1.17 Diterpenes

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1.17.1 Introduction

A variety of cyclic diterpenes are derived from geranylgeranyl diphosphate (GGPP) through the cyclization of GGPP and the chemical modification of carbon skeletons, such as oxidation, reduction, acetylation, methylation, and glycosylation. Cyclization is an important and interesting process, from the perspective of the formation of a variety of carbon skeletons, and is a major branch of the biosynthesis of cyclic diterpenes. In 1994, the casbene synthase gene from caster bean (*Ricinus communis*) was first reported as a diterpene cyclase (see Section 1.17.4.2),[1] as well as the *ent*-copalyl diphosphate (*ent*-CPP) synthase (see Section 1.17.2.1) from *Arabidopsis thaliana*, which functions in gibberellin biosynthesis.[2] As for labdane-related diterpene cyclases, cDNAs encoding the *ent*-kaurene synthase (see Section 1.17.2.3) in pumpkin (*Cucurbita maxima*) and the abietadiene synthase (see Section 1.17.2.2.1) in grand fir (*Abies grandis*), which function in gibberellin biosynthesis and abietic acid biosynthesis, respectively, were isolated in 1996.[3,4] The taxadiene synthase gene (see Section 1.17.4.1), which functions in taxol biosynthesis, was isolated from Pacific yew (*Taxus brevifolia*) in 1996.[5] MacMillan and Beale,[6] in their introduction to diterpene cyclases, described cyclizations initiated by ionization of the diphosphate of GGPP as type A and cyclizations initiated by protonation at the 14,15-double bond of GGPP as type B, based mainly on information of labdane-related diterpene cyclases. According to their classification, casbene synthase and *ent*-kaurene synthase are of type A, *ent*-CPP synthase is of type B, abietadiene synthase is of type B-A, and taxadiene synthase is of type A-B (**Figure 1**). Abietadiene synthase catalyzes the conversion of GGPP into (+)-CPP (type B) and successively converts (+)-CPP into abietadiene (type A). The cyclization of GGPP by taxadiene synthase is initiated by ionization of the diphosphate of GGDP

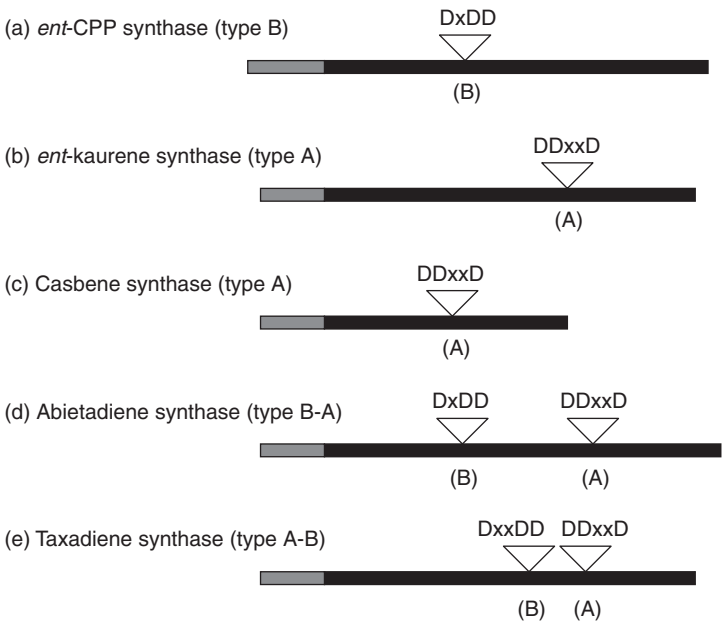


Figure 1 Schematic diagrams of the primary structures of representative diterpene cyclases in plants: (a) *ent*-CPP synthase, (b) *ent*-kaurene synthase, (c) casbene synthase, (d) abietadiene synthase, and (e) taxadiene synthase. Black bars and gray bars indicate putative pseudomature enzymes and transit peptides for plastid targeting, respectively. Type A and type B cyclizations are initiated by ionization of allylic diphosphate group and by protonation of double bond, respectively. Reverse triangles indicate each motif. The type B motif in taxadiene synthase is a modified one (DxxDD).

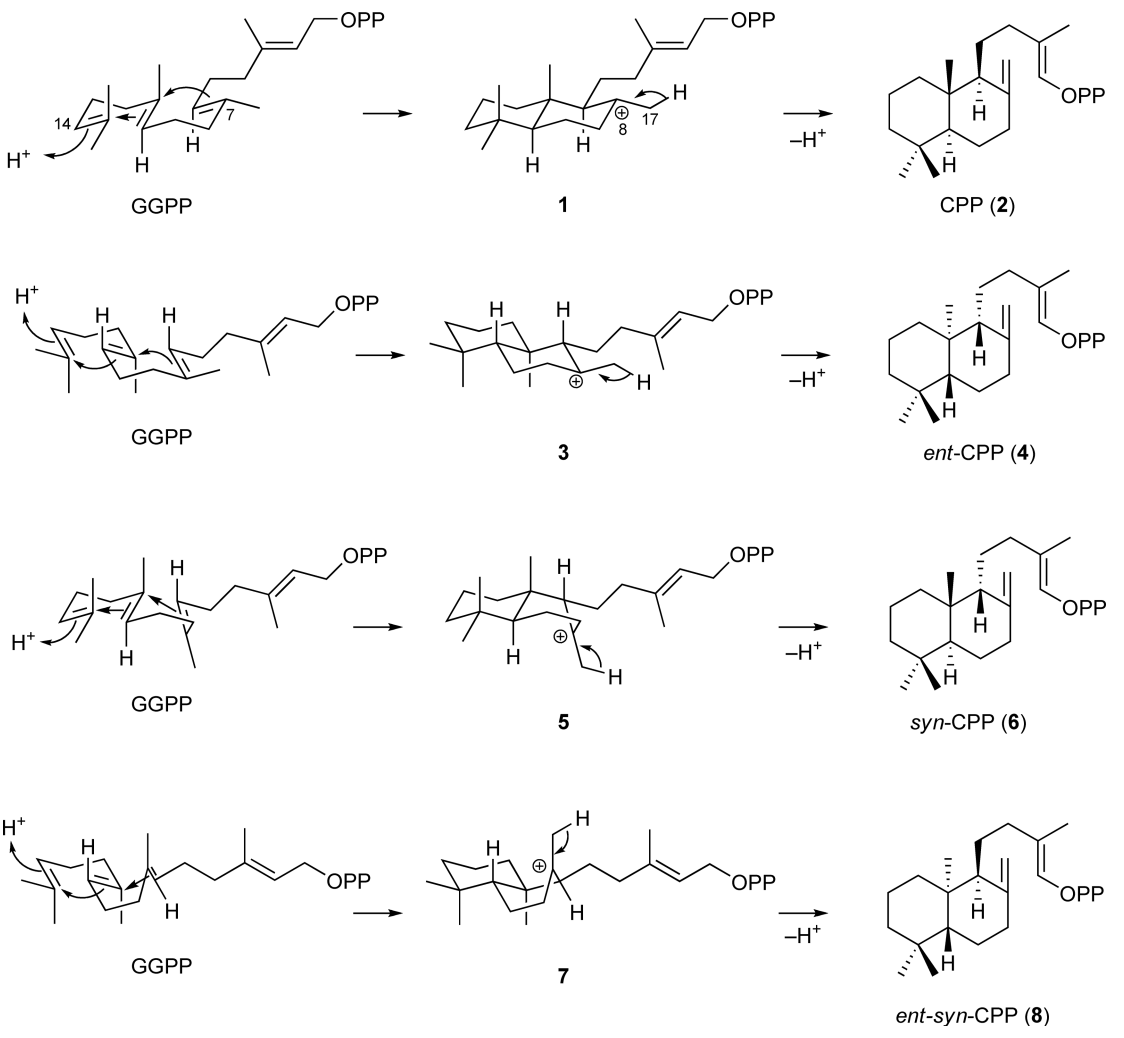
(type A), and further cyclization of the cyclic hydrocarbon intermediate is initiated by protonation at the 7,8-double bond (type B). The details of these reactions are described later. It has been shown that both types of reactions require the D/E-rich motif, as illustrated in **Figure 1**. All diterpene cyclases isolated from higher plants possess transit peptide-like sequences at their N-termini (**Figure 1**), suggesting that the cyclization of GGPP occurs at the plastid in plant cells.

To date, a variety of diterpene cyclase genes have been isolated from higher plants and other taxa, including bryophytes, fungi, and bacteria. An unusual diterpene cyclase gene fused with the GGPP synthase gene was recently isolated from a fungus (see Section 1.17.4.3). This chapter describes the reactions catalyzed by diterpene cyclases that have been isolated so far. In particular, the labdane-related diterpene cyclases are described in more detail. Modification enzymes, which function in the biosynthesis of some diterpenes, such as gibberellins, steviol, and taxol, are also described.

1.17.2 Labdane-Type Diterpenes

1.17.2.1 Formation of Four Copalyl Diphosphate Stereoisomers

The first step in the biosynthesis of labdane-related diterpene hydrocarbons from GGPP is the formation of CPP stereoisomers. The reaction is initiated by protonation of the 14,15-double bond of GGPP, followed by an attack of C-10 on C-15, then C-6 on C-11, affording four stereoisomers of 8-carbonium ions **1**, **3**, **5**, and **7** (**Scheme 1**). The chair–chair conformation of GGPP gives **1** and the chair–boat conformation gives **5**, and their antipodal conformations give **3** and **7**, respectively. The successive deprotonation of 17-H in **1**, **3**, **5**, and **7** gives CPP (**2**), *ent*-CPP (**4**), *syn*-CPP (**6**), and *ent-syn*-CPP (**8**), respectively (**Scheme 1**). The *ent*-CPP synthase gene, which is responsible for gibberellin biosynthesis, was first isolated from *Arabidopsis* (*AtCPS* encoding 802 amino acid residues)[2] and its orthologs have been isolated from several plant species. In higher plants, *ent*-kaurene, a biosynthetic hydrocarbon intermediate of gibberellin, has been indicated to be converted from GGPP by two distinct cyclases, *ent*-CPP synthase and *ent*-kaurene synthase (see Section 1.17.2.3).[7] On the other hand,



Scheme 1

ent-kaurene is converted from GGPP by a single cyclase in moss[8] (see Section 1.17.2.3.2) and in gibberellin-producing fungi (see Section 1.17.2.3.4).[9,10] These cyclases possess type B and type A motifs at their N- and C-termini, respectively, similar to abietadiene synthase (**Figure 1(d)**). Abietadiene synthase converts GGPP into abietadiene via CPP (**2**).[4] Mutational studies have indicated that the type B motifs in these bifunctional cyclases are responsible for the conversion of GGPP into *ent*-CPP (**4**) or CPP (**2**).[8,11,12] Moreover, an *ent*-CPP synthase gene was also identified from the eubacterium *Streptomyces* sp. strain KO-3988 (ORF2).[13,14] The primary structure of the eubacterial *ent*-CPP synthase ORF2 (511 amino acids) is more similar to the primary structure of terpentedienol diphosphate synthase (see Section 1.17.3.1) in *Streptomyces griseolosporeus* MF730-N6 than to the primary structure of eukaryotic *ent*-CPP synthases.

The rice (*Oryza sativa* L. cv. Nipponbare) genome includes two *ent*-CPP synthase genes. *OsCPS1* is responsible for gibberellin biosynthesis, whereas *OsCPS2* functions in the biosynthesis of oryzalexins A–F and phytocassanes A–E, both of which are classes of rice phytoalexins (see Section 1.17.2.3.1).[15] Loss-of-function mutants for *OsCPS1* displayed severe dwarf phenotypes.[16] *OsCPS2* transcription was drastically upregulated upon ultraviolet (UV) treatment in rice leaves.[15] AMO-1618 is a quaternary ammonium compound that not only inhibits the activities of plant *ent*-CPP synthases for gibberellin biosynthesis, but also the type B activity of fungal *ent*-kaurene synthase.[11] Interestingly, OsCPS2 activity was not suppressed in the presence of

Table 1 Labdane-related diterpene cyclase genes in rice cv. Nipponbare

Gene name	*a*	*b*	*Main product*	*References*
OsCPS1	Os02g17780	Os02g0278700	*ent*-CPP	15, 16
OsCPS2	Os02g36210	Os02g0571100	*ent*-CPP	15
OsCPS4	Os04g09900	Os04g0178300	*syn*-CPP	15
OsKS1	Os04g52230	Os04g0611800	*ent*-kaur-16-ene	16, 18
OsKSL4	Os04g10060	Os04g0179700	*syn*-pimara-7,15-diene	19
OsKSL5	Os02g36220	Os02g0571300	*ent*-pimara-8(14),15-diene	20
OsKSL6	Os02g36264	Os02g0571800	*ent*-kaur-15-ene	20
OsKSL7	Os02g36140	Os02g0570400	*ent*-cassa,12,15-diene	21
OsKSL8	Os11g28530	Os11g0474800	stemar-13-ene	18, 22
OsKSL10	Os12g30824	Os12g0491800	*ent*-sandaracopimaradiene	19

Possible pseudogenes are omitted. Loci in Nipponbare by (a) Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) or (b) Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/) are shown.

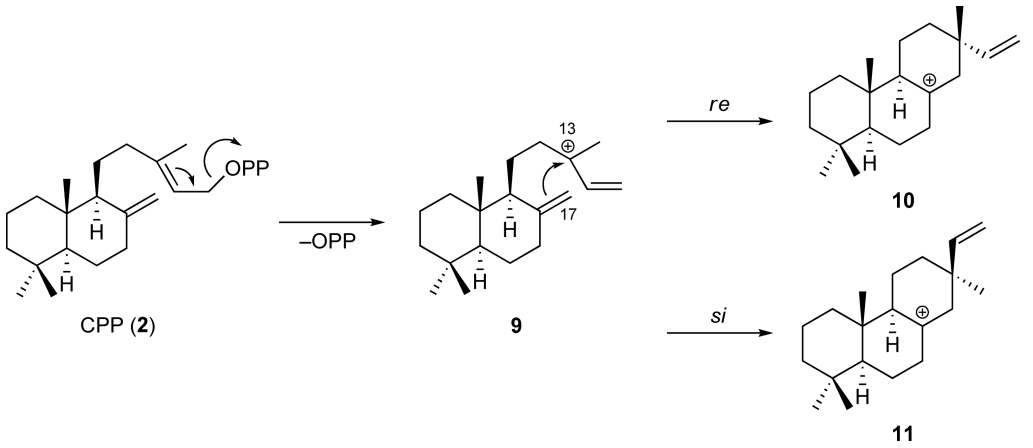
1×10^{-4} mol l^{-1} AMO-1618, under which condition OsCPS1 activity was suppressed.[17] From rice, the *syn*-CPP synthase gene, *OsCPS4*, was also identified as a phytoalexin biosynthetic gene,[15] which functions in the biosynthesis of momilactones A and B and oryzalexin S (see Section 1.17.2.4.1). The primary structure of OsCPS4 (767 amino acids) is similar to those of eukaryotic *ent*-CPP synthases, all of which possess the type B motif, as shown in **Figure 1(a)**. The type A and type B labdane-related cyclase genes in rice are summarized in **Table 1**.[23,24] The *OsCPS1*, *OsCPS2*, and *OsCPS4* genes were also identified in the *indica* rice cultivar IR24.[25,26] Type A rice cyclases are discussed in detail in Sections 1.17.2.3.1 and 1.17.2.4.1.

The cDNAs encoding CPP synthase and *ent-syn*-CPP synthase have not been identified in any organism. The recombinant protein encoded by *PaDC2* (984 amino acids) from the fungus *Phomopsis amygdali* converted GGPP into CPP (**2**) as an end product.[27] However, this reaction may have been due to a naturally occurring mutation at the C-terminal region of the fungal bifunctional diterpene cyclase (see Section 1.17.2.2.2), which may eliminate the activity for conversion of CPP (**2**) into cyclic hydrocarbons. Artificial mutants for the bifunctional abietadiene synthase, in which ^{621}D in the type A motif was substituted with alanine, exhibited a loss of type A activity and also converted GGPP into CPP (**2**).[28]

1.17.2.2 From Copalyl Diphosphate

1.17.2.2.1 Diterpene resin acids in conifers

Abietadiene synthase (868 amino acids) from grand fir (AgAS) produces abietadiene (**16**), levopimaradiene (**17**), and neoabietadiene (**18**) as major products, as well as plaustradiene (**19**), sandaracopimaradiene (**12**), and pimara-8(14),15-diene (**22**) as minor products; these compounds are produced from GGPP via CPP (**2**; see **Schemes 2–4**).[4,29] Abietadiene (**16**) is a biosynthetic intermediate of abietic acid (**20**), a compound that confers resistance against herbivory and pathogen attacks. The P-450 monooxygenase responsible for abietic acid (**20**) biosynthesis (PtAO, CYP720B1) was identified.[30] Cyclization mechanisms of these products are as follows: after ionization of the diphosphate of CPP (**2**), carbocation **9** is cyclized by the attack of C-17 at the *re*- or *si*-face of C-13, giving carbocation **10** or **11** (**Scheme 2**). The deprotonation of 14-H in carbocation **10** gives **12**, successive protonation of the 15,16-double bond of **12** gives carbocation **14**, and a 1,2-shift of the methyl from C-13 to C-15 gives carbocation **15** (**Scheme 3**). The deprotonation of 7-H, 12-H, 15-H, and 9-H in **15** gives compounds **16**, **17**, **18**, and **19**, respectively (**Scheme 3**), whereas the deprotonation of 14-H in carbocation **11** gives **22** (**Scheme 4**). As described in Section 1.17.1, AgAS possesses the type A and type B motifs (**Figure 1(d)**), and the type A motif DDxxD is required for the above reaction.[28] Other required amino acid residues were identified via mutational studies.[31] Notably, the two cyclization sites of AgAS cannot be dissected into catalytically distinct domains,[32] unlike the fungal *ent*-kaurene synthase (see Section 1.17.2.3.4). From Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*), the cDNAs *PaLAS* and *PtLAS* for levopimaradiene/abietadiene synthases were cloned, respectively,[33,34] and a cDNA encoding levopimaradiene synthase



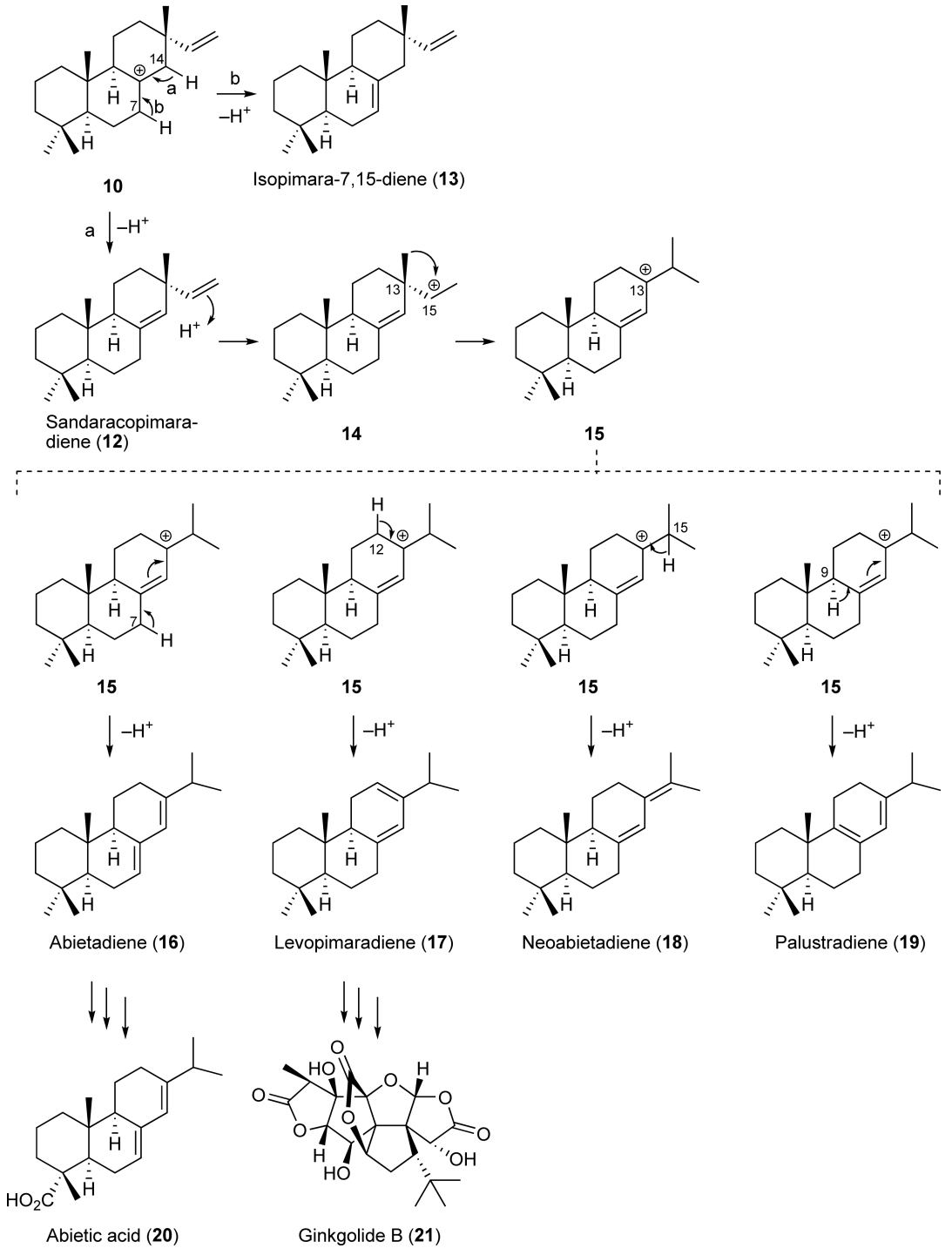
Scheme 2

(GbLS, 873 amino acids) was isolated from *Ginkgo biloba*.[35] Levopimaradiene (**17**) is a possible biosynthetic precursor of ginkgolides (e.g., **21**), which are potent platelet-activating factor (PAF) antagonists isolated from *G. biloba*. Another cyclase gene was isolated from spruce.[33] Isopimaradiene synthase in spruce (PaIso) produced one product, isopimara-7,15-diene (**13**), via the deprotonation of 7-H in carbocation **10**. The details of the biosynthesis of diterpene resin acids in conifers were previously reviewed by Keeling and Bohlmann.[36]

Conifer diterpene cyclases are highly homologous. Domain swapping and site-directed mutagenesis were carried out to identify amino acid substitutions that lead to alternative products. Mutant AgAS, in which ^{723}A was substituted with serine, produced mainly isopimara-7,15-diene (**13**) and sandaracopimaradiene (**12**).[37] The substitution of ^{713}A of PaLAS with serine (PaLAS A713S) similarly changed the products.[38] On the other hand, the PaIso S721A mutant produced mainly **13** and a small amount of **12**; the quadruple mutant, PaIso L687W/H694Y/S721A/L725V, produced abietadiene (**16**)/levopimaradiene (**17**).[38] The catalytic activities of these mutants did not differ significantly compared to the wild type. The results of substituting the alanine with serine suggest that the introduced hydroxyl group stabilizes carbocation **10** long enough for deprotonation to occur. Similar speculation on the rice type A cyclases is presented in Section 1.17.2.3.1.

1.17.2.2.2 Phyllocladane-related diterpenes in a fungus

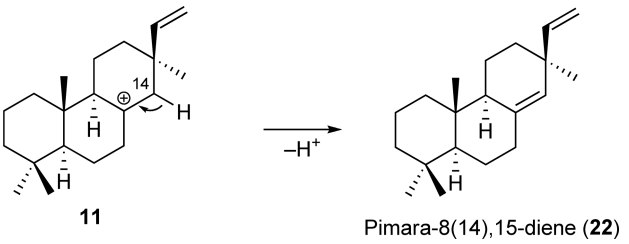
Phomopsis amygdali is a plant-pathogenic fungus that produces fusicoccins (see Section 1.17.4.3). In addition to fusicoccane-related hydrocarbons, a series of phyllocladane-related hydrocarbons were identified from the mycelia of *P. amygdali* F6 strain.[39] Two labdane-related cyclase genes, *PaDC1* and *PaDC2*, were isolated from the F6 strain. The recombinant PaDC1 converted GGPP to mainly phyllocladan-16α-ol (**26**; **Scheme 5**) via CPP.[27] Minor products included sandaracopimaradiene (**12**), isopimara-8,15-diene (**24**), phyllocladene (**27**), pimara-8(14),15-diene (**22**), pimara-8,15-diene (**30**), and kaurene (**32**). During the rearrangement of **10**, carbocation **25** is formed via **23**, and the deprotonation of 17-H in **25** gives phyllocladene (**27**), while stereoselective attack by a water molecule generates phyllocladan-16α-ol (**26**; **Scheme 5**). Compounds **12** and **24** are produced by the deprotonation of 14-H and 9-H in **10**, respectively (**Scheme 5**). Sequential rearrangement of **11** via **29** gives carbocation **31**, and the deprotonation of 17-H in **31** yields **32** (**Scheme 6**). The deprotonation of 14-H and 9-H in compound **11** yields compounds **22** and **30**, respectively (**Scheme 6**). PaDC1 (1017 amino acids) possesses the type B motif DxDD (positions 321–324) and the type A motif DExxE (positions 667–671), similar to AgAS (**Figure 1(d)**). The primary structure of PaDC1 is homologous to those of other fungal labdane-related cyclases (see Sections 1.17.2.3.4 and 1.17.2.4.2). As described in Section 1.17.2.1, recombinant PaDC2 converted GGPP into CPP, but did not catalyze the further cyclization of CPP, which may have been due to a naturally occurring mutation in the type A domain. With the presence of both a type B



Scheme 3

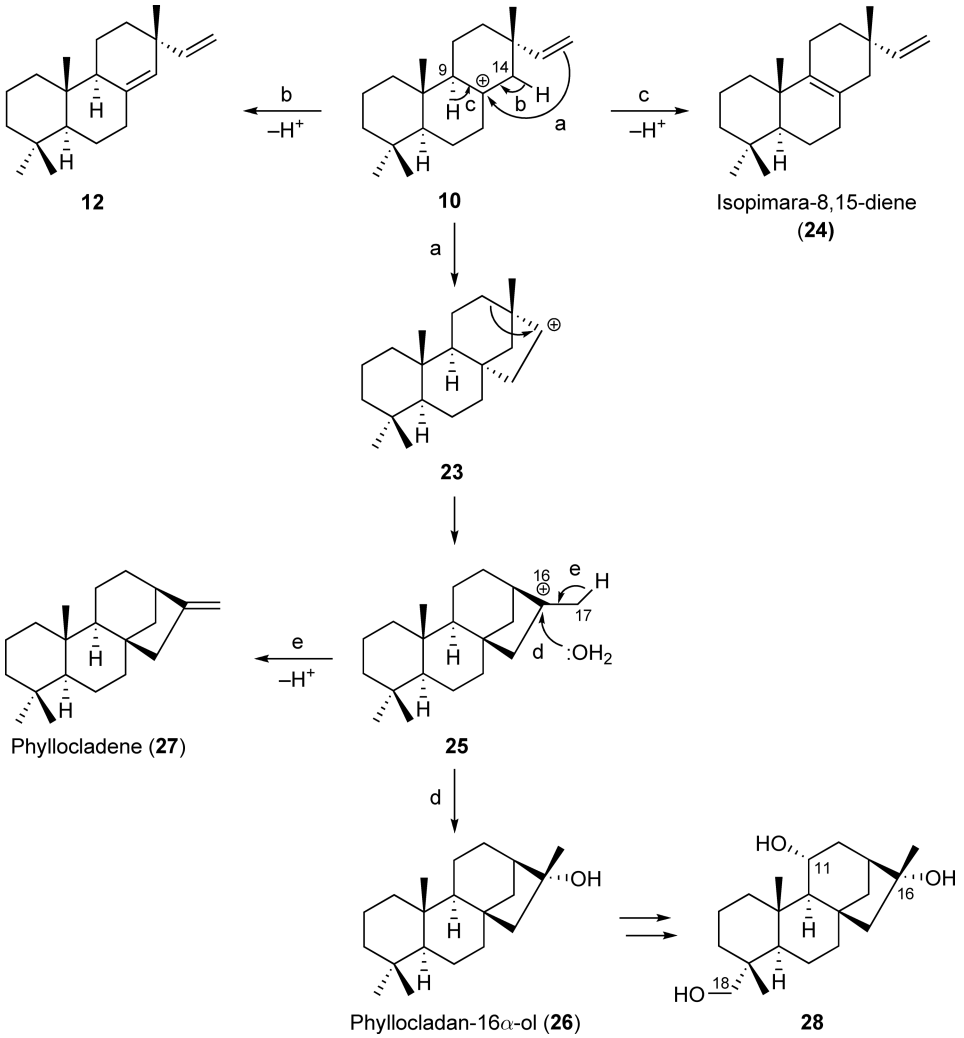
motif (positions 318–321) and a type A motif (positions 656–660) in PaDC2, a C-terminal region other than that of the type A motif may be mutated, causing a loss of the type A function.[27]

Since the discovery of the gibberellin biosynthetic gene cluster in *Gibberella fujikuroi* (see Section 1.17.2.3.4), it has been shown that diterpene biosynthetic genes are clustered within the fungal genome. Five P-450



11 Pimara-8(14),15-diene (**22**)

Scheme 4



12 **10** Isopimara-8,15-diene (**24**)

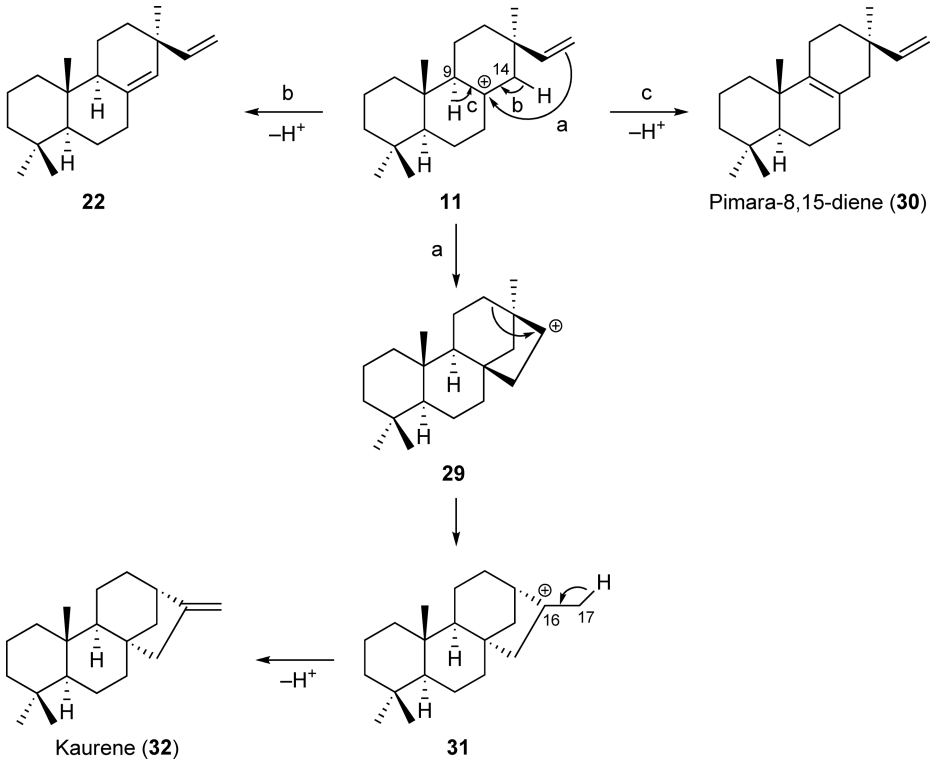
23

Phyllocladene (**27**) **25**

Phyllocladan-16α-ol (**26**) **28**

Scheme 5

monooxygenase-like genes, one dehydrogenase-like gene, and one dioxygenase-like gene are located around *PaDC1* in the *P. amygdali* genome.[27] From the F6 mycelia, phyllocladan-11α,16α,18-triol (**28**) was identified as a possible hydroxylated metabolite of **26**.[27] Two P-450-like genes, among the five genes around *PaDC1*, may be responsible for the hydroxylation of C-11α and C-18 in **26**. Highly oxygenated phyllocladanes, which have not been identified from the *P. amygdali* mycelia, may be generated by other oxygenases.

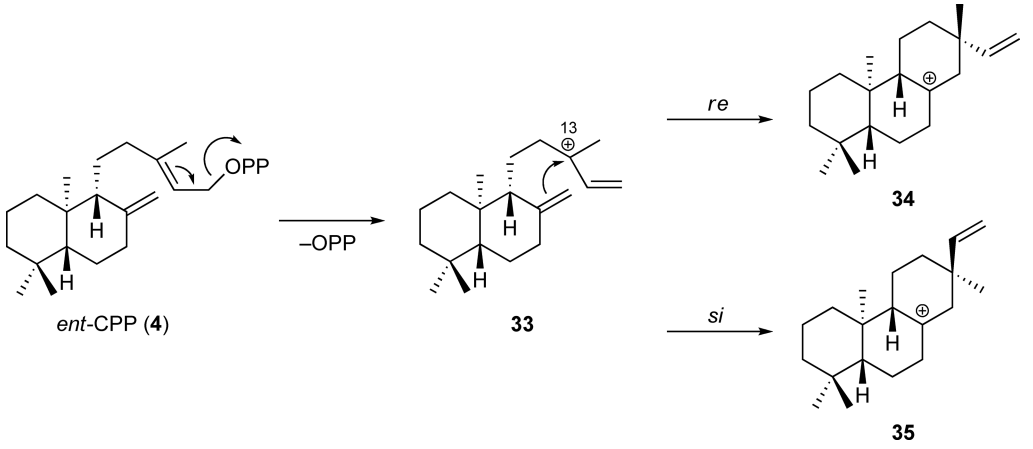


Scheme 6

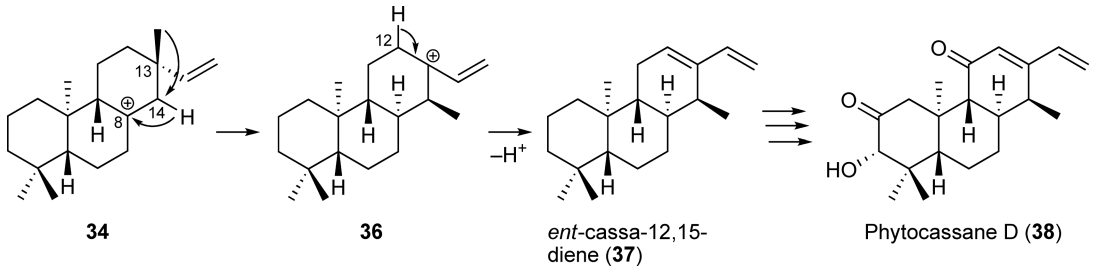
1.17.2.3 From *ent*-Copalyl Diphosphate

1.17.2.3.1 ent-*Labdane-related diterpenes in rice*

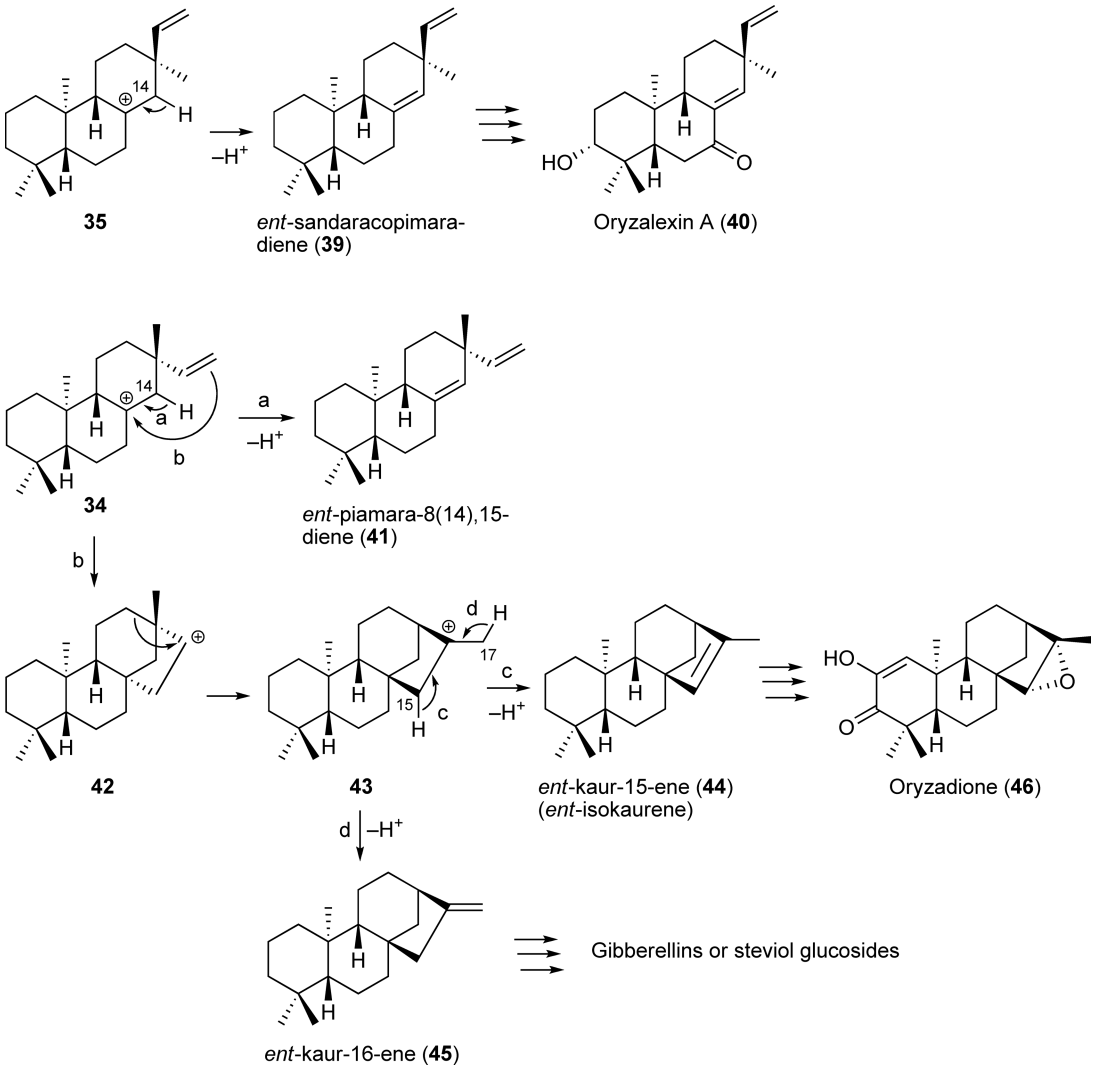
As type A *ent*-labdane-related diterpene cyclase genes, *OsKS1*, *OsKSL5*, *OsKSL6*, *OsKSL7*, and *OsKSL10* were identified in both *japonica* (Nipponbare) and *indica* (IR24) rice cultivars. These genes are homologs of the *ent*-kaurene synthase possessing a type A motif (**Figure 1(b)**). *ent*-Cassa-12,15-diene (**37**) and *ent*-sandaracopimaradiene (**39**), possible biosynthetic intermediate cyclic hydrocarbons of phytocassanes A–E (e.g., **38**) and oryzalexins A–F (e.g., **40**), are converted from *ent*-CPP (**4**) by OsKSL7 and OsKSL10, respectively (**Schemes 7–9** and **Table 1**).[18,19,21] As in the case of CPP (**2**; **Scheme 2**), the C-13 carbocation **33**, derived from *ent*-CPP



Scheme 7



Scheme 8



Scheme 9

(**4**) after the ionization of diphosphate, gives carbocation **34** or **35** via the attack of C-17 at the *re*- or *si*-face of C-13, respectively (**Scheme 7**). The 1,2-hydride shift from C-14 to C-8 and successive 1,2-shift of a methyl group from C-13 to C-14 in carbocation **34** gives carbocation **36**. The deprotonation of 12-H in **36** gives *ent*-cassa-12,15-diene (**37**), whereas the deprotonation of 14-H in **35** gives *ent*-sandaracopimaradiene (**39**);

ent-pimara-8(14),15-diene (**41**) was also identified as a minor product of *ent*-CPP (**4**), catalyzed via OsKSL10.[19] Compound **41** is generated by the deprotonation of 14-H in **34**. The transcription of *OsKSL7* and *OsKSL10*, as well as *OsCPS2* and *OsCPS4*, was induced by UV irradiation in rice leaves or by chitin-elicitor treatment in cell suspensions.[18,19,21]

OsKSL5 and OsKSL6 in Nipponbare converted *ent*-CPP (**4**) into *ent*-pimara-8(14),15-diene (**41**) and *ent*-kaura-15-ene (*ent*-isokaurene, **44**), respectively (**Schemes 7 and 9** and **Table 1**).[20] Compound **44** is a possible precursor of oryzalide-related compounds (e.g., oryzadione **46**), which serve as antifungal agents.[40] Sequential rearrangements of **34** via **42** yields carbocation **43**, and the deprotonation of 15-H in **43** gives **44** (**Scheme 9**). Interestingly, both OsKSL5 and OsKSL6 in IR24 converted **4** into **44** (**Scheme 9**).[18] The comparison of the OsKSL5 amino acid sequence in IR24 with that in Nipponbare led to the identification of specific residues responsible for this difference in compounds produced.[41] The product of catalysis by the I664T mutant of OsKSL5 in IR24 was **41**, and conversely, the T661I mutant of OsKSL5 in Nipponbare produced mainly **44**. These results suggest that the introduced hydroxyl group stabilized carbocation **34**, similar to conifer diterpene cyclases (see Section 1.17.2.2.1). The hypothesis is consistent with the case of OsKSL6, OsKS1, and AtKS.[41]

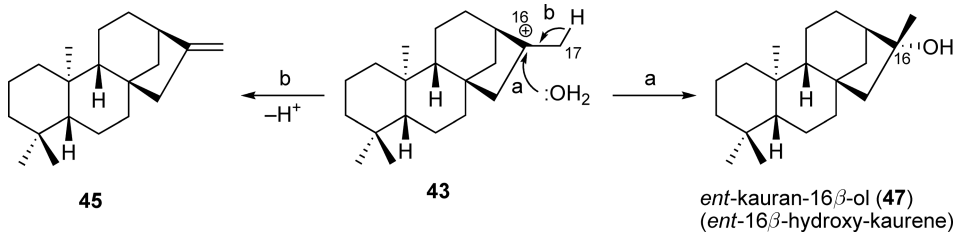
Loss-of-function mutants for *OsKS1* exhibited a severe dwarf phenotype, similar to *OsCPS1* mutants.[16] The recombinant OsKS1 protein converted *ent*-CPP (**4**) into *ent*-kaur-16-ene (**45**; **Table 1**).[18] Compound **45** is produced via the deprotonation of 17-H in carbocation **43** (**Scheme 9**). The orthologs of *ent*-kaurene synthase that function in gibberellin biosynthesis have been isolated from several higher plants, including pumpkin and *Arabidopsis*.[3,7] Compound **45** is metabolized into steviol and its glycosides in *Stevia rebaudiana* (see Section 1.17.2.3.5).[42]

1.17.2.3.2 ent-*Kaurane-related diterpenes in moss*

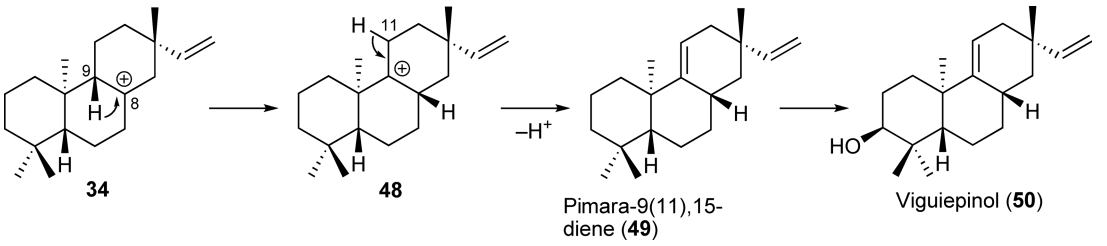
A cDNA encoding a bifunctional type B-A diterpene cyclase (PpCPS/KS, 881 amino acids) has been isolated from moss (*Physcomitrella patens*). PpCPS/KS converted GGPP into mainly *ent*-kauran-16β-ol (*ent*-16β-hydroxy-kaurene, **47**) via *ent*-CPP (**4**).[8] Stereoselective attack of a water molecule on the C-16 cation in **43** gives **47** (**Scheme 10**). As a by-product, *ent*-kaur-16-ene (**45**) was produced by the deprotonation of 17-H in **43**. The type B and type A motifs in PpCPS/KS are required to convert GGPP into *ent*-CPP (**4**), and **4** into **47** and **45**,[8] respectively, similar to AgAS (see Section 1.17.2.2.1) and fungal *ent*-kaurene synthase (see Section 1.17.2.3.4). The metabolism of gibberellins from **45** in bryophytes remains unexplained.

1.17.2.3.3 ent-*Pimarane-related diterpenes in eubacteria*

ORF1, ORF3, and ORF4 were identified near ORF2 of the *ent*-CPP synthase gene (see Section 1.17.2.1) in the genome of eubacteria (*Streptomyces* sp. strain KO-3988).[13] ORF1 encodes a P-450-like protein, whereas ORF4 encodes a GGPP synthase. The recombinant protein from ORF3 (295 amino acids) converted *ent*-CPP (**4**) into pimara-9(11),15-diene (**49**, **Scheme 11**).[43] The 1,2-hydride shift from C-9 to C-8 in carbocation **34** and successive deprotonation of 11-H in **48** gives **49** (**Scheme 11**). The primary structure of ORF3 shows 37% similarity to Cyc2, a terpentetriene synthase (see Section 1.17.3.1). Both enzymes possess a type A motif. The *Streptomyces lividans* TK23 strain, in which the genomic DNA including ORFs 1–4 was introduced, produced viguiepinol (**50**).[14] This result indicates that ORFs 1–4 are clustered for the biosynthesis of **50**, and that ORF1 may be responsible for the 3β-hydroxylation of **49**.



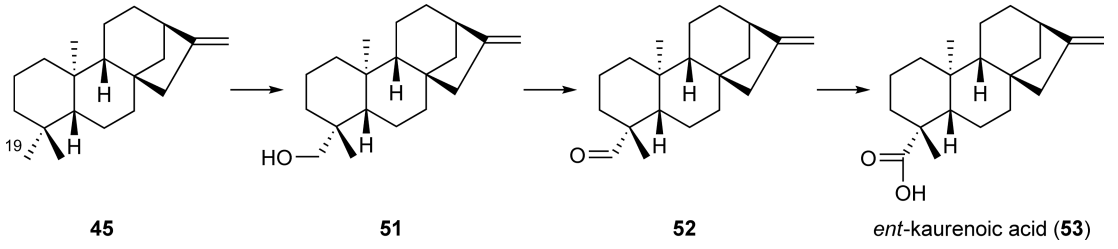
Scheme 10



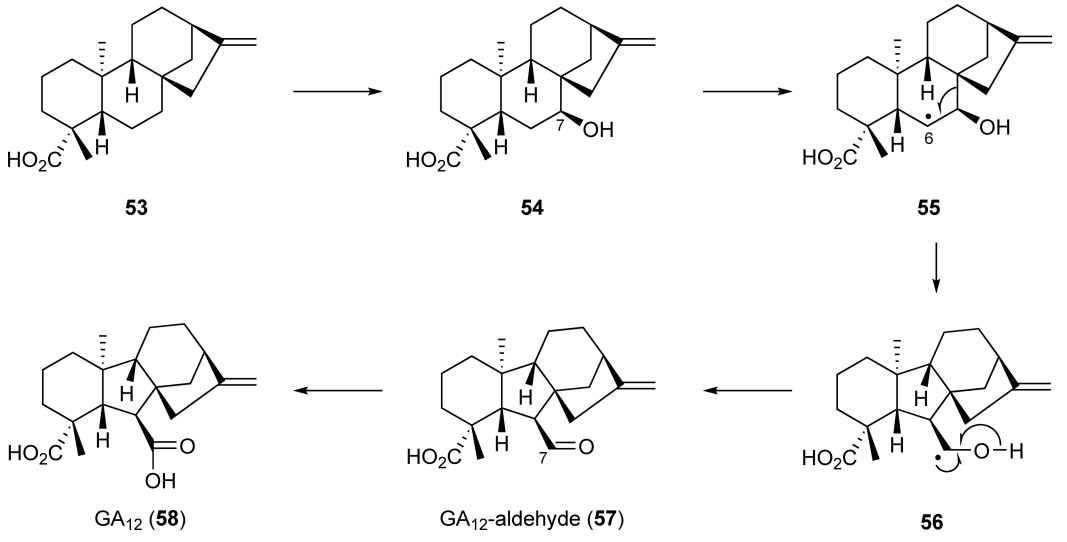
Scheme 11

1.17.2.3.4 Gibberellins

Gibberellins are phytohormones that regulate various aspects of plant growth.[7] Gibberellin-deficient mutants have demonstrated the importance of these compounds in plant growth regulation. Gibberellin metabolism genes have been identified in various higher plants and fungi.[7] As described above, *ent*-kaur-16-ene (**45**) is an intermediate of gibberellins and is produced by two distinct plastid-localized cyclases, *ent*-CPP synthase (see Section 1.17.2.1) and *ent*-kaurene synthase (see Section 1.17.2.3.1) in higher plants. A loss-of-function mutant of the *ent*-kaurene synthase gene in *Arabidopsis* resulted in severe dwarf phenotypes,[44] similar to rice (see Section 1.17.2.3.1). C-19 in **45** is successively oxidized to produce *ent*-kaurenoic acid (**53**) via *ent*-kaurenol (**51**) and *ent*-kaurenal (**52**; **Scheme 12**) by *ent*-kaurene oxidase (KO), which belongs to the P-450 monooxygenase family. The cDNA encoding KO was first cloned from *Arabidopsis* and characterized (CYP701A5).[45,46] Furthermore, **53** is successively oxidized to GA_{12} (**Scheme 13**) via *ent*-7α-hydroxy-kaurenoic acid (**54**) and GA_{12}-aldehyde (**57**)



Scheme 12



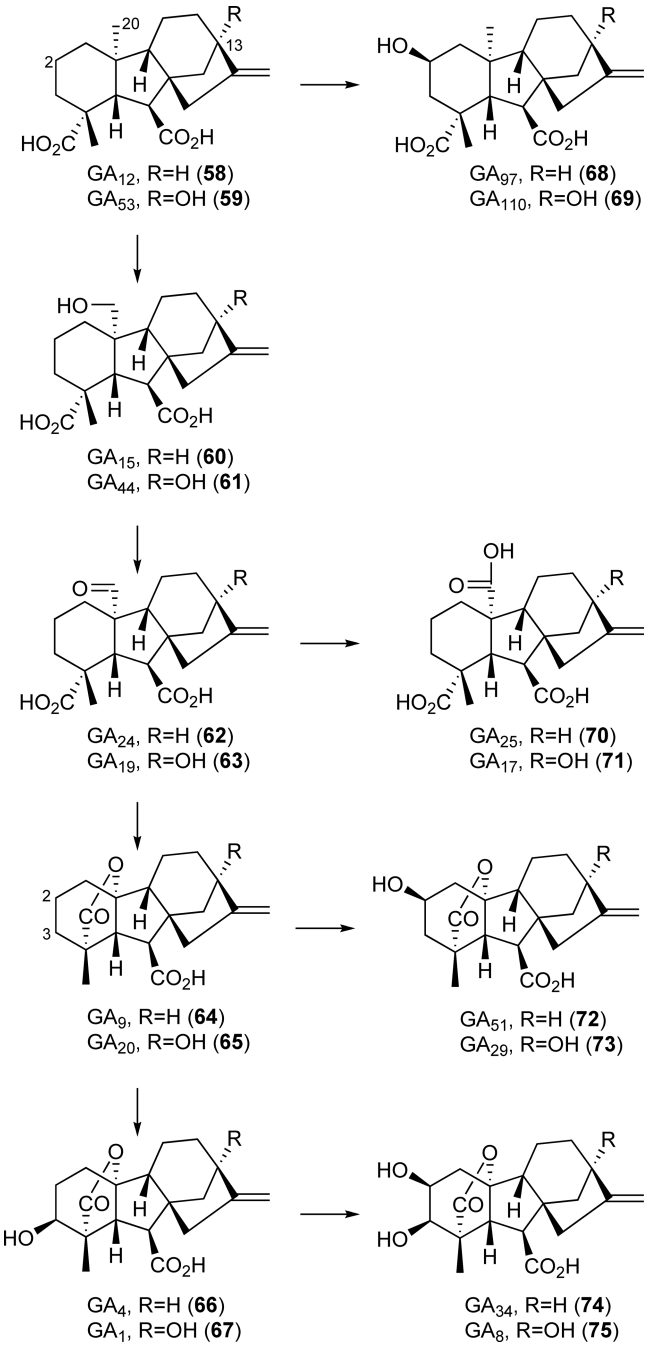
Scheme 13

by *ent*-kaurenoic acid oxidase (KAO), which is also a member of the P-450 monooxygenase family. KAO was first identified in barley (*Hordeum vulgare*) and *Arabidopsis* (CYP88A3 and CYP88A4).[47] The radical (**55**) produced by the extraction of 6β-H undergoes rearrangement, whereby the 7–8 bond migrates to C-6 to form GA_{12}-aldehyde (**57**) via **56**. In pumpkin, gibberellin 7-oxidase (GA7ox), which is a member of the 2-oxoglutarate-dependent soluble dioxygenase family, was identified.[48] GA7ox converts GA_{12}-aldehyde (**57**) to GA_{12} (**58**). Orthologs of the *GA7ox* gene have not been identified in *Arabidopsis* or rice.

Physiologically active gibberellins are biosynthesized from GA_{12} (**58**) through two primary parallel pathways in higher plants, the early-nonhydroxylation pathway and early-13-hydroxylation pathway, which lead to GA_4 (**66**) and GA_1 (**67**), respectively (**Scheme 14**). These steps are catalyzed by 2-oxoglutarate-dependent dioxygenases, although gibberellin 13-hydroxylase has not been identified so far in higher plants. Gibberellin 20-oxidase (GA20ox) converts GA_{12} (**58**) and GA_{53} (**59**) into GA_9 (**64**) and GA_{20} (**65**), respectively, by the oxidation of C-20. The γ-lactone formation type GA20ox, which leads to bioactive gibberellins, was first identified in *Arabidopsis*.[49] Pumpkin GA20ox converted GA_{12} (**58**) and GA_{53} (**59**) into mainly GA_{25} (**70**) and GA_{17} (**71**), tricarboxyl-type gibberellins, respectively.[50] Finally, 3β-hydroxylation of GA_9 (**64**) and GA_{20} (**65**) produces physiologically active gibberellins GA_4 (**66**) and GA_1 (**67**), respectively. Bioactive GA_4 (**66**) and GA_1 (**67**) are deactivated through 2β-hydroxylation, which produces GA_{34} (**74**) and GA_8 (**75**), respectively. These steps are catalyzed by gibberellin 3-oxidase (GA3ox)[51,52] and gibberellin 2-oxidase (GA2ox),[53] respectively. Loss-of-function mutants for *GA3ox* exhibited a dwarf phenotype, and those of *GA2ox* displayed an elongated phenotype.[7] These results indicate that GA_4 (**66**) and GA_1 (**67**) are physiologically active gibberellins in higher plants. GA2ox enzymes also convert GA_9 (**64**) and GA_{20} (**65**), direct precursors of GA_4 (**66**) and GA_1 (**67**), into GA_{51} (**72**) and GA_{29} (**73**). A novel class of GA2ox, which prefers C_{20}-gibberellins to γ-lactone-type C_{19}-gibberellins as substrates, was identified in *Arabidopsis*, and converts GA_{12} (**58**) and GA_{53} (**59**) into GA_{97} (**68**) and GA_{110} (**69**).[54]

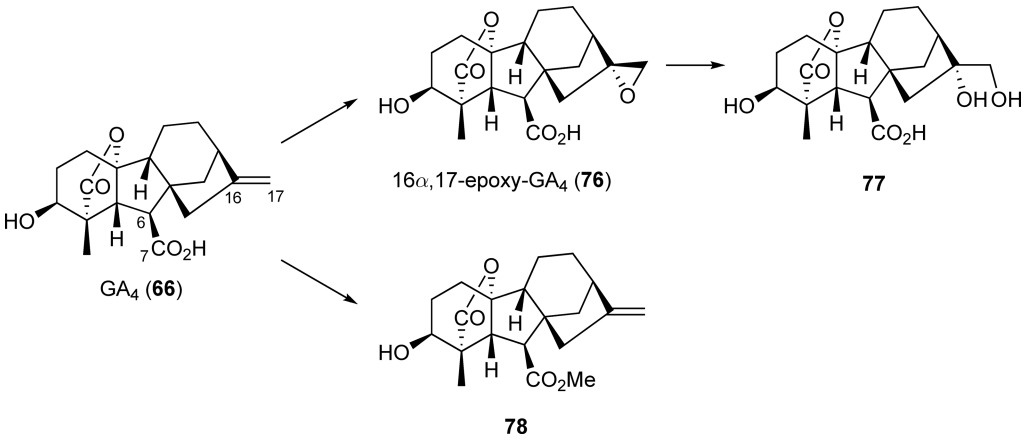
Recently, other types of gibberellin metabolic enzymes were identified in rice and *Arabidopsis*. Rice *eui* mutants possessing an impaired P-450 monooxygenase gene exhibited an elongated phenotype. The P-450 enzyme (CYP714D1) converts non13-hydroxylated C_{20}- and C_{19}-gibberellins (e.g., **66**) into their 16,17-epoxide types (e.g., **76**; **Scheme 15**).[55] The 16α,17-dihydrodiols (e.g., **77**) may be nonenzymatically produced by hydration of the epoxides (e.g., **76**). Compounds **76** and **77** are not physiologically active. Taken together, these results suggest that CYP714D1 serves in gibberellin deactivation. Methyltransferases, which prefer γ-lactone-type gibberellins (e.g., **66**) as substrates to produce methylated gibberellins (e.g., **78**; **Scheme 15**), were identified in *Arabidopsis* (GAMT1 and GAMT2).[56] Transgenic *Arabidopsis*, in which GAMT1 or GAMT2 was overexpressed, exhibited severe dwarf phenotypes, suggesting that methylation is a deactivation step for gibberellins, although methylated gibberellins have not been identified in higher plants.

Gibberellins were originally isolated as secondary metabolites of a fungus, *G. fujikuroi*. The biosynthetic pathway of gibberellins from GA_{12} (**58**) to bioactive GA_3 (**81**) in *G. fujikuroi* is shown in **Scheme 16** by bold arrows. On the other hand, *Phaeosphaeria* sp. L487 produces GA_1 (**67**), as indicated by normal arrows in **Scheme 16**. Several steps from GGPP to GA_{12} (**58**) via *ent*-kaurene (**45**) are common to both fungi. As described, *ent*-kaurene (**45**) is converted from GGPP via *ent*-CPP (**4**) by a single enzyme (PhCPS/KS or GfCPS/KS) in both fungi.[9,10] The fungal bifunctional cyclases possess both type A and type B motifs, similar to AgAS (**Figure 1(d)**). Site-directed mutagenesis of PhCPS/KS demonstrated that the type B motif is required for the conversion of GGPP into *ent*-CPP (**4**), and that the type A motif is required to convert **4** into *ent*-kaurene (**45**).[11] Furthermore, experiments using truncated mutants for PhCPS/KS revealed that the N- and C-terminal domains, respectively, possess *ent*-CPP synthase and *ent*-kaurene synthase activity.[11] The genes for the enzymes responsible for the steps from *ent*-kaurene (**45**) to GA_3 (**81**) were identified in *G. fujikuroi* by gene walking from the GfCPS/KS gene. One GGPP synthase-like gene and four P-450-like genes (P-450-1, P-450-2, P-450-3, and P-450-4) were found in the flanking region of genomic DNA near the *GfCPS/KS* gene.[57] P-450-4 (CYP503)[58] and P-450-1 (CYP68A)[59] are responsible for the conversion of *ent*-kaurene (**45**) to *ent*-kaurenoic acid (**53**) and **53** to GA_{14} (3β-hydroxylated GA_{12}, **79**), respectively. The primary structures of these two fungal P-450 enzymes are not highly homologous to the corresponding P-450 enzymes in higher plants. Interestingly, fungal KAO not only converts *ent*-kaurenoic acid (**53**) into GA_{12} (**58**), but also catalyzes the 3β-hydroxylation of GA_{12}. This is because the early-3-hydroxylation



Scheme 14

gibberellin biosynthetic pathway operates in *G. fujikuroi*. Unlike higher plants, fungal GA20ox (P-450-2) in *G. fujikuroi* is a member of the P-450 family (CYP68B).[60] Finally, GA_4 desaturase, which was found in subsequent gene walking studies, converts GA_4 (**66**) to GA_7 (**80**), and P-450-3 (CYP69A1) hydroxylates C-13 in GA_7 (**80**) to produce GA_3 (**81**).[61] This information indicates that gibberellin biosynthetic genes are clustered within the *G. fujikuroi* genome (**Figure 2**). A possible gibberellin biosynthetic gene cluster was also suggested in *Phaeosphaeria*.[62]



Scheme 15

1.17.2.3.5 Steviol glycosides

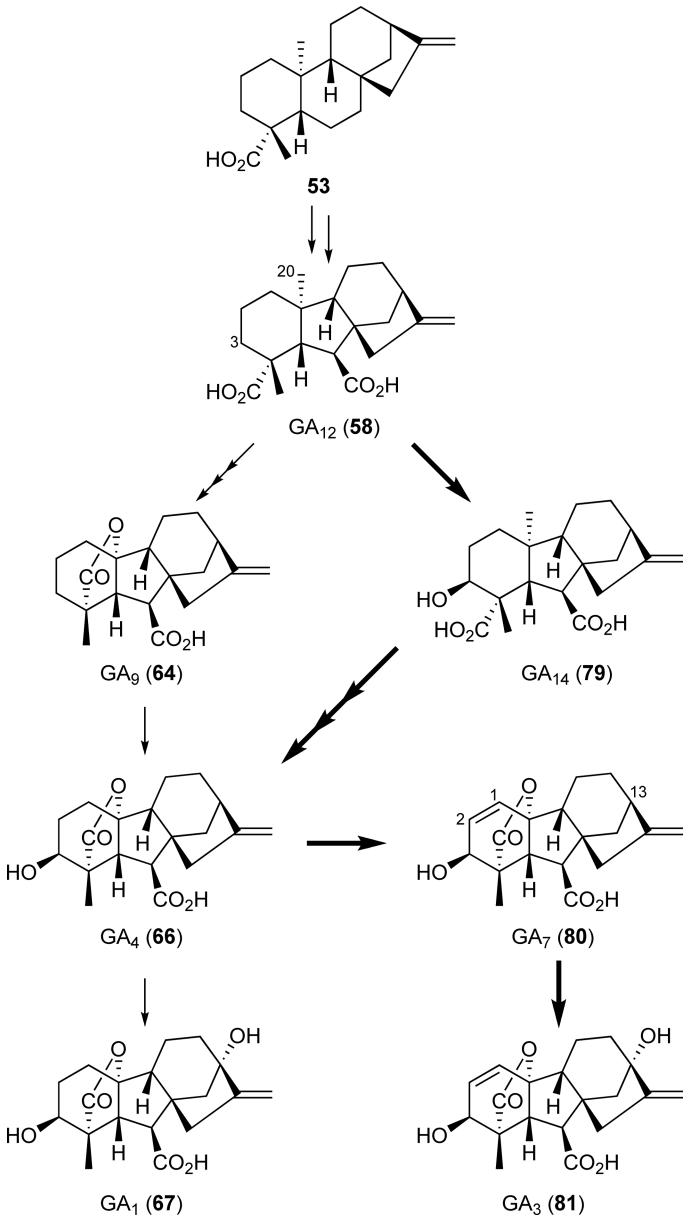
Stevia rebaudiana produces a series of steviol glycosides in its leaves (e.g., **85** in **Scheme 17**). These compounds have an intensely sweet flavor, and their biosynthesis was reviewed by Brandle and Telmer.[63] The aglycon of the steviol glycosides is steviol (**82**), which is derived from GGPP via *ent*-CPP (**4**), *ent*-kaurene (**45**), and *ent*-kaurenoic acid (**53**; **Schemes 1**, **7**, **9**, and **12**). From *S. rebaudiana*, one *ent*-CPP synthase gene and two *ent*-kaurene synthase genes were cloned and characterized.[64] KO in *S. rebaudiana* catalyzed the conversion of **45** into **53**,[65] whereas 13-hydroxylase, which converts **53** into **82**, has not been identified. The UDP-glucosyltransferases (UGTs) in *S. rebaudiana* have been characterized.[66] UGT85C2 transfers glucose to C-13 in steviol (**82**) to produce 13-*O*-β-glucosylated steviol (steviolmonoside, **83**). UGT74G1 and UGT76G1 are responsible for the addition of glucose to the hydroxyl of the C-4 carboxyl group in steviolbioside (**84**) to produce stevioside (**85**) and for the transfer of glucose to C-3′ of the 13-*O*-glucosyl moiety in **85** to produce rebaudioside (**86**), respectively. The UGT, which transfers glucose to C-2′ of the 13-*O*-glucosyl moiety in **83** to produce **84**, has not been identified.

1.17.2.4 From *syn*-CPP

1.17.2.4.1 syn-*Labdane-related diterpenes in rice*

In rice, *syn*-pimara-7,15-diene (**90**) and stemar-13-ene (**98**) are the possible biosynthetic intermediate hydrocarbons of momilactones A (**92**) and B (**93**), and oryzalexin S (**99**), respectively, and are derived from *syn*-CPP (**6**), as shown in **Schemes 18–20**. Compounds **92**, **93**, and **99** serve as phytoalexins in rice. OsKSL4 and OsKSL8, type A cyclases in *japonica* Nipponbare rice, catalyze the conversion of **6** into mainly **90** and **98** (**Table 1**).[18,19,22] The *re*- and *si*-face attacks of C-17 on C-13 in **87** yields the C-8 carbocations **88** and **89**, respectively (**Scheme 18**), whereas the deprotonation of 7-H in **89** yields **90** (**Scheme 19**). The OsKSL4 in *indica* IR24 rice also catalyzes the same reaction.[67] The 1,2-hydride shift from C-9 to C-8 in **88** gives the C-9 carbocation **94**, and the successive attack of C-16 on C-9 gives the C-15 carbocation **95** (**Scheme 20**). The rearrangement (pathway a in **Scheme 20**) and successive deprotonation of 14-H in **95** gives **98** via **96**. An alternative rearrangement (pathway b in **Scheme 20**) of **95** gives the C-13 carbocation **97**. The deprotonation of 17-H and 12-H in **97** gives stemod-13(17)-ene (**100**) and stemod-12-ene (**101**), respectively. The identified OsKSL8-catalyzed products were **98** (major) and **101** (minor).[18,22] The recombinant protein from *OsKSL11*, a possible allele of *OsKSL8* in IR24, produced mainly **100** and trace amounts of **98** and **101**.[68]

The genomic region flanking rice *OsKSL4* encodes not only *OsCPS4*, a *syn*-CPP synthase (see Section 1.17.2.1), but also two P-450-like genes (*CYP99A2* and *CYP99A3*) and one dehydrogenase-like gene (AK103462). The transcription of the three modification enzyme genes, as well as two cyclase genes, was drastically upregulated in rice suspension cells after elicitor treatment. The RNA interference (RNAi)-mediated



Scheme 16

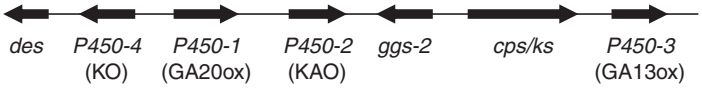
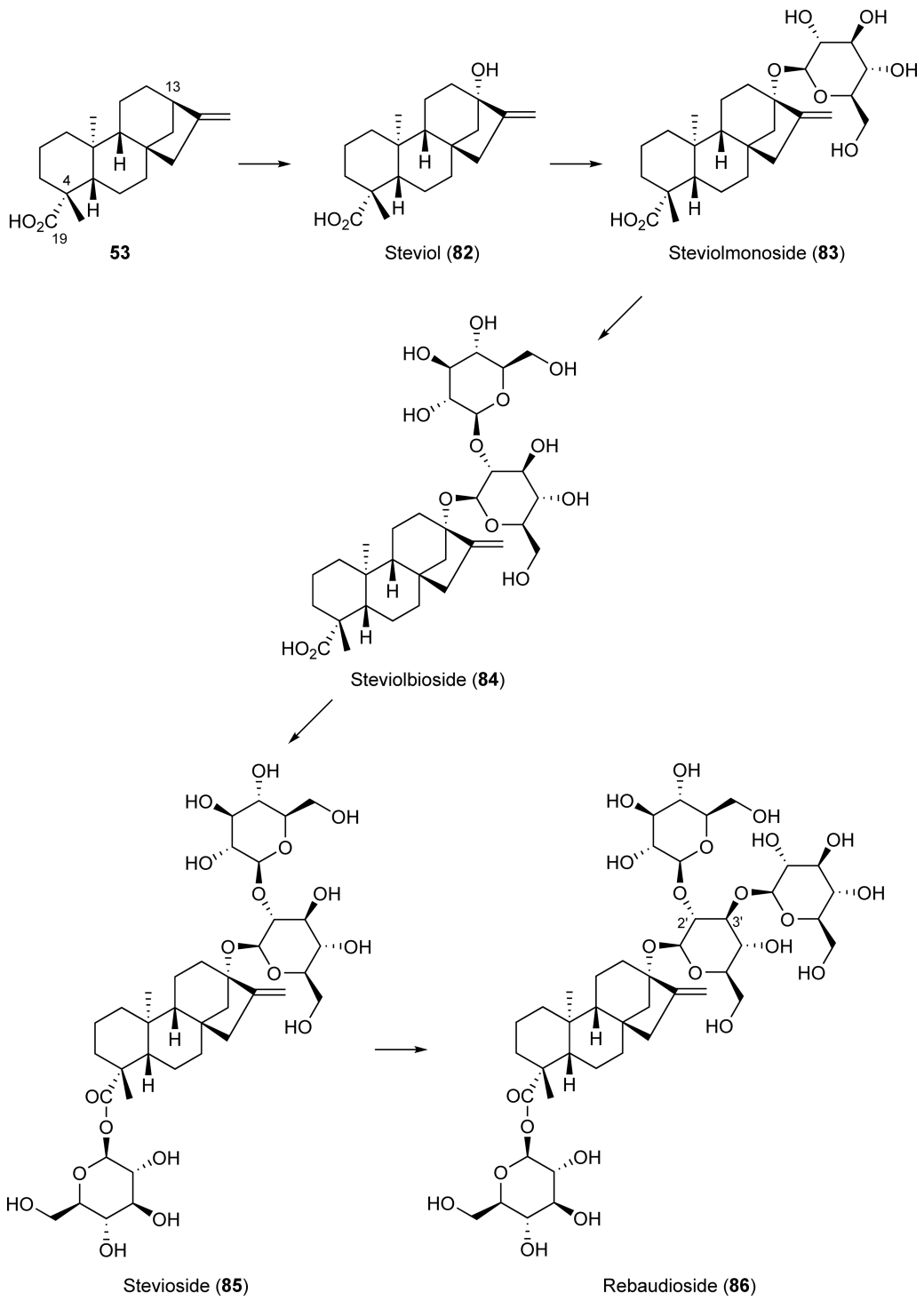


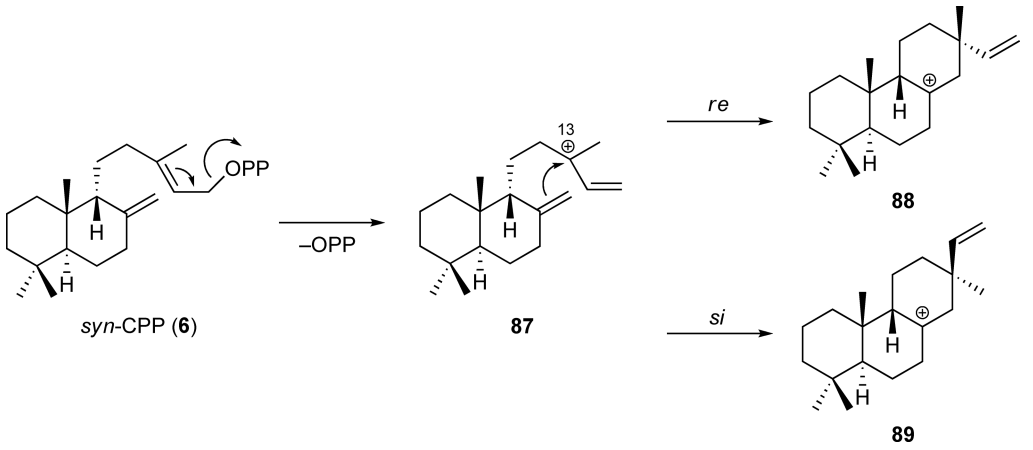
Figure 2 Gibberellin biosynthetic gene cluster in *Gibberella fujikuroi*. GGPP synthase, *ent*-CPP/*ent*-kaurene synthase, *ent*-kaurene oxidase, *ent*-kaurenoic acid oxidase, gibberellin 20-oxidase, gibberellin 13-hydroxylase, and GA_4 desaturase are encoded by *ggs-2*, *cps/ks*, *P-450-4*, *P-450-2*, *P-450-1*, *P-450-3*, and *des*, respectively.

downregulation of both *CYP99A2* and *CYP99A3*, which possess similar nucleotide sequences, resulted in the reduced production of **92** and **93** in rice cells, suggesting that *CYP99A2* and *CYP99A3* genes are responsible for the biosynthesis of momilactones. Furthermore, recombinant AK103462 catalyzed the conversion of

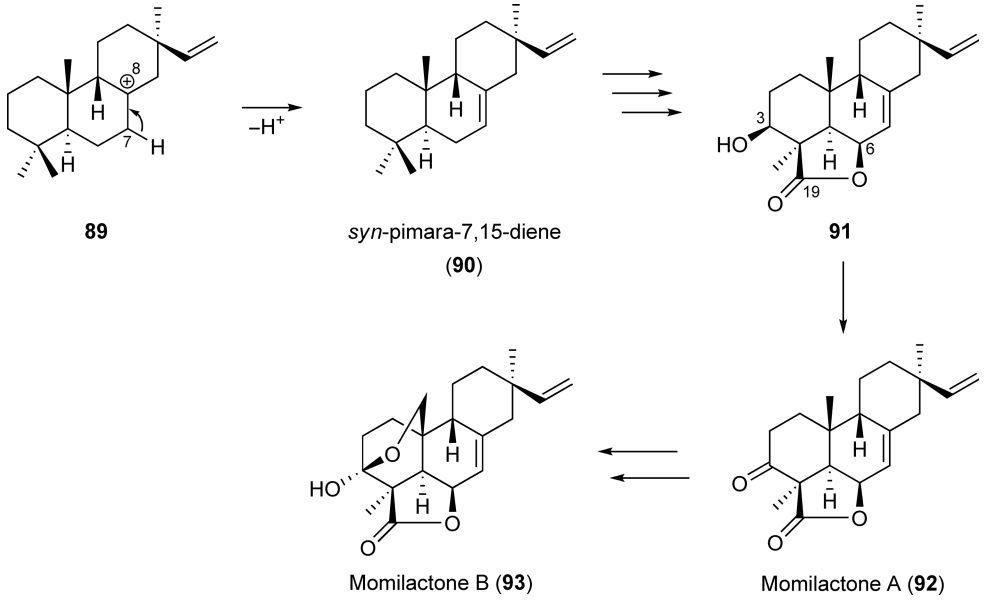


Scheme 17

3β-hydroxy-9β*H*-pimara-7,15-dien-19,6β-olide (**91**) into **92**, indicating that AK103462 encodes momilactone A synthase. These results strongly suggest that the biosynthetic genes of momilactones are clustered within the rice genome,[69] as well as in bacteria and fungi.



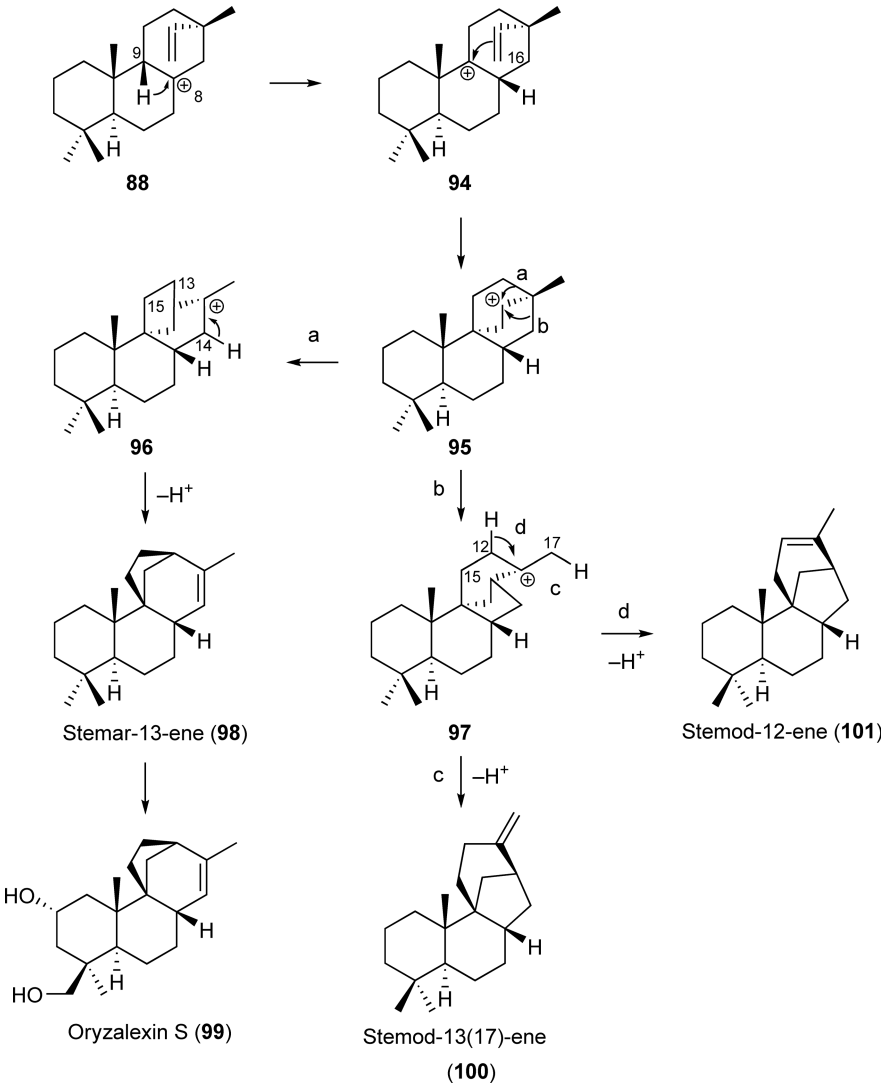
Scheme 18



Scheme 19

1.17.2.4.2 Aphidicolane-related diterpenes in a fungus

The plant-pathogenic fungus *Phoma betae* produces aphidicolin (**107** in **Scheme 21**), derived from *syn*-CPP (**6**). In physiological studies, aphidicolin (**107**) is used to induce cell cycle arrest via the specific inhibition of DNA polymerase α. A cDNA (*PbACS*) encoding the diterpene cyclase responsible for aphidicolin biosynthesis was isolated from the mycelia of *P. betae*.[70] PbACS (944 amino acids) converted GGPP into mainly aphidicolan-16β-ol (**104**) via *syn*-CPP (**6**). Aphidicol-16-ene (**105**) and aphidicol-15-ene (**106**) were also identified as minor by-products. The 1,2-hydride shift from C-9 to C-8 and successive attack of C-16 on C-9 in the C-8 carbocation **89**, derived from *syn*-CPP as shown in **Scheme 18**, yields the C-15 carbocation **102** (**Scheme 21**). The rearrangement of **102** produces the C-13 carbocation **103**. Quenching through the stereoselective capture of water (pathway a in **Scheme 21**) in **103** gives **104**. The deprotonation of 17-H (pathway b) and 12-H (pathway c) in **103** gives **105** and **106**, respectively (**Scheme 21**). These results suggest that pathway



Scheme 20

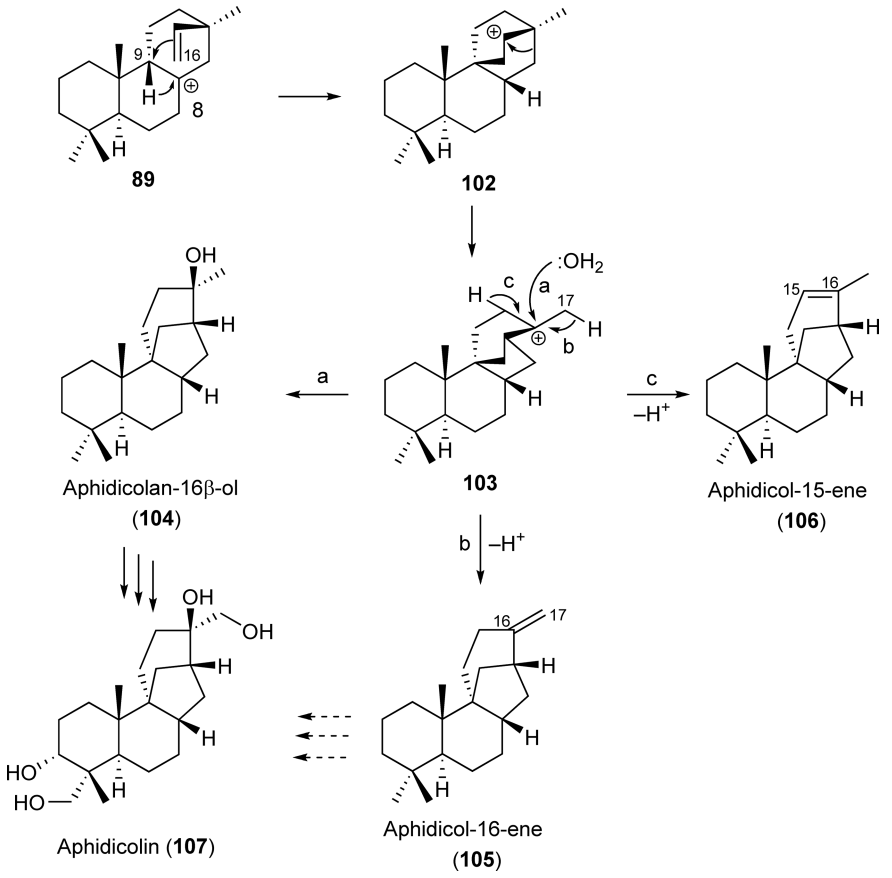
a is the major and pathway b is the minor pathway in the biosynthesis of **107**. The cyclization mechanism of PbACS was proposed based on experiments on biomimetic cyclization and *ab initio* calculation.[71]

Gene walking was used to identify two P-450-like genes and one GGPP synthase-like gene near the *PbACS* gene,[72] suggesting that the genes involved in aphidicolin biosynthesis are clustered within the *P. betae* genome, although the functions of these additional genes have not yet been determined.

1.17.3 Clerodane- and Halimane-Type Diterpenes

1.17.3.1 Clerodane

Terpentecin (**113**), a diterpene antibiotic, was identified as a metabolite in the eubacterium *S. griseolosporeus* MF730-N6. Through the identification of a gene cluster responsible for biosynthesis of **113**, two cyclase-like sequences (Cyc1 and Cyc2) were also found.[73] Cyc1 converted GGPP into terpentedienol diphosphate

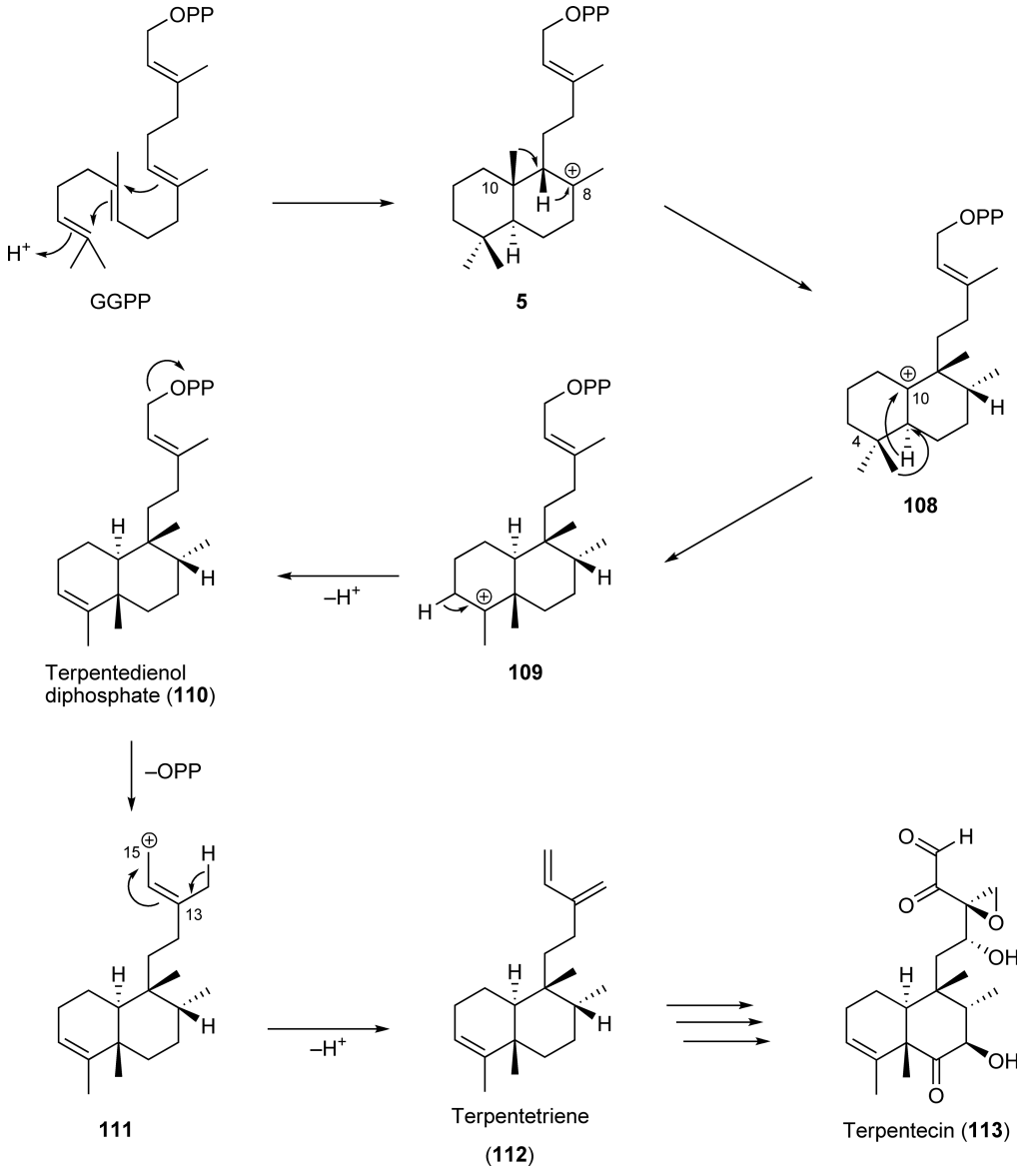


Scheme 21

(**110**), and Cyc2 converted **110** into terpentetriene (**112**) via the C-15 carbocation **111** (**Scheme 22**).[74] The amino acid sequences of Cyc1 (499 amino acids) and Cyc2 (311 amino acids) include a type B motif (DxDD) and a type A motif (DDxxD), and were similar to those of ORF2 (*ent*-CPP synthase; see Section 1.17.2.1) and ORF3 (*ent*-pimara-9(11),15-diene synthase; see Section 1.17.2.3.3) in *Streptomyces* sp. strain KO-3988, respectively. The type A Cyc2 possessed phosphatase activity, and used not only **110** but also GGPP and farnesyl diphosphate (FPP) as substrates. The proposed mechanism of the reaction from GGPP to **110** catalyzed by type B Cyc1 is as follows: the 1,2-hydride shift from C-9 to C-8 in the C-8 carbocation **5**, derived from GGPP (see **Scheme 1**), and successive 1,2-methyl shift from C-10 to C-9 gives the C-10 carbocation **108**. The 1,2-hydride shift from C-5 to C-10 in **108** and successive 1,2-methyl shift from C-4 to C-5 gives the C-4 carbocation **109**. Finally, **110**, a *syn-trans*-clerodane, is formed by the deprotonation of 3-H in **109**.

1.17.3.2 Halimane

A type B cyclase (Rv3377, 501 amino acids), whose primary structure was similar to that of eubacterial Cyc1 (see Section 1.17.3.1), was identified in *Mycobacterium tuberculosis* H37.[75] As shown in **Scheme 23**, Rv3377 converted GGPP into tuberculosinol diphosphate (**116**) via the C-8 carbocation **1** (see **Scheme 1**). The 1,2-hydride shift from C-9 to C-8 in **1** and 1,2-methyl shift from C-10 to C-9 produces the C-10 carbocation **114**. The 1,2-hydride shift from C-5 to C-10 in **114** gives the C-5 carbocation **115** and, finally, the deprotonation of 6-H in **115** gives **116**.

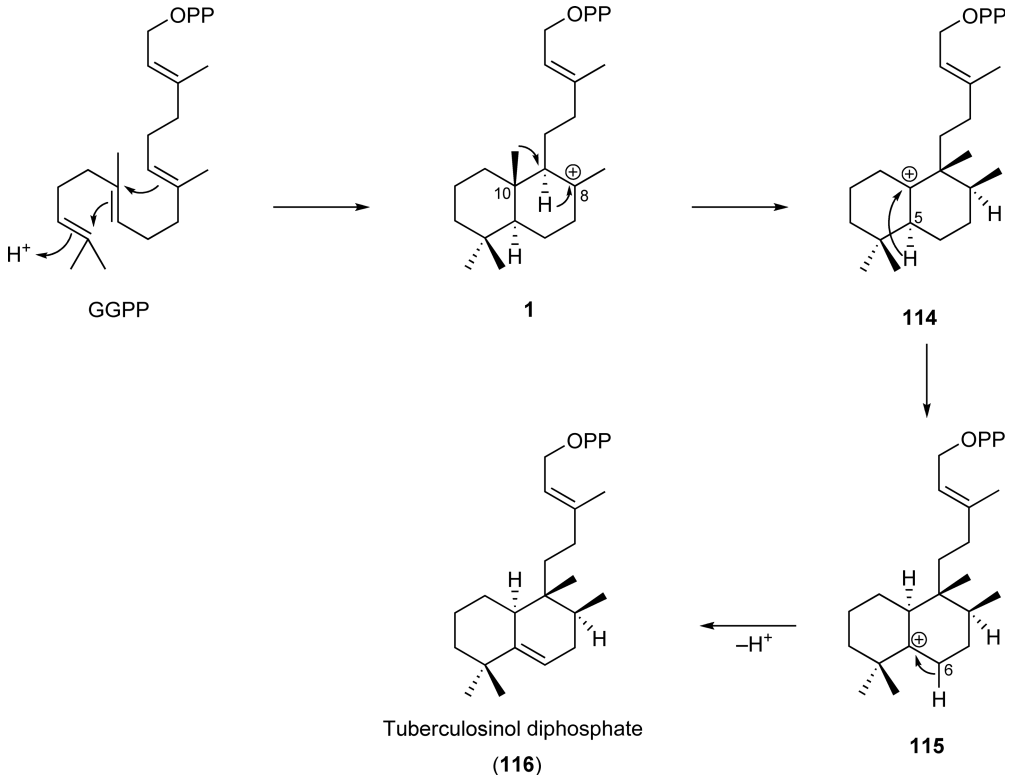


Scheme 22

1.17.4 Other types of Diterpenes

1.17.4.1 Taxane and Phomactane

Taxol (**126** in **Scheme 24**) is a potent anticancer drug that was originally isolated from the Pacific yew. It was shown that **126** is biosynthesized from GGPP through several steps via taxa-4(5),11(12)-diene (**120**) as an actual intermediate cyclic diterpene hydrocarbon, as reviewed by Croteau *et al.*[76] As described in Section 1.17.1, the taxadiene synthase (862 amino acids) gene was isolated from *T. brevifolia* (**Figure 1(e)**),[5] and several orthologs have been identified from other yew species, such as *Taxus chinensis*[77] and *Taxus* × *media*.[78] The mechanism of the reaction catalyzed by taxadiene synthase has been elucidated (**Scheme 24**).[76] The attack of C-14 on C-1 and successive attack of C-10 on C-15 after ionization of the diphosphate in GGPP (type A) produces a verticillen-12-yl cation (**117**). Intramolecular proton 1,5-migration from C-11 to C-7 gives the C-8



Scheme 23

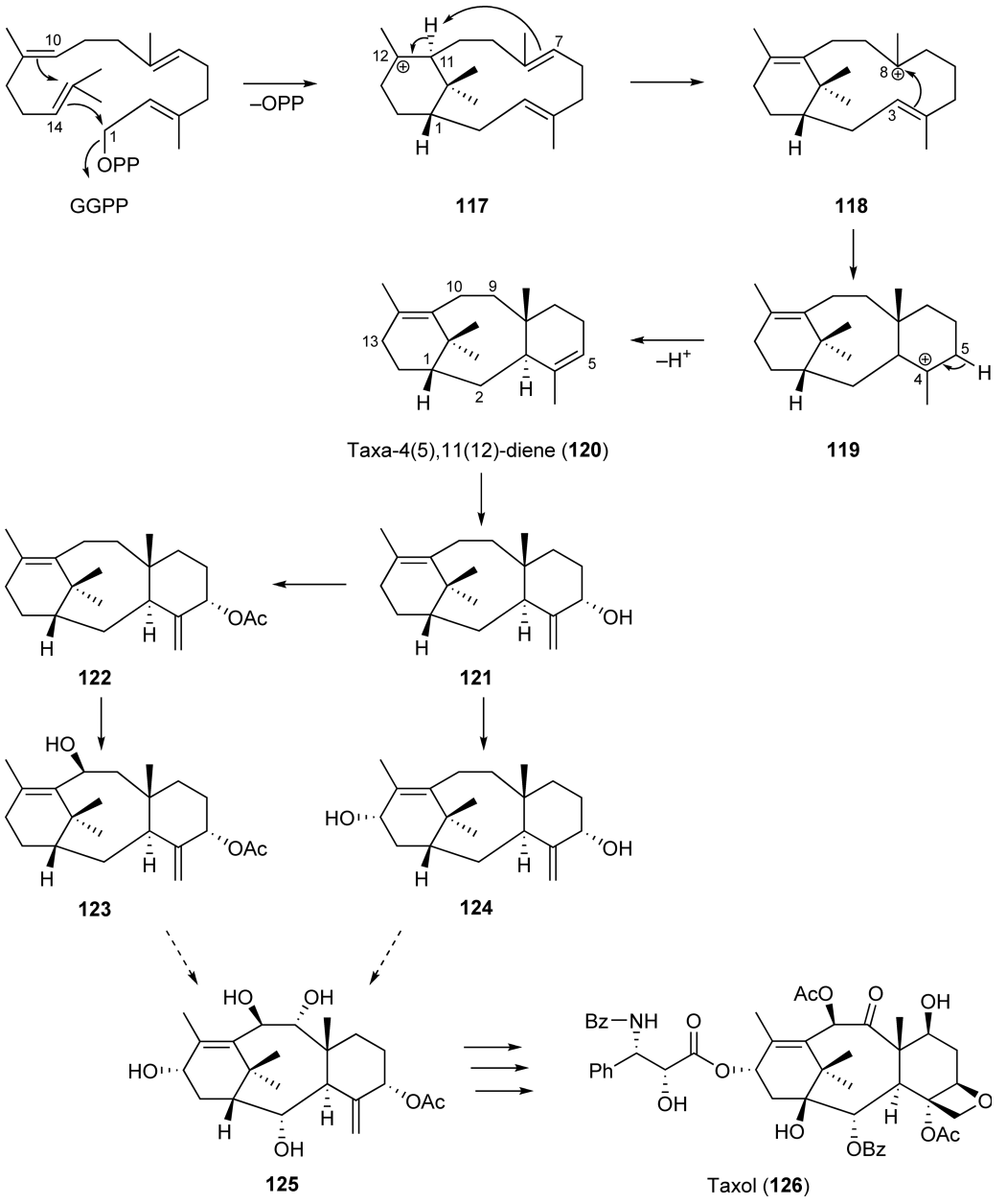
carbocation **118**. This step includes type B cyclization. Another pathway was recently suggested by theoretical calculations that a subsequent intramolecular proton migration from C-11 to C-3, and from C-3 to C-7 gives **118**.[79] The attack of C-3 on C-8 in **118** gives the C-4 carbocation **119**, and the deprotonation of 5-H in **119** provides **120**.

On the other hand, phomacta-1(14),3,7-triene (**130**) is also converted from verticillen-12-yl cation **117**, and the mechanism of this reaction has been investigated (**Scheme 25**).[80,81] Compound **130** is a biosynthetic precursor of phomactins (e.g., **131**), which are potent PAF antagonists. The 1,2-hydride shift from C-11 to C-12 in the intermediate compound **117** gives the C-11 carbocation **127**, and a 1,2-methyl shift from C-15 to C-11 in **127** provides the C-15 carbocation **128**. The 1,2-hydride shift from C-1 to C-15 in **128** gives the C-1 carbocation **129**, and the deprotonation of 14-H in **129** provides **130**. However, the diterpene cyclase responsible for the conversion of **130** from GGPP has not yet been identified.

Chemical modification enzymes, which are also involved in taxol (**126**) biosynthesis, have been identified (**Scheme 24**). Taxadiene hydroxylase, a member of the P-450 monooxygenase family, converts **120** into taxa-4(20),11(12)-dien-5α-ol (**121**) by 5α-hydroxylation.[82] An acetyltransferase that converts **121** to taxa-4(20),11(12)-dien-5α-ol-acetate (**122**) and a P-450 enzyme that converts **122** into taxa-4(20),11(12)-dien-5α,10β-diol-5-acetate (**123**) by 10β-hydroxylation have been cloned.[83,84] Moreover, P-450 taxoid 13α-hydroxylase, which converts **121** into taxa-4(20),11(12)-dien-5α,13α-diol (**124**), was also identified.[85] However, taxoid 10β-hydroxylase could not use **124** as a substrate and, in contrast, taxoid 13α-hydroxylase could not use **123**. Therefore, the precise order of hydroxylation and acetylation remains unclear, although **125** has been suggested as a hypothetical intermediate of **126**.[76]

1.17.4.2 Casbene and Cembratriene

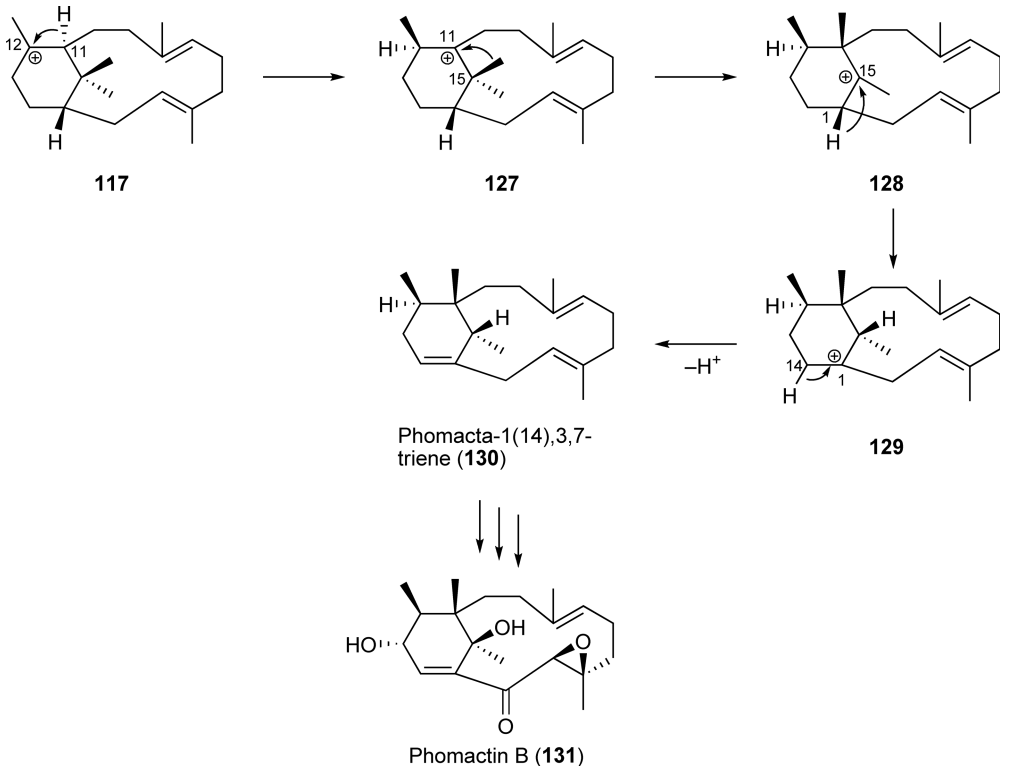
Casbene (**133** in **Scheme 26**) is a novel bicyclic diterpene hydrocarbon that serves as a phytoalexin in castor bean (*R. communis*). As described in Section 1.17.1, the cDNA for the casbene synthase that catalyzes the conversion of GGPP into **133** (**Scheme 26**) has been cloned (**Figure 1(c)**).[1] Following ionization of the



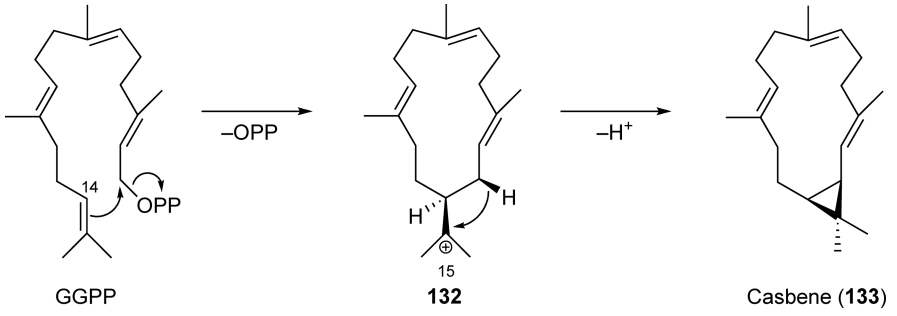
Scheme 24

diphosphate in GGPP, an attack on the *re*-face of the 14,15-double bond at C-1 provides the C-15 carbocation **132**. The stabilization of the intermediate **132** via proton abstraction gives **133**.

The type A diterpene cyclase gene, *CYC-1*, which is responsible for the biosynthesis of cembratriene 4,6α-diol isomers (**137** in **Scheme 27**), was identified from tobacco (*Nicotiana tabacum*).[86] Cembratriene-related compounds accumulate in trichomes. RNAi experiments have clearly indicated that CYC-1 (597 amino acids) converts GGPP into cembratriene 4-ol isomers (**136**). The 1,3-hydride shift from C-2 to C-15 of the intermediate compound **134** provides the C-4 carbocation **135**, and the nonstereospecific capture of water by **135** gives **136**. RNAi experiments have also shown that CYP71D16, a member of the P-450 monooxygenase family, catalyzes the conversion of **136** into **137** by 6α-hydroxylation.[86] The transcripts of both these genes accumulate substantially in tobacco trichomes.



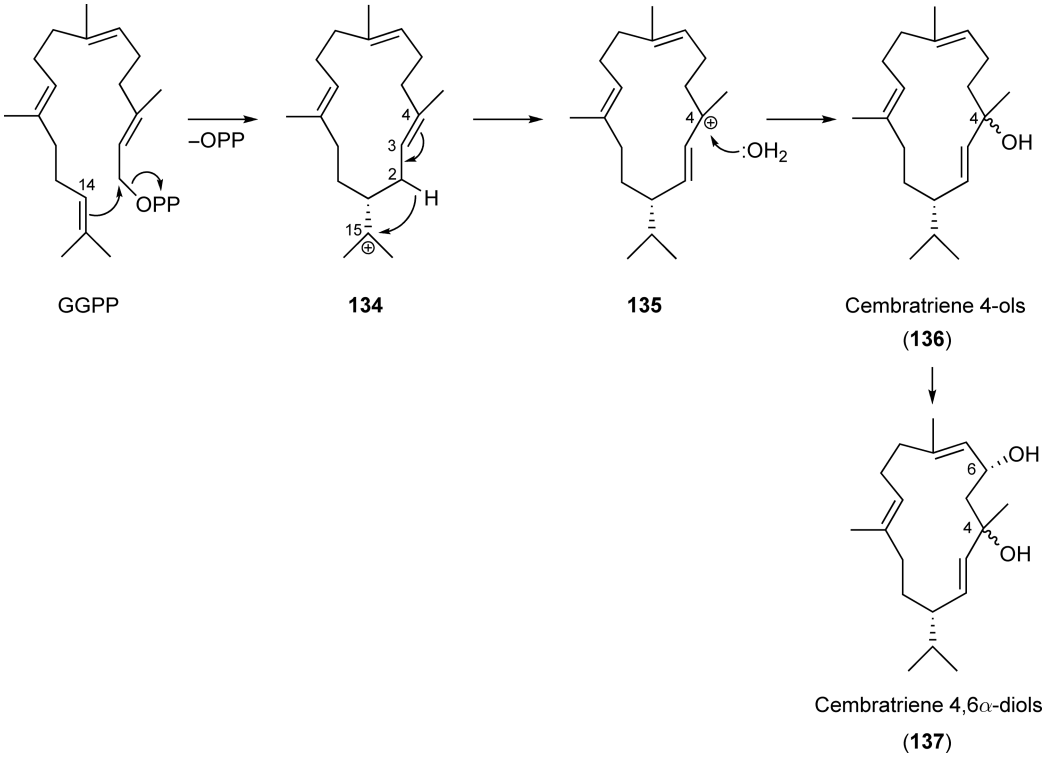
Scheme 25



Scheme 26

1.17.4.3 Fusicoccane

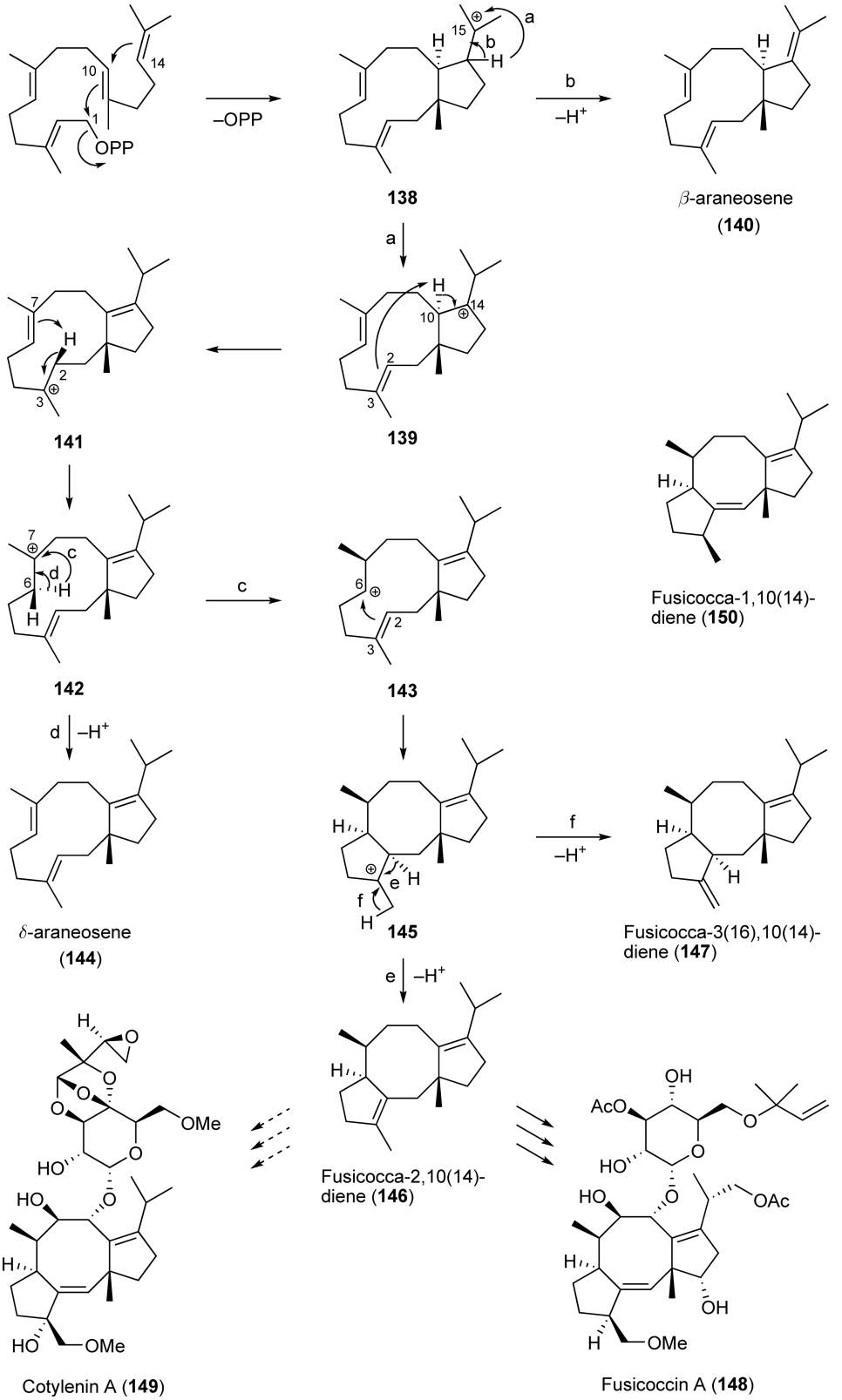
Fusicoccins (e.g., **148** in **Scheme 28**) are diterpene glucosides, produced by the plant-pathogenic fungus *P. amygdali*. Compound **148** exhibits auxin-like activity on plant cells via the continuous activation of proton-ATPase through the formation of a ternary complex consisting of 14-3-3 protein, **148**, and a phosphopeptide from the C-terminus of proton-ATPase.[87] Cotylenin A (**149**), a fusicoccane-related metabolite produced by *Cladosporium* sp. 501-7W, not only shows phytohormone-like activity, but also induces the differentiation of a series of human cells.[88] It was previously thought that **148** was derived from GGPP via fusicocca-1,10(14)-diene (**150**), a possible intermediate tricyclic hydrocarbon, as reviewed by MacMillan and Beale.[6] However, a detailed search of cyclic hydrocarbons and feeding experiments using labeled compounds in the mycelia of *P. amygdali* indicated that fusicocca-2,10(14)-diene (**146**) is a true biosynthetic intermediate hydrocarbon of **148**.[89–91] How **146** might also be an intermediate of **149** remains unexplained. Furthermore, a cDNA encoding fusicoccadiene



Scheme 27

synthase (PaFS, 719 amino acids) was isolated from the mycelia of *P. amygdali* and characterized.[92] PaFS converted GGPP into mainly **146**. The minor by-products β-araneosene (**140**), δ-araneosene (**144**), and fusicocca-3(16),10(14)-diene (**147**) were also produced, all of which were isolated from the mycelia. Experiments using recombinant PaFS and labeled GGPPs, together with previous information, have led to a newly proposed mechanism for the formation of **146** from GGPP (**Scheme 28**).[93] After ionization of the diphosphate in GGPP, the attack of the 10,11-double bond on C-1 and successive attack of the 14,15-double bond on C-10 gives the C-15 carbocation **138**. The 1,2 hydride shift from C-14 to C-15 in **138** (pathway a) gives the C-14 carbocation **139** or, alternatively, the deprotonation of 14-H in **138** (pathway b) gives **140**. A subsequent intramolecular proton 1,4-migration from C-10 to C-2β and from C-2β to C-6β gives the C-7 carbocation **142** via **141**. The possibility of alternative pathway cannot be excluded that 1,5-proton migration from C-10 to C-6β gives **142**. The 1,2-hydride shift from C-6α to C-7α in **142** (pathway c) gives the C-6 cation **143**, whereas the deprotonation of 6α-H in **142** (pathway d) gives **144**. The attack of C-2 on C-6 in **143** provides the C-3 carbocation **145**, and the subsequent deprotonation of 2-H (pathway e) and 16-H (pathway f) in **145** gives **146** and **147**, respectively.

PaFS possesses an unusual primary structure and multifunctionality.[92] It consists of two domains, the diterpene cyclase domain at the N-terminus and the prenyltransferase domain at the C-terminus, that are responsible for the synthesis of **146** from GGPP and GGPP synthesis from isoprene units, respectively (**Figure 3**). The amino acid sequence of the diterpene cyclase domain is more similar to those of aristolochene synthases in fungi, rather than other identified fungal diterpene cyclases, and includes a type A motif. The prenyltransferase domain has a DDxxD motif, which is generally included in the GGPP synthases. PaFS can produce **146** in any cell in which isoprene units or FPP for sterol synthesis is present, due to the ability to supply a GGPP substrate by itself. Homologous genes of a chimeric *PaFS* are found in the databases of fungi other than *P. amygdali*. In the flanking region of genomic DNA near the *PaFS* gene, a P-450-like gene, a dioxygenase-like gene, and a short-chain dehydrogenase/reductase-like gene were found, suggesting that some fusicoccin biosynthetic genes are clustered within the genome of *P. amygdali*.[92]


–OPP
b
–H+
138
β-araneosene
(140)
a
141
139
Fusicocca-1,10(14)-
diene (150)
c
142
143
d –H+
δ-araneosene
(144)
f
–H+
145
Fusicocca-3(16),10(14)-
diene (147)
e –H+
Fusicocca-2,10(14)-
diene (146)
Cotylenin A (149)
Fusicoccin A (148)

Scheme 28

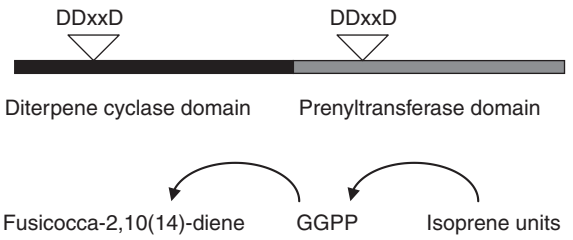
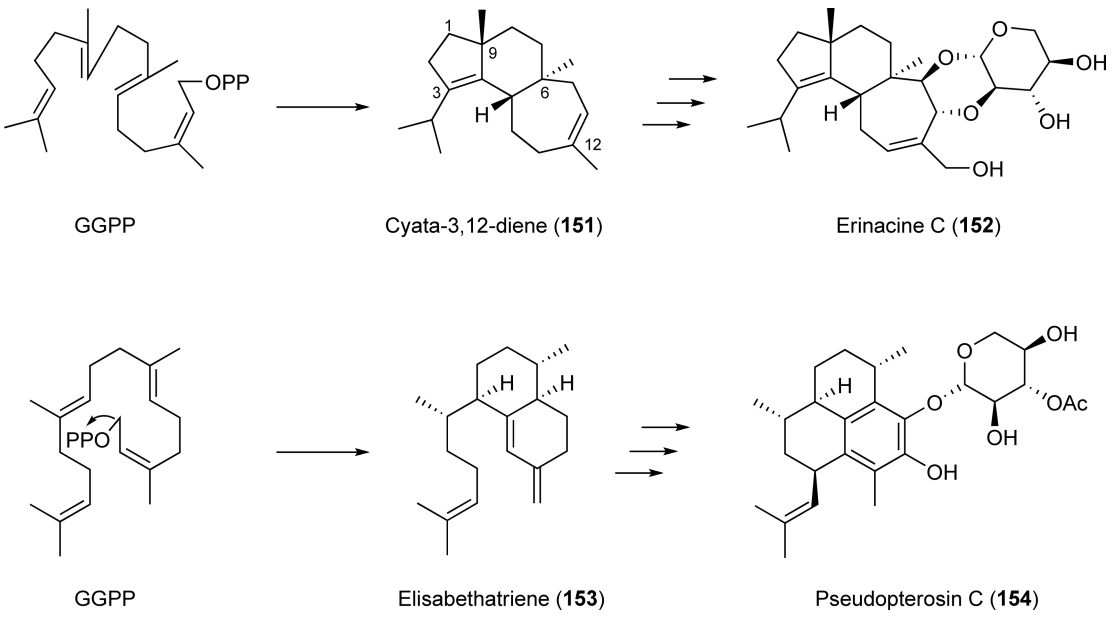


Figure 3 Schematic diagram of the primary structure of fusicoccadiene synthase (PaFS) in *Phomopsis amygdali*. Black bar and gray bar indicate diterpene cyclase domain and prenyltransferase domain, which are responsible for fusicocca-2,10(14)-diene synthesis and GGPP synthesis, respectively. Reverse triangles indicate aspartate-rich motifs.

1.17.5 Summary and Future Prospects

A large number of diterpenes have been isolated from various organisms. These diterpenes include not only antibiotics, but also physiologically active substances, such as phytohormones and drugs. Structural and functional diversity is achieved through variations in carbon skeleton formation and chemical modification. As described in this chapter, a variety of biosynthetic enzymes and genes for diterpene biosynthesis have been identified. In particular, a wide variety of diterpene carbon skeletons are derived from the common precursor GGPP by their corresponding cyclases. Accumulated information on diterpene synthases gives us two important insights. (1) Diversity: A single cyclase produces several by-products in addition to its main product, and a change in one or several amino acid residues contributes to a change in the products. This means that minor changes in the primary structure of a cyclase can yield cyclases that produce different products. In fact, rice produces a variety of diterpenes by the action of a multigene family of diterpene cyclases that may have evolved through gene duplication. (2) Efficiency: Several gene clusters for diterpene biosynthesis have been identified in bacteria, fungi, and rice, a higher plant. Diterpene cyclase fused with GGPP synthase was found in fungi.

A further insight into diterpene biosynthesis will require the identification of other diterpene cyclases, such as cyata-3,12-diene (**151**) synthase and elisabethatriene (**153**) synthase (**Scheme 29**). Compounds **151** and **153** are the possible biosynthetic intermediate hydrocarbons of erinacines (e.g., **152**) in *Hericium erinaceum*[94] and of



Scheme 29

pseudopterosins (e.g., **154**) in *Pseudopterogorgia elisabethae*,[95] respectively. To date, the X-ray structures of these diterpene cyclases have not been determined. More information, including 3D structures of cyclases, will provide a new insight into catalytic mechanisms and how to manipulate the products or stereochemical outcome. The identification of biosynthetic genes will enable us to produce diterpenes, which are produced in very small quantities in a given organism, using heterologous expression systems in familiar bacteria and yeast. Moreover, finding a gene cluster can lead to the discovery of a series of biosynthetic genes, the manipulation of which in heterologous or homologous cells will allow the production of structurally and functionally modified diterpenes.

Abbreviations

CPP	copalyl diphosphate
FPP	farnesyl diphosphate
GA2ox	gibberellin 2-oxidase
GA20ox	gibberellin 20-oxidase
GA3ox	gibberellin 3-oxidase
GA7ox	gibberellin 7-oxidase
GGPP	geranylgeranyl diphosphate
KAO	kaurenoic acid oxidase
PAF	platelet-activating factor
RNAi	RNA interference
UGT	UDP-glucosyltransferase
UV	ultraviolet

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Biographical Sketches

Tomonobu Toyomasu is an Associate Professor at Yamagata University in Japan. He was born in 1966. He received his B.S. (1989), M.S. (1991), and Ph.D. (1994) from the University of Tokyo. His Ph.D. thesis titled 'Studies on Mechanism of Germination of Photoblastic Lettuce Seed' investigated the regulation by light of endogenous levels of gibberellins and abscisic acid in lettuce seeds. He did postdoctoral research at RIKEN Frontier Research Program in 1995, and studied gibberellin biosynthetic enzyme genes in rice. He was appointed Assistant Professor at Yamagata University in 1995, and promoted to Associate Professor in 1998. He was involved in three main projects at Yamagata University: (1) regulation by light of metabolism of gibberellins and abscisic acid in photoblastic lettuce seeds; (2) biosynthesis of diterpene phytoalexins in rice; and (3) isolation and characterization of diterpene cyclase genes in fungi. He received The Japanese Society of Chemical Regulation of Plants Award for the Encouragement of Young Scientists in 2004 (project 1), and The Japan Bioscience, Biotechnology, and Agrochemistry Society Award for the Encouragement of Young Scientists in 2007 (projects 2 and 3).

Takeshi Sassa is a Professor Emeritus at the Faculty of Agriculture, Yamagata University. He received his Ph.D. (1969) in agricultural organic chemistry from Nagoya University. He spent 3 years (1967–69) at Nagoya University, working on microbial plant growth regulators, and moved as an Associate Professor to Faculty of Agriculture, Yamagata University in 1970. He worked as a Visiting Researcher with Professor R. P. Pharis at Calgary University in 1975. He received an award for the encouragement of young scientists for studies on plant growth substances produced by microorganisms from The Japan Society of Bioscience, Biotechnology, and Agrochemistry in 1975 and The Japanese Chemical Regulation of Plants Society Award for chemical studies on plant growth-regulating substances produced by true fungi in 1993. In 1977, he was promoted to Professor at the same Faculty. He retired in 2005, and then spent 2 years as a Visiting Professor at the same Faculty to continue the study on the biosynthesis including cyclases of bioactive diterpenoids. His present research focuses on the creation of a novel differentiation-inducing anticancer drug by chemical modification of fungal diterpene glycosides, cotylenin and fusicoccin.