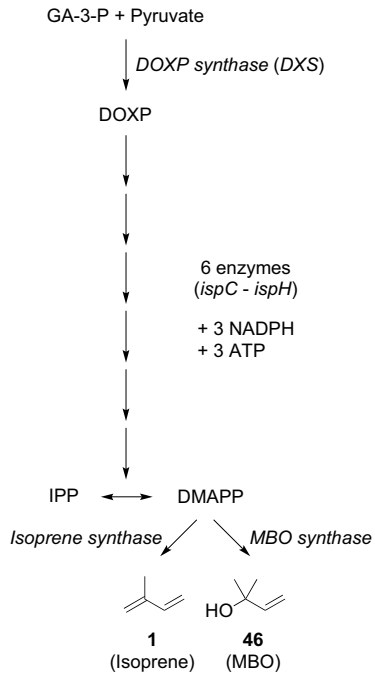
photosynthetic light reactions the three ATP and three NADPH required for plastidic IPP biosynthesis. Under light conditions green plant cells use this efficient IPP biosynthesis program of chloroplasts to also provide, via a fast export system, the C_5 building blocks (IPPs) required for the cytosolic sterol and sesquiterpene biosynthesis.[42,43] In contrast, in the dark and apparently also in most cases in non-green plant tissues, the cytosolic acetate/mevalonate pathway is fully responsible for supplying the IPP molecules for sterol and sesquiterpene biosynthesis. However, there are exceptions to this general rule. Thus, Dudareva *et al.*[47] unambiguously demonstrated that, in the chlorophyll-free epidermis of snapdragon petals, the DOXP/MEP pathway of the non-green plastids (leucoplasts) provided the IPP precursors not only for the synthesis of volatile plastidic monoterpenes, but also for the biosynthesis of cytosolic volatile sesquiterpenes. In addition, Hampel *et al.*[48] demonstrated in carrot leaves and carrot roots that monoterpenes are synthesized exclusively via the DOXP/MEP pathway, whereas the C_5 carbon skeletons of cytosolic sesquiterpenes are generated via the classical MVA pathway as well as via the plastidic DOXP/MEP pathway.

2.8 Biosynthesis and Emission of Volatile Isoprene at High Irradiance

The branched chain hydrocarbon isoprene (**1**) is emitted by a great number of higher plants and also by mosses, ferns and gymnosperms.[6] The isoprene emission by plants was independently discovered in the late 1950s and at the beginning of the 1960s by Sanadze and by Went.[49] In evolutionary terms, isoprene emission appears to be a very old trait of plants that may have been common to all photosynthetically active algae and the first land plants. During further evolution isoprene emission was apparently lost in many plants, but was retained in other plants as a major mechanism for survival during high-temperature, high-irradiance and other stress conditions. Its emission from plants amounts to hundreds of millions of metric tonnes to the global atmosphere; the estimates range from 180 to 450×10^{12} g carbon $year^{-1}$ worldwide.[50] It is of great interest in this respect that more organic carbon is lost from plants as isoprene than as any other volatile plant molecule.[8] The light- and temperature-dependent isoprene emission by leaves preferentially occurs at high rates at temperatures above 28 °C and at high irradiance, such as full sun light, when the photosynthesis process with its light reactions and associated electron transport reactions is almost fully light-saturated.

Trees are responsible for the highest rates of isoprene emission of green leaves, for example *Acacia nigrescens* (African acacia), *Eucalyptus globulus* (blue gum), *Liquidambar styraciflua* (sweet gum), *Populus nigra* (black poplar) and other *Populus* species, *Quercus robur* (European oak, pedunculate oak), *Quercus coccinea* (scarlet oak), *Salix babylonica* (weeping willow) and other plants such as *Arundo donax* (giant reed), *Myrtus communis* (myrtle) and *Pueraria* spp. (kudzu), with emission rates of 30 to 300 µg isoprene g^{-1} dw h^{-1}.[4,51]

Isoprene is synthesized via the DOXP/MEP pathway and requires three ATP and three NADPH molecules (Scheme 2.4).[29,52] With its two conjugated double bonds it has a typical UV spectrum, and its time-dependent continuous emission at high light conditions can easily be measured in plant leaves via a photometric UV cuvette test system using leaf pieces (Figure 2.7).[53] A leaf section in the closed UV cuvette with its main leaf vein



Scheme 2.4 *Biosynthesis of the volatile hemiterpene isoprene in chloroplasts from pyruvate and GA-3-P via the DOXP/MEP pathway. Seven enzymes (genes) are involved in the biosynthesis of the active isoprenoid C_5 unit IPP, and the cofactor requirements are three ATP and three NADPH. Isoprene (**1**) and MBO (**46**) are set free from the IPP isomer DMAPP through the action of plastidic hemiterpene synthases*

immersed in water is exposed from the side, perpendicular to the leaf surface, to high irradiance (above 2000 µmol m^{-2} s^{-1}) and a temperature above 30 °C (e.g. water bath). In a spectrophotometer one can register, via the measuring light beam that passes through the cuvette unhindered by the leaf, the continuous emission of isoprene after one, two or four hours. In this test system one can investigate the leaf capacity of different plants for isoprene emission. In addition, it can be checked if leaves of particular plants are able to produce isoprene or not. Another method to prove the isoprene emission of leaves, including various conifer needles, is the collection of the emitted isoprene gas in a closed system and its injection into a gas chromatography system combined with a mass spectrometer (GC-MS).[53]

There occurs a rapid path of photosynthetically fixed carbon into isoprenoid hydrocarbons, as shown by the rapid appearance of ^{14}C activity (after applying $^{14}CO_2$) in prenyl chains, such as carotenoids and the phytyl chain of chloroplasts, for example as shown for the green alga *Chlorella*.[54] This also applies to isoprene. Under high irradiance and at a temperature above 30 °C isoprene is instanteously formed de novo. In fact, the fast appearance of ^{13}C-labelling in isoprene from photosynthetically fixed $^{13}CO_2$ suggested that isoprene biosynthesis must be closely connected to intermediates of the photosynthetic

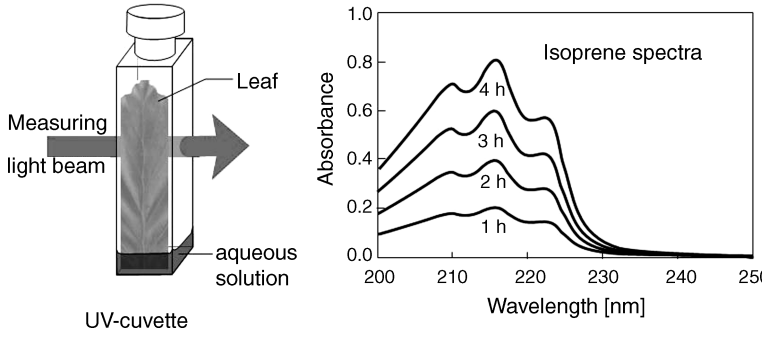


Figure 2.7 *Measurement of isoprene emission by leaves via the UV cuvette system. The isoprene emitted at high light (> 1500 μmol m^{-2} s^{-1}) and high temperature (> 30 °C) conditions is collected in a closed UV cuvette and its time-dependent emission measured in a spectrophotometer*[53,60]

CO_2 fixation pathway, known as the Calvin cycle.[55,56] In 1997 we demonstrated that the biosynthesis of isoprene proceeds via the plastidic DOXP/MEP pathway for isoprenoid biosynthesis from GA-3-P and pyruvate.[29,52] GA-3-P is an early product and an intermediate in the photosynthetic Calvin cycle. In several plants, for example spinach, pyruvate can directly be formed in chloroplasts from phosphoglyceric acid (PGA, **44**, Figure 2.8), another early product of the Calvin cycle.[57]

After detecting the plastidic DOXP/MEP pathway of IPP formation, we showed the specific incorporation of deuterium-labelled 1-deoxy-D-xylulose (^{2}H-DOX, ^{2}H-**42**) in the form of its xyluloside (methylglycoside) into isoprene and also into phytol, the prenyl side chain of chlorophylls (Scheme 2.5). This incorporation was verified via GC-MS and via high-resolution NMR spectroscopy.[29,52,53] DOX, the nonphosphorylated precursor substance, is rapidly phosphorylated in the plant cell by a cytosolic enzyme into DOXP (**31**), the actual intermediate precursor of the plastidic isoprenoid pathway.[58] DOXP can be transported from the cytosol into chloroplasts by the xylulose-5-phosphate translocator, a recently discovered plastidic transporter for pentose phosphates.[59]

Deuterium-labelled 1-deoxy-D-xylulose (^{2}H-DOX) was readily incorporated into isoprene by the green leaves of *Chelidonium majus* (greater celandine), *Eucalyptus globulus* (blue gum), *Platanus x acerifolia* (plane tree), *Populus nigra* (poplar), *Robinia pseudoacacia* (locust) and *Salix viminalis* (common willow), whereby a labelling degree of 30–80% was achieved.[60] This was proved using the GC-MS technique, whereby isoprene and other volatile

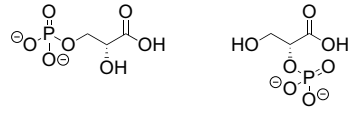
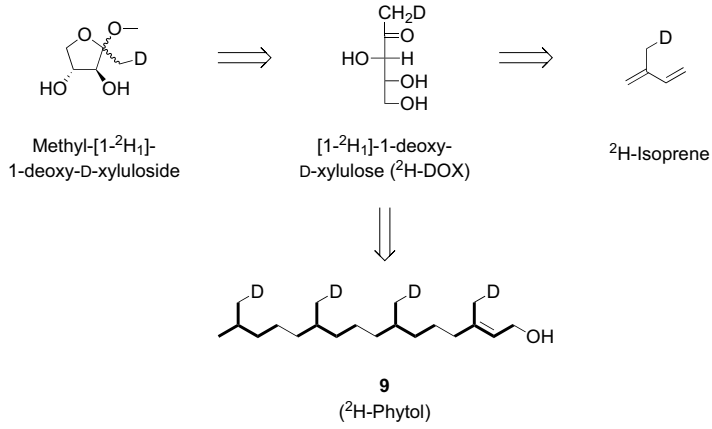


Figure 2.8 *Chemical structures of 3- and 2-phosphoglyceric acid (PGA, **44a** and **44b**)*



Scheme 2.5 *Specific incorporation of deuterium-labelled* [1-^{2}H]*-deoxyxylulose (D-DOX) into the volatile hemiterpene isoprene and phytol (side chain of chlorophylls). The deuterium label was found in the expected and specific methyl group of isoprene and in the four methyl groups of phytol, which proved DOX and DOXP to be a precursor and an intermediate, respectively, of the pastidic DOXP/MEP pathway*[25,52] *(D = deuterium label)*

isoprenoids emitted were collected by means of the solid phase microextraction (SPME) method with one drop of decane hanging in the closed experimental cuvette. In contrast, in blue gum leaves ^{2}H-DOX was not incorporated into the monoterpene 1,8-cineol which, after isoprene, was the next most frequent volatile isoprenoid.[60] This indicates that isoprene is spontaneously formed and emitted, whereas the emitted monoterpene cineol had been synthesized before and was released at high-light and high-temperature conditions apparently from a depot within the leaves.

The final step of the DOXP/MEP pathway yields both isoprenoid C_5 diphosphates, IPP and its isomer DMAPP. Isoprene is set free from DMAPP in a single enzymatic step via the plastidic isoprene synthase.[61] The latter exists in a thylakoid-bound form[62] and also as isoforms in the chloroplast stroma.[63] The enzyme isoprene synthase, a hemiterpene synthase, is related to monoterpene synthases found in other plants.[64] Its K_m is 10- to 100-fold higher for its DMAPP substrate than the related monoterpene synthases for their substrate geranyl diphosphate (GPP).[65] These findings indicate that isoprene biosynthesis apparently starts only when, under particular environmental conditions (e.g. high irradiance, heat stress), the concentration of DMAPP in the chloroplast rises to considerably higher levels. This assumption would also mean that isoprene biosynthesis starts when other processes that use either GA-3-P and pyruvate or IPP and DMAPP slow down, with the consequence that higher levels of IPP and DMAPP accumulate, finally starting the isoprene synthase.

2.8.1 Regulation of Isoprene Emission

The regulation of the light- and temperature-dependent isoprene emission apparently proceeds via the relative activity of the DOXP/MEP pathway and possibly via the

concentration of DMAPP.[65,66] Moreover, in grey poplar leaves isoprene synthase and the second enzyme of the DOXP/MEP pathway, DXR, show distinct seasonal patterns and peak in the summer,[67] thus suggesting that the metabolic carbon flux through the DOXP/MEP pathway and isoprene emission are closely intercoordinated. Studies on the natural ^{13}C-carbon isotope composition of isoprene in several plants confirmed that isoprene is synthesized de novo from the currently formed primary photosynthates, yet a low percentage of carbon came from another carbon source,[68] possibly from cytosolic pyruvate imported to the chloroplast to be joined with GA-3-P in order to form DOXP, the first intermediate in the DOXP/MEP pathway (see Scheme 2.3). When photosynthetic carbon fixation was inhibited by CO_2-free air in these studies, the contribution of this alternative carbon source increased. Also, other leaf internal carbon pools (e.g. starch or xylem-fed labelled glucose) can be used as alternative carbon sources for isoprene emission, especially when, after abscisic acid application, the stomata close and CO_2 for photosynthetic carbon fixation is missing.[56] Xylem-transported glucose as an additional carbon source for leaf isoprene formation was also verified in pedunculate oak *Quercus robur*.[69] In this oak it was also demonstrated that isoprene synthase activity correlated well with the isoprene emission rates observed.[70] Recent research deals with various aspects of isoprene formation and the regulation of isoprene enzymes. Thus, the relationship of IPP isomerase activity (transforming IPP to DMAPP) with isoprene emission have been studied,[71] as well as the circadian rhythms of isoprene biosynthesis in grey poplar leaves[72] and the fact that transgenic, non-isoprene-emitting poplars 'don't like it hot'.[73] These transgenic non-isoprene-emitting poplars showed reduced rates of net assimilation and photosynthetic electron transport during heat stress, but not in the absence of stress, whereby the decrease in the efficiency of photochemistry was inversely correlated with the increase in heat dissipation of absorbed light energy, measured as nonphotochemical quenching (NPQ) of chlorophyll fluorescence. The downregulation of isoprene emission affected the thermotolerance of photosynthesis and induced an increased energy dissipation and, as expected, an increased formation of the xanthophyll cycle pigment zeaxanthin. In addition, studies were performed to link isoprene emission with plant thermotolerance, antioxidants and monoterpene emission.[74]

2.9 Inhibition of Isoprene Biosynthesis

2.9.1 Fosmidomycin and 5-Ketoclomazone

Biosynthesis and emission of isoprene in illuminated plant leaves is efficiently blocked by the herbicide fosmidomycin (**36**, see Figure 2.5) that specifically inhibits the second enzyme DXR of the plastidic DOXP/MEP pathway of IPP and isoprenoid formation, as verified also in the UV cuvette test system shown in Figure 2.9.[30] 5-Ketoclomazone (**37**), the specific inhibitor of the plastidic DOXP synthase, also efficiently blocks isoprene formation, as shown in the UV cuvette test system.[26,33,34,60] In these tests the leaf section is dipped either into the cuvette for 6–10 h at low light and room temperature conditions (below 24 °C) or overnight into the inhibitor solution and the controls into water. At temperatures above 30 °C and high irradiance (above 1200 $\mu mol\ m^{-2}\ s^{-1}$) a dose-dependent inhibition of isoprene emission is detected.

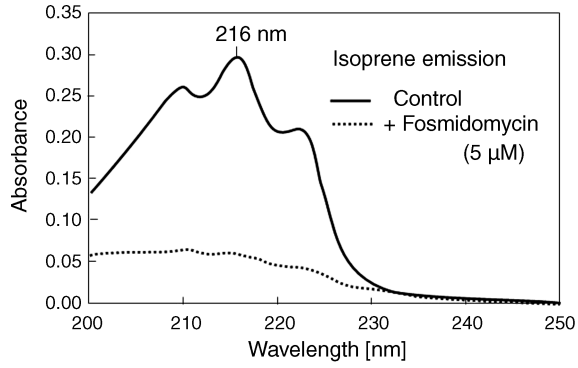


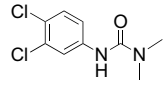
Figure 2.9 *Inhibition of light-induced isoprene emission in leaf pieces of Platanus x acerifolia by fosmidomycin (target: DOXP-reductase). The inhibitor (5 µM) was applied to the leaf in the dark in the cuvette test 12 h before starting the illumination. The amount of isoprene emission can be determined by measuring its UV spectrum in the closed cuvette*

2.9.2 Diuron

Using the herbicide diuron (DCMU, **45**, Figure 2.10), we demonstrated that the photosynthetic light reactions and associated electron transport reactions with their ATP and NADPH synthesis are as essential for isoprene biosynthesis and emission as the precursor substances GA-3-P and pyruvate. Diuron is known to specifically block the electron transport at the photosynthetic photosystem 2 by binding to the D1-protein instead of the endogenous electron acceptor plastoquinone-9,[75] an inhibition that blocks the formation of ATP and NADPH. The effect of diuron on isoprene emission was tested in the leaves of several plants, such as *Platanus x acerifolia*, *Chelidonium majus*, *Populus nigra* and *Populus alba x tremula*. After 24 h pre-incubation with diuron, by dipping the lower leaf part into a 10^{-5} M diuron solution, the isoprene emission was 70–80% inhibited. When the leaves were dipped into a 10^{-4} M diuron solution, the inhibition of the high-irradiance-induced isoprene emission was 100%.[60]

2.10 Inhibition of Carotenoid and Chlorophyll Biosynthesis by Fosmidomycin and 5-Ketoclomazone

The photosynthetic pigments, chlorophylls and carotenoids, are bound to thylakoids, the photosynthetic biomembranes.[76] In this context, it is of interest that, similar to isoprene



45
(Diuron)

Figure 2.10 *Chemical structure of the herbicide diuron (**45**)*

inhibition, the two specific inhibitors of the DOXP/MEP pathway of IPP biosynthesis, fosmidomycin (**36**) and 5-ketoclomazone (**37**), also affect and block the biosynthesis of carotenoids and chlorophylls (phytyl side chain) in chloroplasts in a dose-dependent manner.[30,33,34] This was investigated in etiolated barley seedlings which were dipped overnight with their cut ends into inhibitor solutions and the controls into water. During subsequent light exposure both inhibitors caused a dose-dependent, considerable reduction of the biosynthesis and accumulation of carotenoids and chlorophylls. In fact, the greening process of the leaves was retarded and an incomplete photosynthetic apparatus was obtained.[43] The latter is in the biosynthetic transition stage, as indicated by high values of the ratio of chlorophyll *a* to chlorophyll *b* (Chl *a*/*b* ratio) of 6.0 to 10.0, whereas normal values of green leaf tissue are in the range 2.7 to 3.3. The inhibition also causes a considerable increase in the weight ratios of total chlorophylls $(a + b)$ to total carotenoids $(x + c)$, with x corresponding to xanthophylls; and the values of $(a + b)/(x + c)$ range from 10.0 to 19.0. Green leaf tissue exhibits values of $(a + b)/(x + c)$ in the range 4.3 to 7.0, indicating an even stronger inhibition of carotenoid accumulation. For comparison, the normal pigment ratios of photosynthetic pigments in green leaf tissue,[42,77] and the changes in pigment ratios during the greening process are reported in the literature.[78]

2.11 Biosynthesis and Emission of Methylbutenol at High Irradiance

The needles of several pines of western North America (*Pinus ponderosa*, *P. contorta*, *P. sabiniana*) do not emit isoprene itself, but its partially oxidized form, the hemiterpene 2-methylen-3-buten-2-ol (MBO, **46**).[79] The MBO structure is shown in Scheme 2.4. Like isoprene, MBO is volatile and emitted in a light- and temperature-dependent manner at higher temperatures (heat stress) and high irradiance. Similar to isoprene, MBO can have a significant influence on the oxidative capacity of the atmosphere, for example through the consumption of hydroxyl radicals.

MBO is synthesized via the plastidic DOXP/MEP pathway, which was demonstrated by a high-rate incorporation of deuterium-labelled deoxy-D-xylulose (^{2}H-DOX) into MBO, as verified by mass spectrometry.[60,80] Incorporation of ^{2}H-DOX into MBO was studied in needles of *Pinus ponderosa* after 14 days preincubation with ^{2}H-DOX. The degree of MBO-labelling was at least 50% and was determined via collection of MBO with the SPME drop method followed by GC-MS. In addition, the formation of MBO is inhibited by fosmidomycin, which specifically blocks the DOXP reductase, the second enzyme in the plastidic DOXP/MEP pathway,[30] and also via 5-ketoclomazone, which specifically blocks the DOXP synthase.[26] Moreover, when offering ^{13}C-labelled MVA to ponderosa pine needles we did not find any incorporation of the ^{13}C-label into MBO, as checked using mass spectrometry. Like isoprene, the hemiterpene MBO is not formed from IPP but from its isomer DMAPP; this occurs in one step by the action of the enzyme MBO synthase. The two hemiterpene synthases, isoprene synthase and MBO synthase, use the same substrate DMAPP (Scheme 2.4), but the chemical mechanism for cleavage of the C_5 carbon structure from the diphosphate of DMAPP is different,[42] resulting in different endproducts.

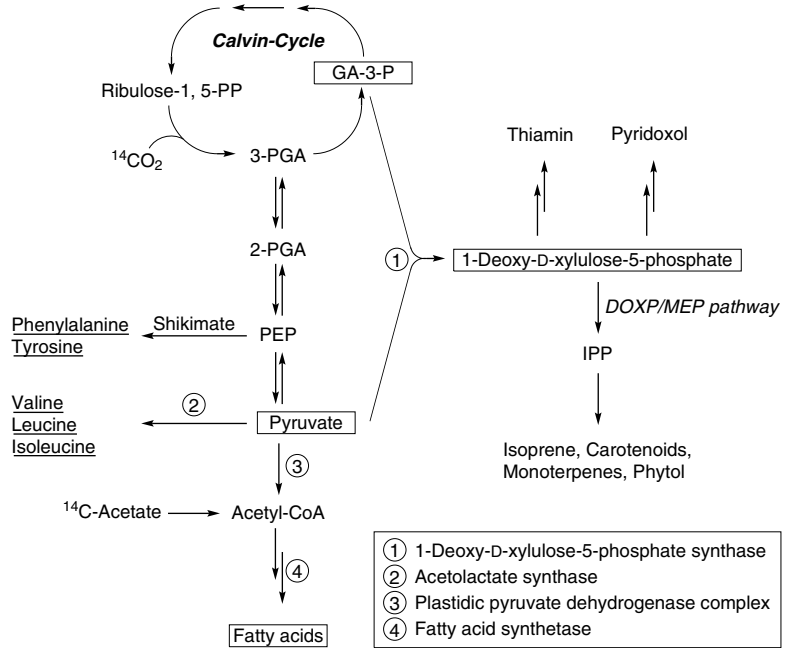
Various conditions for MBO emission have been studied in detail. At temperatures above 25 °C the MBO emission and photosynthetic rates of ponderosa pine needles increased

with light intensity and neither process showed any light saturation, even at an irradiance of 2000 $\mu mol\ m^{-2}\ s^{-1}$, which is close to full sunlight.[81] Water stress (closure of stomata) strongly reduced the photosynthetic rates but had no effect on MBO emission.

2.12 Source of Pyruvate for Isoprene and Methylbutenol Biosynthesis

Isoprene and methylbutenol are both synthesized from DMAPP produced via the DOXP/MEP pathway in chloroplasts. As volatile compounds they are not stored in the leaf. The rather large amounts of isoprene and MBO emitted by herbaceous plants as well as broadleaf trees and pine forests at higher irradiances and elevated summer temperatures derive from spontaneous de novo biosynthesis under photosynthetic conditions starting from GA-3-P, an intermediate of the Calvin cycle, and pyruvate (Scheme 2.6). Pyruvate in chloroplasts can derive from three sources. It can be formed in the chloroplast, at least in some plants such as spinach, directly from photosynthetically fixed carbon, that is from the first photosynthetic product 3-phosphoglyceric acid (3-PGA, **44a**) via 2-phosphoglyceric acid (2-PGA, **44b**) and phosphoenol pyruvate (PEP, **47**, Figures 2.8, 2.11).[57] In addition, pyruvate can be imported to chloroplasts from the cytosol where it is formed in the glycolytic pathway. As long as photosynthetic CO_2 fixation works well, both volatile hemiterpenes are formed from newly synthesized GA-3-P (Calvin cycle) and pyruvate that derives from its successive oxidation and transformation. However, pyruvate is also formed as a byproduct of ribulosebisphosphate carboxylase/oxygenase (RubisCO) activity.[82] Theoretically there is the possibility that, at higher temperatures and a certain shortage of CO_2, the enzyme RubisCO may yield a higher amount of pyruvate. This means that a higher yield of pyruvate by an enhanced RubisCO activity could stimulate the DOXP/MEP pathway as well as the biosynthesis and emission of isoprene and MBO. In fact, when the endogenous level of pyruvate in chloroplasts is increased, as shown below in the experiment with the inhibitor acetylmethyl phosphinate (AMPI, **48**, Figure 2.11),[60] this resulted in enhanced isoprene emission. However, the hypothesis that RubisCO at high-irradiance conditions, higher temperatures and lower CO_2 levels produces more pyruvate has yet to be analytically investigated and proved.

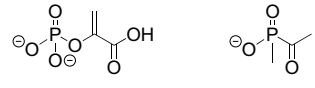
However, the particular environmental conditions of isoprene and MBO emission lead sooner or later to a certain water stress and, as a consequence, to partial or full closure of the leaf stomata. Since, under water stress and stomata closure, the CO_2 assimilation via the Calvin cycle is successively reduced, the major part of the starter molecules of the DOXP/MEP pathway (i.e. GA-3-P and pyruvate) must then come from sources other than the process of photosynthetic carbon fixation and reduction. In contrast to photosynthetic CO_2 assimilation, the two photosynthetic light reactions and associated electron transport reactions that provide ATP and NADPH for the DOXP/MEP pathway still take place even at water stress and stomata closure. At such conditions the main carbon sources for isoprene and MBO biosynthesis are: (*a*) GA-3-P from the breakdown of starch and (*b*) pyruvate that is formed by a breakdown of GA-3-P. Most probably pyruvate is formed in the cytosol by glycolytic breakdown of GA-3-P and is imported by chloroplasts. The question has yet to be determined as to whether chloroplasts of isoprene-emitting leaves and ponderosa pine needles possess the competence to form pyruvate directly from GA-3-P, as may be possible in some plants.



Scheme 2.6 *Biosynthetic pathways in chloroplasts with branching points for photosynthetic intermediates and products. The flow is indicated for carbon metabolites from the photosynthetic CO_2 reduction cycle (Calvin cycle) into different end-products, such as IPP and isoprenoids, or into fatty acids and amino acids, as well as from DOXP into thiamine and pyridoxal. The central role of GA-3-P and pyruvate in the DOXP/MEP pathway for biosynthesis of IPP and plastidic isoprenoids is emphasized. In some plants pyruvate can directly be made in chloroplasts from photosynthetically formed 3-PGA; in others it comes from the cytosol and is transported into the chloroplast*[25]

2.13 Branching Point of DOXP/MEP Pathway with Other Metabolic Chloroplast Pathways

Besides the photosynthetic carbon reduction cycle (Calvin cycle, Scheme 2.6), the performance of the photosynthetic light reactions (ATP, NADPH) and the DOXP/MEP pathway for isoprenoid biosynthesis, chloroplasts possess several other biosynthetic activities, such



47 (PEP) **48** (AMPI)

Figure 2.11 *Chemical structures of phosphoenol pyruvate (PEP, **47**) and acetylmethyl phosphinate (AMPI, **48**)*

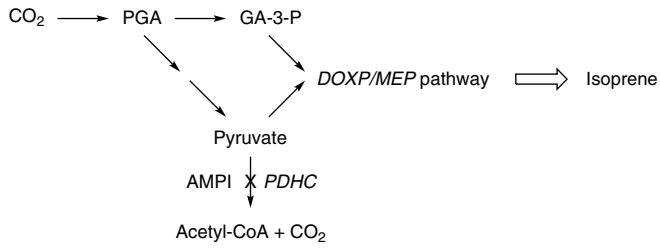
as the biosynthesis of chlorophyll (porphyrin ring), de novo fatty acid biosynthesis[83] and also the biosynthesis of particular amino acids, such as aromatic amino acids and branched-chain amino acids. Some of these pathways compete for the same substrates and are dependent on the carbon metabolite flow from intermediates or products of the Calvin cycle (Scheme 2.6).

In view of the operation of the plastidic DOXP/MEP pathway, in which GA-3-P and pyruvate are direct substrates of DOXP synthase, the early observations are understood very well today, confirming that ^{14}C-labelled CO_2, GA-3-P and pyruvate are better precursors of chloroplast isoprenoids than ^{14}C-MVA or ^{14}C-acetate.[84] The two latter compounds are no substrates of the DOXP/MEP pathway, and within the chloroplasts ^{14}C-acetate is rapidly incorporated into fatty acids via the plastidic de novo fatty acid synthetase.[83] In contrast, photosynthetically fixed $^{14}CO_2$ is rapidly transformed in the Calvin cycle to 3-PGA and reduced to GA-3-P.[85] For the reasons mentioned above, the ^{14}C-label of $^{14}CO_2$ rapidly appears via GA-3-P and pyruvate in β-carotene and various other plastidic isoprenoids, as described by various authors.[54] Also, the fast ^{13}C-labelling of isoprene from photosynthetically fixed $^{13}CO_2$ is easily understood in view of the DOXP/MEP pathway that is fed by products of the Calvin cycle.[55,56]

Two other biosynthetic pathways in chloroplasts compete for distant products of the carbon reduction cycle. Thus, PEP (**47**) is a substrate of the shikimic acid pathway that yields the aromatic amino acids phenylalanine and tyrosine. Pyruvate, in turn, is also a substrate of the biosynthesis of branched-chain amino acids, such as isoleucine, leucine and valine, a pathway that also proceeds in chloroplasts. Moreover, when the plastidic pyruvate dehydrogenase complex yielding acetate is blocked by the specific inhibitor AMPI (**48**),[86] as shown in Scheme 2.7, biosynthesis and emission of the isoprene is enhanced by 30% at 5×10^{-4} M AMPI and by 60% at 10^{-4} M AMPI applied to leaf pieces.[60] These results indicate that there exist several branching points in the metabolite flow of Calvin cycle products to pyruvate and different other chloroplast products. This requires a very special fine regulation of chloroplast metabolism concerning the use of the photosynthetic products. This fine regulation must respond in the daily changing climatic conditions (temperature, irradiance) to the availability of CO_2 (e.g. open and closed stomata). In any case, enhanced or reduced isoprene emission can be a very effective valve for a fine-tuning of carbon flow and chloroplast metabolism.[42] In addition, there also exist particular regulations of the activity of the DOXP/MEP pathway and its enzymes, as recently reviewed.[87]

2.14 Is There a Physiological Function of Isoprene and MBO Emission?

The biosynthesis and emission of both volatile plant hemiterpenes depend on the chloroplastidic production of DMAPP. In fact, plant species with the highest potential for isoprene and MBO production also possess an elevated light-dependent production of DMAPP.[66] The physiological meaning of the emission of isoprene and MBO is not yet fully clear. Isoprene provides the leaves with a certain thermotolerance to heat damage.[88] In addition, by functioning as a potential scavenger of radicals both volatile hemiterpenes can protect thylakoid lipids and various chloroplast constituents from ozone and other reactive oxygen species,[68,89–91] thereby preventing photo-oxidation of the photosynthetic apparatus at high irradiance conditions. When in *Phragmites australis* leaves isoprene formation was



Scheme 2.7 *Enhanced flow of pyruvate into isoprene when the plastidic pyruvate dehydrogenase is inhibited by AMPI,*[60,86] *a specific inhibitor of the plastidic pyruvate dehydrogenase*

blocked by the inhibitor fosmidomycin, leaves became sensitive to ozone, as seen in the decline of photosynthesis, stomatal conductance and chlorophyll fluorescence parameters and the start of membrane lipid peroxidation.[90] These results clearly indicate that isoprene biosynthesis and emission exert a protective effect on the photosynthetic apparatus and its biomembranes.

Concerning the mechanism how isoprene exerts this protective effect, one also has to consider that the enhanced de novo biosynthesis of isoprene and MBO requires a continuous supply of ATP and NADPH being formed in the photosynthetic light reactions (see Scheme 2.4), a process which keeps the two photosynthetic photosystems 'busy' and intact by avoiding overreduction and photo-oxidative damage under excess light conditions. Thus, isoprene and MBO emissions may be a 'safety valve', similar to the process of photorespiration, to protect the photosynthetic pigment apparatus with its photosystems and light-harvesting pigment proteins[92] from photo-oxidation. The only disadvantage is that isoprene and MBO emissions are a waste of the previously photosynthetically fixed reduced carbon. Although the ATP and NADPH consumption through isoprene and MBO biosynthesis should not be overestimated, it essentially contributes, besides other mechanisms, to the stability of the photosynthetic pigment apparatus under high irradiance conditions.

Recently Rosenstiel *et al.*[66] proposed a special hypothesis why plants emit isoprene: the isoprene synthase converting DMAPP to isoprene and pyrophosphate would prevent DMAPP to rise to such high levels that would unnecessarily sequester phosphate. However, this concept is not convincing and should be ignored. The starting point of this hypothesis is the assumption that plants would accumulate more and more DMAPP at high-light and high-temperature conditions. However, this hypothesis fully neglects the fact that, from DMAPP and its isomer IPP, various other isoprenoid compounds can be and are produced de novo under high light- and heat-stress conditions, such as β-carotene (**15**) and the xanthophyll zeaxanthin (**16**), which accumulate at high rates, as has been proved for several plants.[42] Zeaxanthin is known to play an essential role in the heat dissipation of absorbed light energy by disconnecting the photosynthetic light-harvesting pigment proteins from the photosynthetic reaction centres. Moreover, various plants that have no competence for isoprene emission in their leaves accumulate nonvolatile diterpenes under such conditions, for example different ginkgolides in the green leaves of ginkgo trees or various other diterpenes. Also, the high export rates of IPP from chloroplasts to the cytosol for sterol formation[41] need to be considered. In any case, the available data show that, depending on

the type of plant under high incident photon flux and elevated temperatures, rather high amounts of photosynthetically fixed carbon are channelled within the chloroplasts into the biosynthesis of either volatile isoprene and MBO or carotenoids, volatile monoterpenes as well as sterols, volatile sesquiterpenes or other isoprenoid compounds. All those biosynthetic programmes consume the ATP and NADPH produced during the course of the photosynthetic light reactions, which keeps those light reactions going. Thus, the emission and/or accumulation of volatile and nonvolatile isoprenoids preserve the two photosynthetic photosystems PS1 and PS2 under light- and heat-stress conditions when (e.g. at closed stomata) the photosynthetic CO_2 assimilation and the performance of the Calvin cycle are blocked.

This is an extremely essential physiological function of the emission of isoprene and other volatile isoprenoids and also of the accumulation of nonvolatile isoprenoid compounds in plants with no competence for isoprene emission. Also, several other metabolic processes occur, particularly in plants without isoprene emission, such as photorespiration, efficient quenching of excess light (heat emission of absorbed light energy) or the process of photoinhibition, all of which have in common that they more or less protect the photosynthetic photosystems from destruction and photo-oxidation. Thus, as soon as the stress conditions are over, the leaves can immediately restart photosynthesis and photosynthetic carbon fixation because the two photosystems PS1 and PS2 still exist. This immediate switch from the carbon-wasting emission of isoprene (and other isoprenoids) to a functional, photosynthetic carbon gain is an essential advantage for the leaves of green plants and thus has been preserved and even enhanced during evolution. Other plants that lost their isoprene synthase and isoprene emission capacity during evolution had to develop other mechanisms to protect their two photosynthetic photosystems.

2.15 Biosynthesis and Emission of Monoterpenes, Sesquiterpenes and Diterpenes

There are various plant families that produce particularly volatile monoterpenes and sesquiterpenes as part of their essential oils ('etherische Öle') in specialized cells, such as the glandular cells or trichomes, secretory cells in flower petals, inflorescences, fruits, orange peels, rhizomes or seeds (e.g. Lamiaceae, Lauraceae, Myrtaceae, Pinaceae, Rosaceae, Rutaceae, Umbelliferae). There are schizogenic and lysogenic compartments for the deposition of the essential oils. Many of these volatile isoprenoids are monoterpenes, such as the *acyclic monoterpenes* geraniol (**3**), linalool (**4**), citronellol (**49**), nerol, (**50**), citral (**51**) and myrcene (**52**, Figures 2.1, 2.12). *Monocyclic monoterpenes* are menthol (**5**), cineol (**6**), limonene (**53**), α- and β-phellandrene (**54**, **55**), α-terpineol (8-terpineol, **56**), menthone (**57**), pulegone (**58**), *p*-cymene (**59**), thymol (**60**) and carvacrol (**61**). There also exist *bicyclic monoterpenes*, such as sabinene (**62**), thujol (**63**), carene (**64**), 1,8-cineol (eucalyptol, **65**), α- and β-pinene (**66**, **67**), borneol (**68**) and camphor (**69**). *Sesquiterpenes* in essential oils are farnesol (**7**), bisabolol (**8**), nerolidol (**70**), zingiberene (**71**) and caryophyllene (**72**, see Figures 2.1, 2.13). Rosemary leaves primarily contain 1,8-cineol and borneol and, to a lesser degree, camphor and limonene. Lavender flowers possess linalool as a major volatile ingredient, together with cineol and geraniol. Peppermint leaves exhibit menthol as a major substance, together with menthone, cineol, limonene and α-pinene. The

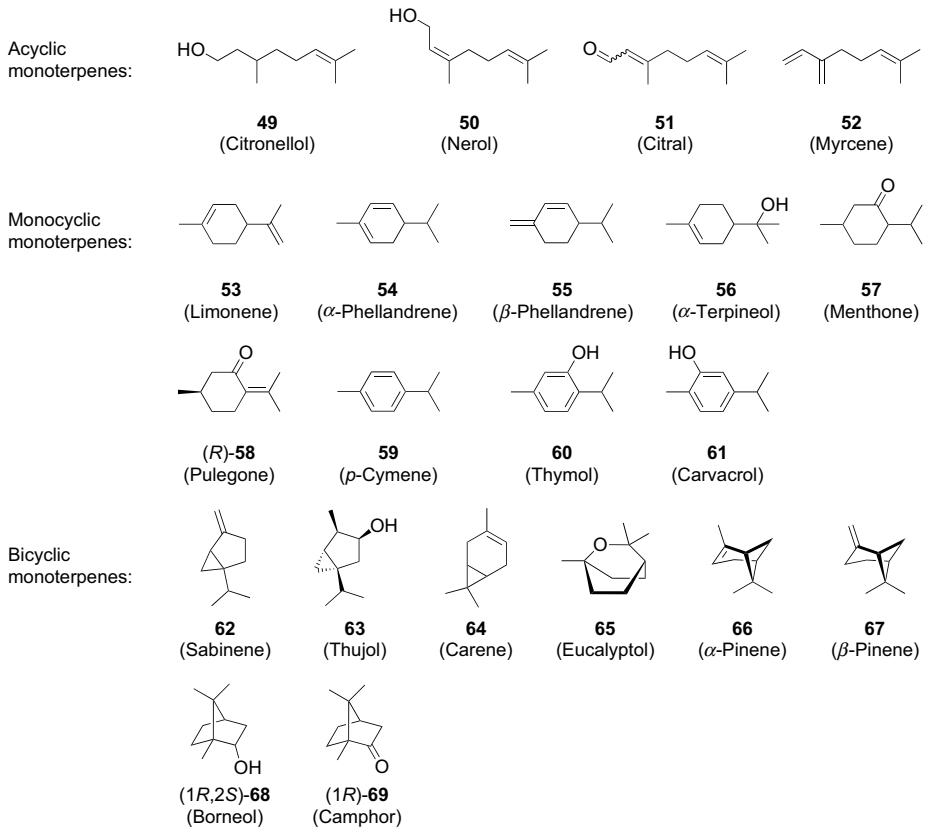


Figure 2.12 *Chemical structures of a series of acyclic, monocyclic and bicyclic monoterpenes*

scales and peels of citrus fruits contain high levels of limonene and, in lower amounts, geraniol, nerol, linalool and citral. The essential oil of rose flowers consists of citronellol (40–50%) and geraniol (20%). Eucalyptus leaves possess high amounts of the monoterpene 1,8-cineol. Only a few volatile diterpenes exist in plants. These are acyclic C_{20} isoprenoids (e.g. phytol derivatives), monocyclic (e.g. camphorene, **10**) or dicyclic isoprenoids (e.g. geranyllinalool, **73**). Most diterpene derivatives are, however, nonvolatile diterpenic carbonic acids as components of bitter substances ('Bitterstoffe') and resins. Monoterpenes, sesquiterpenes and diterpenes are formed and accumulated in particular cell structures of inflorescences, leaves and seeds or in special compartments between the cell walls of neighbouring cells. Since such volatile isoprenoids are formed only in members of certain plant families and in specialized cells, their contribution to the total amount of volatile organic compounds emitted into the air by plants is much smaller than the amount of isoprene, which is formed in many plants from different systematic positions and not only in specialized leaf cells but in the cells of all green leaves. An exception to this rule, that primarily isoprene is the main source of organic plant volatiles, may be oak forests in Mediterranean areas and eucalyptus forests in Australia, where monoterpene and other

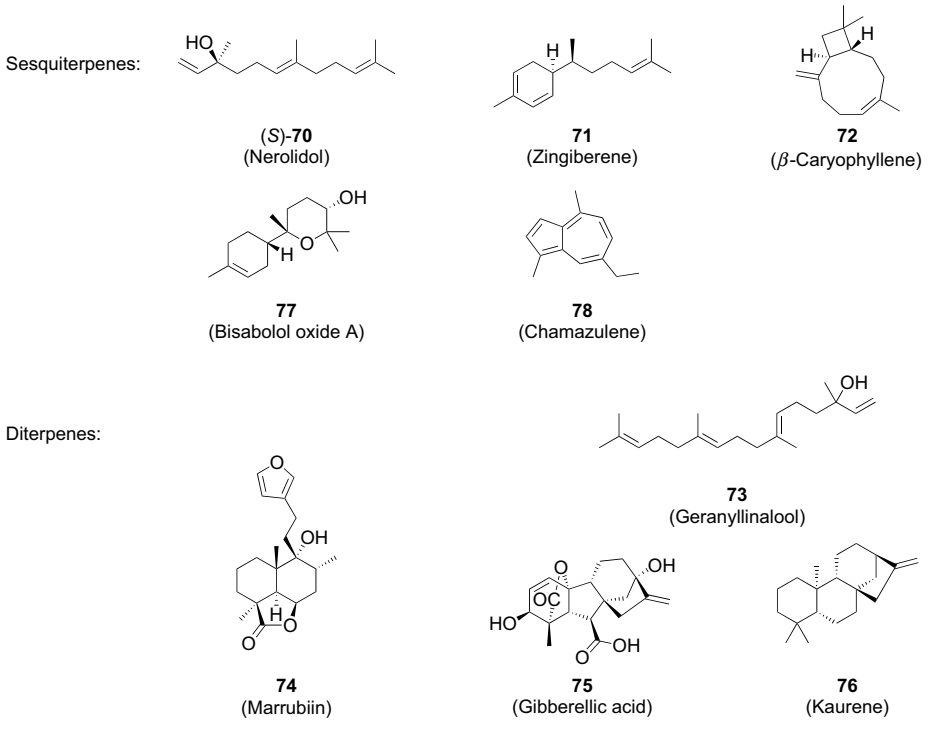


Figure 2.13 *Chemical structures of selected sesqui- and diterpenes*

isoprenoids, besides isoprene, may essentially contribute to the emitted phytogenic organic compounds in the atmosphere.

2.15.1 Monoterpenes

The biosynthesis of monoterpenes also proceeds in plastids via the DOXP/MEP pathway of IPP. After detection of the DOXP/MEP pathway, this was verified by different working groups for various monoterpenes, such as menthone, pulegone, geraniol and thymol.[47,93] It was shown that limonene synthase, the enzyme responsible for the synthesis of the monoterpene limonene (**53**), is localized in peppermint in the leucoplasts of the oil gland secretory cells.[94] Additional enzymes of monoterpene biosynthesis have been cloned and characterized in peppermint, such as menthone reductase[95] as well as geranyl diphosphate synthase, limonene-6-hydroxylase, isopiperitenol dehydrogenase and pulegone reductase.[96] Geranyl diphosphate synthase functions as a heterodimer.[97] This has also been documented in snapdragon (*Antirrhinum majus*) and fairyfan (*Clarkia breweri*) flowers.[98] Other publications describe seasonal changes of monoterpene emission and monoterpene synthase activities.[99] A recent investigation concentrated on the emission of biogenic volatile organic compounds in local urban vegetation which contribute to urban ozone and aerosol formation. The monoterpene emissions of 11 ornamental tree species, three conifers and nine angiosperms were dominated by α- and β-pinene, myrcene, α- and β-phellandrene, carene, limonene and eucalyptol.[100]

2.15.2 Diterpenes

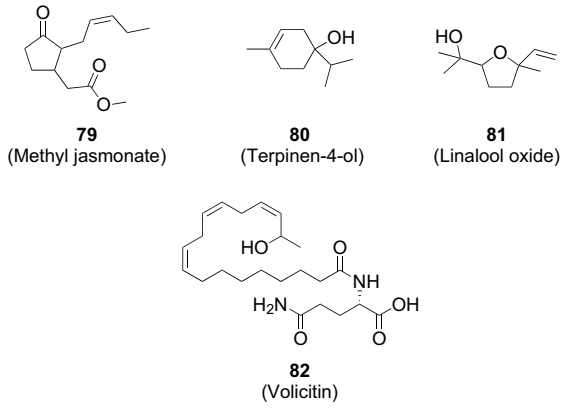
The biosynthesis of diterpenes proceeds within the plastids from IPP produced via the DOXP/MEP pathway. This was first shown for phytol (**8**, side chain of chlorophylls, Scheme 2.5).[23,25] Later it was proved for the diterpenes marrubiin (**74**, Figure 2.13)[101] and taxol (**11**)[102] as well as ginkgolide A (**12**), gibberellins (e.g. gibberellic acid, **75**), kaurene (**76**) and other compounds.[103]

2.15.3 Sesquiterpenes

These are produced in the cytosol and derive from farnesyl diphosphate (FPP, **26**) which is usually made via the acetate/MVA pathway (Scheme 2.1). However, under photosynthetic conditions, the IPP molecules produced in chloroplasts via the DOXP/MEP pathway are exported to the cytosol. They make an essential contribution not only to the biosynthesis of sterols,[41,42] but also to that of sesquiterpenes (e.g. in snapdragon flowers).[44,47] Of the sesquiterpenes, bisabolol oxide A (**77**, Figure 2.13) and chamazulene (**78**) from chamomile, 50% of the isoprenic units came from the acetate/MVA pathway, whereas the other 50% were derived from the DOXP/MEP pathway of IPP formation.[104] Other sesquiterpenes made from IPP of the DOXP/MEP pathway are found in eucalyptus leaves, clematis and in barley roots. Recent research has been concentrating on the characterization and cloning of particular enzymes involved in sesquiterpene biosynthesis, such as the farnesyl diphosphate synthase in the big sagebrush *Artemisia tridentata*.[105]

2.16 Some General Remarks on the Regulation of Terpene Biosynthesis in Plants

Concerning the regulation of monoterpenes, sequiterpenes and diterpenes, it has to be taken into consideration that their biosynthesis is differently regulated from that of the spontaneously formed and immediately emitted highly volatile hemiterpenes isoprene and MBO. The C_{10}, C_{15} and C_{20} prenyl derivatives are usually synthesized and accumulated during or after full development of the proper plant tissues where they are formed more or less continuously. Their synthesis can also be induced as a defensive response. Their biosynthesis is not as specifically bound to high temperatures and high light conditions as that of isoprene and MBO. Thus, at de novo biosynthesis conditions of isoprene in blue gum (*Eucalytus globulus*), as proved by specific incorporation of deuterium ^{2}H-DOX into isoprene, there was no incorporation into the monoterpene cineol.[60] Moreover, the biosynthesis of monoterpenes and diterpene resin acids are induced in the stems of lodgepole pine (*Pinus contorta* var. *latifolia*) saplings when wounded and inoculated with the blue-stain fungus *Ceratocystis clavigera* or treated with carbohydrate elicitors.[106] In addition, the biochemical regulation of monoterpene, sequiterpene and diterpene formation, accumulation and release from conifer needles of Norway spruce (*Picea abies*) can be induced as a defensive response against potential herbivores and pathogens via methyl jasmonate (**79**, Figure 2.14).[107] These are just a few examples for the independent regulation of terpene biosynthesis in comparison to that of isoprene and MBO.



79 (Methyl jasmonate) **80** (Terpinen-4-ol) **81** (Linalool oxide)

82 (Volicitin)

Figure 2.14 *Chemical structures of methyl jasmonate (**79**), terpinen-4-ol (**80**), linalool oxide (**81**) and volicitin (**82**)*

2.17 Volatile Terpenoids as Aroma Compounds of Wine

Various kinds of terpenoid compounds have been found in the different grape varieties and in wine, where they are essential components of the wine aroma and wine taste. Most of these compounds are volatile monoterpene alcohols, such as geraniol, linalool, nerol and α-terpineol. However, in certain wines the volatile sesquiterpene alcohol farnesol is also an important constituent of the wine aroma. Although not all terpene volatiles of grapes have been particularly checked, it is clear from specific labelling studies and the localization of special terpene synthase enzymes in plastids that the volatile monoterpenes of ripening grapes are synthesized via the plastidic DOXP/MEP pathway of IPP formation, whereas the sesquiterpenes are formed from IPP molecules deriving from the cytosolic acetate/MVA pathway. Yet under photosynthetic conditions IPP molecules, exported from chloroplasts, can also contribute to the biosynthesis of grape sesquiterpenes.

Essential for the particular aroma of a special grape variety and its wine is apparently the relative level of geraniol with respect to the other terpenes. The grape terpenoids of *Vitis vinifera* are usually synthesized rather late in grape berry development and particularly accumulate during ripening of the grapes. Thus, the very characteristic and rather strong aroma of Muscat wines is due to the three monoterpene alcohols geraniol, linalool and nerol. An investigation of the Albariño wines, found in north-west Spain, showed that the terpenes (here linalool and terpineol) are mostly present in the free alcohol form and very little in a bound or ester form.[108] In a broad investigation of Chardonnay wines from all regions of California, one found particularly linalool and α-terpineol, however in minor amounts also terpinen-4-ol (**80**, Figure 2.14), geraniol, nerol and linalool oxide (**81**).[109] In the present research of wine breeding institutions terpenes are screened as markers for potential new grape wine varieties. Other studies deal with the overexpression of particular enzymes of the isoprenoid and terpene biosynthesis machinery to increase the terpene level of grapes. Since the level of volatile terpenes decreases during wine ageing together with the decline

of aroma and taste, recent research also concerns the search for inhibitors of the disappearance of volatile aroma in wine.[110]

2.18 Function of Terpenes in Plant Defence

Volatile plant terpenes play an essential ecological role and have a variety of functions in mediating antagonistic and beneficial interactions among organisms.[111] The emission of volatile terpenes from flowers to attract insects for pollination is widely known. Volatile terpenes can also be released at low rates from leaves and other vegetative plant tissues such as roots. However, the level and profile of released volatile terpenes from leaves and other plant tissues is markedly different when plant leaves are damaged, for example by insect feeding. Then newly synthesized volatile terpenes can become part of a defensive mechanism, for example either by direct toxic action against the plant pathogen and the feeding insect or by very special interactions. Thus, in the past two decades it has been shown that volatile plant terpenes mediate very specific interactions between plants and herbivores and other organisms, such as arthropods, nematodes and also other plants. Such interactions have been detected and documented in recent years by various groups;[112–114] and certainly many more interactions will be detected in future.

When plants are damaged (e.g. by herbivorus insects) many more terpene volatiles and also non-isoprenoid volatiles are synthesized de novo and set free. Such volatile terpenes attract both parasitic and predatory insects that are natural enemies of herbivores, as described in the update reviews by Paré and Tumlinson[112] and Arimura *et al.*[113] This is then of great advantage for the attacked plant which via the volatile terpene signal 'asks' for help and can indirectly defend itself. In addition, the emitted volatile plant isoprenoids can also induce defensive responses in neighbouring plants. Such specific interactions and communications are often based on volatile mono- and sesquiterpenes. It is of interest in this respect that, in all plants investigated so far, even in phylogenetically distant plants, there exist notable similarities in the structure of volatile terpene and nonterpenoid volatiles that are released from insect-damaged leaves and from leaves distal to the site of the damage.[111,112] The production of such volatile terpenes is generally induced by an interaction of special elicitors such as volicitin (**82**) in the oral secretion of insect herbivores with the damaged leaf tissue. In these elicitor-induced responses for synthesis and release of volatile defensive terpenes various special compounds, such as jasmonates and oxylipins, seem to be involved. More recently, the drimane sesquiterpenes occurring in plants, fungi and some marine organisms have found special attention in defensive responses.[115] So far, very little is known on the production of volatile terpenes in plant roots as indirect defensive compounds against nematodes and other plant herbivores. However, one can expect a function similar to that in leaf tissues.

2.19 Conclusion

Plants possess two systems for the biosynthesis of IPP and isoprenoid formation; and the DOXP/MEP pathway in the chloroplasts operates independently of the acetate/MVA pathway in the cytosol. The DOXP/MEP pathway is required to supply the IPP C_5 units

necessary for the synthesis of functional compounds in the photosynthetic apparatus, such as carotenoids, prenyl side chains of chlorophylls, plastoquinone-9, α-tocopherol and phylloquinone K_1. This plastidic isoprenoid pathway is also involved in the chloroplast adaptation response to high- or low-light conditions by providing the isoprenoid C_5 building blocks to form sun- and shade-type chloroplasts that are characterized by particular chlorophyll *a*/*b* ratios as well as carotenoid and prenylquinone composition.[42] At high photon flux densities the DOXP/MEP pathway also participates in the relatively rapid de novo biosynthesis of high additional amounts of the xanthophyll cycle carotenoids (zeaxanthin, antheraxanthin) that protect the photosynthetic apparatus from photoinhibition as well as from photo-oxidation. In sun-exposed leaves the DOXP/MEP pathway also supplies the active C_5 IPP units for the continuous accumulation of plastoquinone-9 and α-tocopherol in the osmiophilic plastoglobuli.[42] At high irradiances the chloroplast DOXP/MEP pathway operates efficiently and uses photosynthetically formed ATP, NADPH and newly fixed carbon to provide a major part of the C_5 IPP units necessary for the biosynthesis of the cytosolic sterols. In addition, starting with intermediates of the Calvin cycle, the chloroplastidic isoprenoid pathway serves the biosynthesis and emission or accumulation of volatile isoprenoids, such as isoprene, methylbutenol, monoterpenes, diterpenes (see Scheme 2.2), and particular sesquiterpenes, all of which can be regarded as direct primary photosynthetic products and secondary plant products or natural products. The data reviewed above indicate that, depending on the light and temperature conditions, enormous amounts of freshly fixed photosynthetic carbon flow into various volatile and nonvolatile isoprenoid compounds. Thus, chloroplast isoprenoid biosynthesis via the IPP-forming DOXP/MEP pathway appears to be a 'metabolic valve' for regulating the photosynthetic carbon flow as well as fine-tuning for chloroplast and cell metabolism. This chloroplast isoprenoid pathway consumes large amounts of photosynthetically formed ATP and NADPH and may also serve as a 'safety valve' in order to avoid overreduction and photoinhibition of the photosynthetic apparatus as well as photo-oxidation of the photosynthetic pigment systems PS1 and PS2. The essential significance of terpene synthesis in plants is underlined by the fact that terpenoid compounds (prenyl pigments, prenylquinones) not only have specific functions in primary cell metabolism, but that the synthesis and emission of various mono- and sesquiterpenes in plants serve as chemical defensive mechanisms against herbivores, pathogens and competitors. Currently various genetic and metabolic engineering studies are being performed in many laboratories on the biosynthetic isoprenoid pathways of plants, using genetically transformed bacterial and plant test systems. This research is essential in order to better understand the biological function of these plant terpenoids and to modulate the production of carotenoids, vitamin E and particular mono-, sesqui- and diterpenes of commercial and pharmaceutical interest.

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