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Microreview

Isotopic Labellings

Modern Aspects of Isotopic Labellings in Terpene Biosynthesis

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Abstract: In this review article recent isotopic labelling experiments are presented that served in the elucidation of the complex mechanisms of terpene biosynthesis. The article is structured by first presenting the recent advances in the biosynthesis of terpene monomers, followed by sections on monoterpenes, sesquiterpenes and diterpenes. This overview covers a personal selection of eminent examples from the accumulated literature since 2010.

1. Introduction

Terpenes are one of the structurally and biosynthetically most fascinating classes of natural products. All compounds of the family derive from the two building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), reactive isoprene analogues that are produced either by the mevalonate or the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. Historically, the work on both pathways represents – for different reasons – hallmark studies from natural product chemistry with isotopically labelled compounds. Classical labelling experiments with (*R*)- and (*S*)-[$^2H_1$,$^3H_1$]acetate gave precise insights into the stereochemical course of the 3-hydroxy-3-methylglutaryl-CoA synthase, requiring the enantioselective synthesis of the labelled acetate isotopomers, the determination of their absolute configurations and enantiomeric purity,[1] and the structure elucidation of the product generated in the addition to acetoacetyl-CoA.[2] Further seminal labelling experiments by Cornforth and Popják gave insights into the stereochemical courses of the downstream steps of the mevalonate pathway, the isomerisation of IPP to DMAPP, and the C–C bond formation by oligoprenyl diphosphate synthases.[3] Later on, a series of feeding experiments associated with the discovery of the MEP pathway became particularly famous in which the isotopic labelling did not occur in the expected positions,[4] and only the critical evaluation of the obtained data allowed for the identification of a new pathway.[5] With the advent and spectacular rise of gene sequencing and cloning techniques that were established in many natural product chemistry laboratories at the beginning of the new millennium, the focus seemed to have shifted away from classic labelling experiments to the identification of biosynthetic gene clusters, allowing for gene knockout and heterologous expression studies, and in vitro incubation experiments with purified enzymes as powerful techniques to investigate the pathway intermediates and reactions that nature uses to build complex natural products from simple building blocks. Today biosynthetic experiments with isotopes encounter a revival,[6] that may be stimulated by the commercial availability of many new labelled compounds and the rapid developments of highly sensitive analytical techniques. As will be shown in this review, the interplay between the modern techniques from molecular biology, analytical chemistry and isotopic labellings is particularly powerful and was recently applied to unravel the complex cyclisation cascades in enzyme mechanistic studies of several terpene cyclases.

Arguably, terpene cyclases belong to the mechanistically most interesting enzymes. They convert a simple linear precursor such as geranyl diphosphate (GPP, monoterpenes), farnesyl diphosphate (FPP, sesquiterpenes), geranylgeranyl diphosphate (GGPP, diterpenes) or geranylfarnesyl diphosphate (GFPP, sesterterpenes) into their product through substrate ionisation by diphosphate abstraction (type I enzymes and likely the recently discovered UbiA-related terpene cyclases)[7] or protonation of an olefinic double bond (type II).[8] The terpene cyclase's active site forms a hydrophobic cavity from which water is excluded

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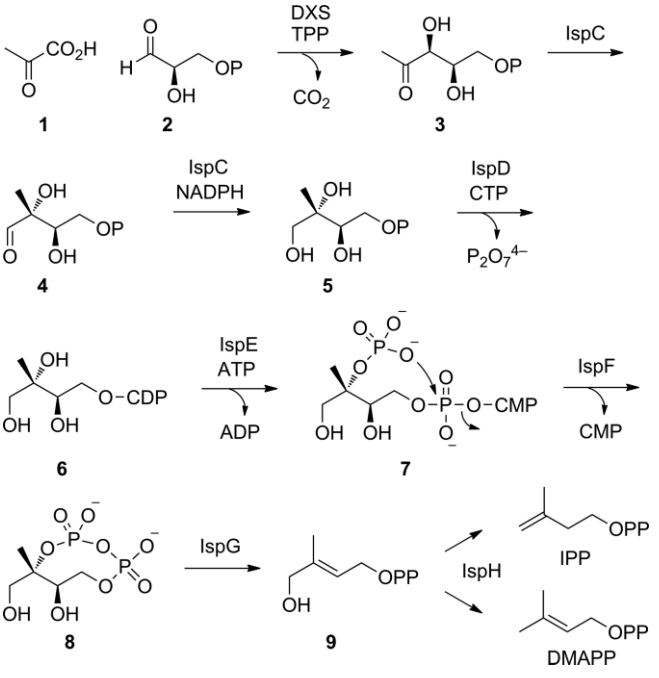
and that serves as a template to force the terpene cyclase into a reactive conformation. Substrate ionisation causes a multistep reaction cascade via cationic intermediates that includes elementary steps such as hydride or proton transfers, Wagner–Meerwein and dyotropic rearrangements, and terminating deprotonations or capture with water. The whole process is guided by the inherent reactivity of the initially generated cation,[9] while the enzyme may stabilise certain cationic intermediates, e.g. by cation–π interactions. In this review recent insights into the complex mechanisms of terpene biosynthesis that were obtained by isotopic labelling experiments will be discussed.

2. Biosynthesis of Terpene Precursors

All terpene precursors are generated by two independent biosynthetic pathways, the mevalonate pathway used by fungi, plants and some bacteria, and the MEP pathway found in most bacteria and plants. As discussed in the Introduction, a series of renowned labelling experiments conducted half a century ago has unravelled the mechanisms and stereochemical courses of the individual steps of the mevalonate pathway, while only recent research clarified important mechanistic aspects of the MEP pathway that will be discussed here.

2.1. MEP Pathway to IPP and DMAPP

The confusing situation associated with the unexpected results from several feeding experiments was resolved in the 1990s and the first years of the new millennium by the discovery of the MEP pathway (Scheme 1). The pathway starts from pyruvate (**1**) and D-glyceraldehyde 3-phosphate (**2**) that are converted into 1-deoxy-D-xylulose 5-phosphate (**3**) by the deoxyxylulose phosphate synthase (DXS) through a biological umpolung of **1** with thiamine diphosphate (TPP) under decarboxylation.[10] The subsequent isomerisation to 2-*C*-methyl-D-erythrose 4-phosphate (**4**) and NADPH-dependent reduction by IspC (also DXR for deoxyxylulose phosphate reductoisomerase) yield 2-*C*-methyl-D-erythritol 4-phosphate (**5**), which is the first and thus eponymous intermediate that is unique for the pathway towards the isoprenoids.[11] A cytidinylation with cytidine triphosphate (CTP) by IspD to 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (**6**)[12] and ATP-dependent phosphorylation by IspE yield 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (**7**).[13] The intramolecular attack of the 2-phosphate at the proximal phosphorus atom of the 4-diphosphocytidyl group catalysed by IspF proceeds with extrusion of cytidine monophosphate (CMP) with formation of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**8**).[14] A reductive dehydration with ring-opening by the [4Fe-4S] protein IspG results in (*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (**9**)[15] that is further reduced by IspH, another [4Fe-4S] enzyme, to a ca. 5:1 mixture of IPP/DMAPP.[16] Two mechanistically challenging steps of the pathway, the IspC and IspH reactions, have been investigated with isotopes and will be discussed here.

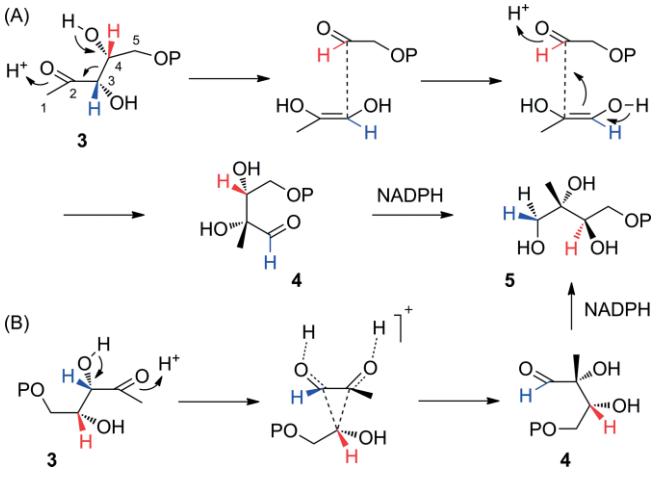


Scheme 1. MEP pathway towards IPP and DMAPP.

2.2. IspC Reaction

The IspC reaction is a mechanistically highly interesting enzyme reaction that has attracted much attention. During the rearrangement step of the transformation the C3–C4 bond in **3** is broken, and a new C2–C4 bond is formed to give **4**. Two possible mechanisms have been discussed for this process (Scheme 2), including a stepwise retroaldol/aldol reaction with intermediate disconnection of a C_2 fragment and a C_3 fragment,[17] or a concerted ketol rearrangement as in the biosynthesis of branched amino acids via a single three-membered transition state intermediate.[11] Since the two mechanisms only differ in their electronics, with the two halfs of the molecule that migrate against each other being more (mechanism A) or less disconnected (mechanism B), but not in their consequences for the constitutional formula of the product **4**, the question which mechanism is correct cannot easily be addressed by isotopic labelling experiments. The results from initial attempts with fluorinated substrate analogues were interpreted in favour of the retroaldol/aldol mechanism, but did not allow for unambiguous conclusions for the natural substrate **3** as the introduction of fluorine may change the electronic situation.[18] Strictly speaking, also the exchange of isotopes, especially in the case of H/D substitutions, influences the electronics of a substrate and may lead to a modified reactivity, but this is an intrinsic problem of all mechanistic investigations using the isotopic labelling technique, and the exchange of isotopes certainly represents the minimal invasive approach. Kinetic experiments with deuterated substrates in which either the red or the blue hydrogen atom in **3** was substituted by a deuterium atom showed a significant secondary kinetic isotope effect, pointing to the rehybridisation of C3 and C4 during the reaction, which

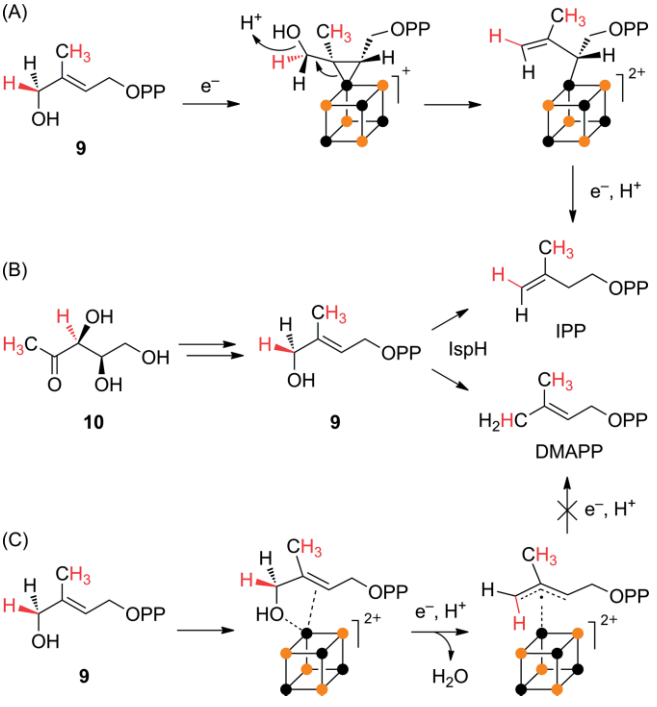
is in favour of mechanism A, while in mechanism B only C3 but not C4 undergoes rehybridisation.[19] It has been well established in deuterium labelling experiments that the attack of hydride from NADPH in the reduction of **4** to **5** proceeds from the *Re* face of the aldehyde function.[20]



Scheme 2. Two possible mechanisms for the isomerisation step of the IspC reaction from **3** to **4**. (A) Retroaldol/aldol reaction, (B) ketol rearrangement. The reduction from **4** to **5** is the same for both mechanisms. Red and blue hydrogen atoms were exchanged by deuterium in labelling studies.

2.3. IspH Reaction

The enzyme mechanism of the [4Fe-4S] protein IspH was subject of a scientific debate essentially since its discovery. One of the suggested mechanisms (Scheme 3A) proceeds from **9** by one-electron reduction via a metallacyclopropane intermediate that was supported by ENDOR spectroscopic data.[21] Its ring opening with elimination of water yields an η^1-allyl complex that upon a second electron transfer and protonation results in IPP and DMAPP. The second possible mechanisms is essentially a Birch reduction of **9** that first coordinates with its hydroxy and alkene functions to one iron atom of the [4Fe-4S] cluster (Scheme 3C).[22] Both mechanisms are in agreement with the observation that the deuterium atoms from (1,1-$^2H_2$)-**9** are fully retained in IPP and DMAPP,[23] but the two mechanisms could be distinguished by a stereospecific deuterium labelling of the *pro-S* hydrogen atom at C4 of **9** (red hydrogen atoms in Scheme 3). For the Birch mechanism the 4-*pro-S* hydrogen atom should end up in the 4*Z* position of IPP, whereas the ring opening of the metallacyclopropane intermediate in the organometallic mechanism can yield IPP with the deuterium atom in the 4*E* position of IPP, if the ring opening reaction proceeds from a conformation as shown. The deuterium label indeed ends up in the 4*E* position, which is in favour of the organometallic mechanism. This was shown in a feeding experiment with (1,1,1,3-$^2H_4$)deoxy-D-xylulose to *Streptomyces avermitilis*.[24] The bacterium can phosphorylate the deoxy sugar and convert it into labelled **9** by the reactions shown in Scheme 1. The stereochemical fate of the deuterium labelling in IPP could be deduced from the incorporation of only eleven deuterium atoms into the sesquiterpene pentalenene for which a stereospecific deprotonation has been well established[25] that could be traced back to the 4*E* hydrogen atom of IPP because of the known stereochemical course of FPP biosynthesis.[3] The additional label in the methyl group of **3** was introduced to give unambiguous evidence for the incorporation of three labelled terpene units.



Scheme 3. Stereochemical course and mechanism of the IspH reaction. (A) Organometallic mechanism, (B) labelling experiment with deuterated deoxy-D-xylulose (**10**), (C) Birch reduction mechanism.

3. Monoterpene Cyclases

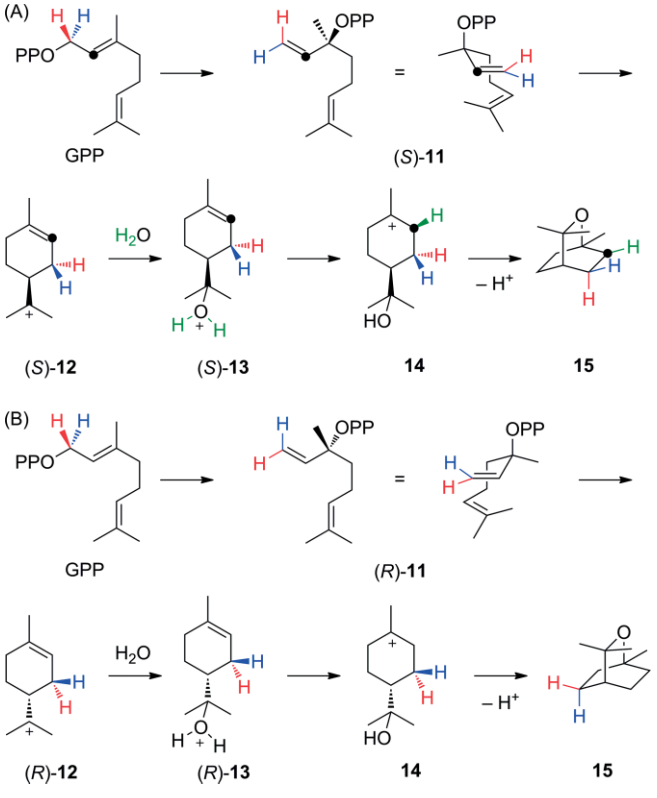
Monoterpene biosynthesis is very well understood, and – apart from the acyclic representatives – all compounds from this class are formed via one of the enantiomers of the α-terpinyl cation.[26] The biosynthesis of the achiral compound 1,8-cineol via this cation is a peculiar problem that will be discussed here.

3.1. Biosynthesis of 1,8-Cineol

The monoterpene ether 1,8-cineol (eucalyptol) is a well-known constituent of many plant essential oils that was first described by Wallach in 1884.[27] More than one century later the development of gene technologies allowed for the discovery of the first 1,8-cineol synthase from *Salvia officinalis* by Croteau and co-workers,[28] followed by the identification of a bacterial 1,8-cineol synthase from *Streptomyces clavuligerus* in Ohnishi's laboratories.[29]

As is demonstrated by the cyclisation mechanisms shown in Scheme 4A, the achiral monoterpene **15** can be formed from GPP by isomerisation to (*S*)-linalyl diphosphate (**11**), followed by cyclisation to the (*S*)-α-terpinyl cation (**12**). The attack of water results in **13**, and a deprotonation/reprotonation se-

quence or an intramolecular proton transfer lead to **14** that upon ring closure yields **15**. Essentially the same mechanism, but via the enantiomeric intermediates including (*R*)-linalyl diphosphate and the (*R*)-α-terpinyl cation, can give rise to the same product **15** (Scheme 4B). Notably, the two different mechanisms can be distinguished using enantioselectively deuterated GPP, with either the 1-*pro*-*S* hydrogen atom (red) or the 1-*pro*-*R* hydrogen atom (blue) being substituted by a deuterium atom, because these deuterium labels end up in diastereotopic positions in **15** (*exo* or *endo*, respectively). Incubation of (*R*)- and (*S*)-(1-^{2}H)GPP with the 1,8-cineol synthase from *S. clavuligerus* revealed a cyclisation mechanism via the intermediates of Scheme 4A.[30] Further experiments with (2-^{13}C)GPP in deuterium oxide revealed that the conversion from **13** to **14** may indeed proceed by intramolecular proton transfer, because the introduced deuterium labelling in **15** (green hydrogen atom) specifically ended up in the *exo* position, as was determined by HSQC spectroscopy (in this experiment the ^{13}C label was introduced for a highly sensitive signal detection). Notably, the 1,8-cineol synthase from *S. officinalis* was shown by similar labelling experiments to catalyse a cyclisation cascade via (*R*)-**11** and (*R*)-**12**, also possibly with intramolecular proton transfer from **13** to **14**,[31] which reflects the observation that terpenes from plants frequently resemble the optical antipodes of bacterial terpenes.[32]



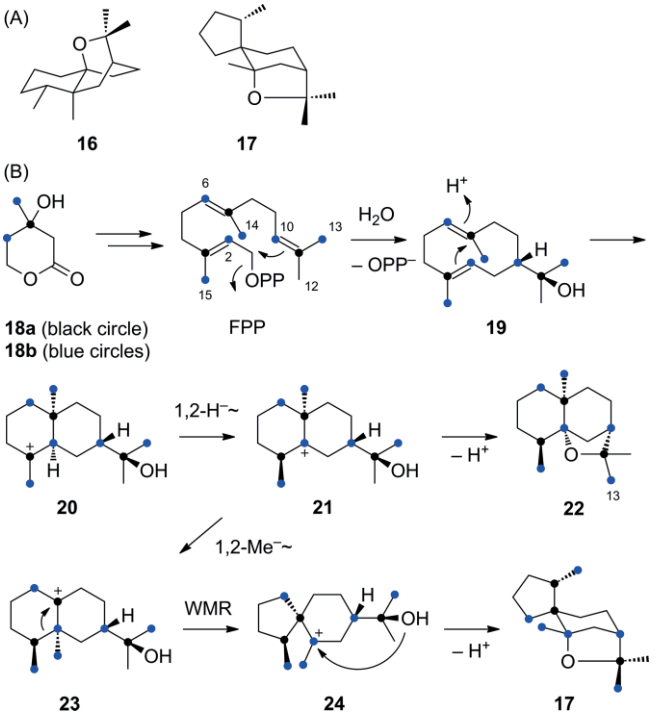
Scheme 4. Stereochemical course of 1,8-cineol biosynthesis. Mechanism (A) proceeds via (*S*)-linalyl diphosphate (**11**) and the (*S*)-α-terpinyl cation (**12**), mechanism (B) proceeds via the corresponding enantiomers. Black dots in (A) indicate ^{13}C-labelled carbon atoms, and the red, blue, and green hydrogen atoms have been exchanged in isotopic labelling experiments with 1,8-cineol synthase from *S. clavuligerus* by deuterium atoms.

4. Sesquiterpene Cyclases

A series of classical isotope labelling experiments that were carried out in vivo or in vitro and that targeted the mechanisms of bacterial and fungal sesquiterpene biosynthesis, including work on pentalenene,[33] trichodiene,[34] aristolochene,[35] *epi*-isozizaene,[36] avermitilol,[37] geosmin,[38,39] and 2-methylisoborneol,[38,40] has been carried out during the past decades with main contributions from the Cane laboratories. These experiments are well known to the scientific community, have been included in previous reviews,[7a,7b,9] and will not be discussed in detail here. Particularly interesting and worth to mention is the fruitful interaction between isotopic labelling experiments and quantum mechanical calculations performed by Tantillo and co-workers that stimulated further research on systems that seemed to have been fully understood, but led in some cases as for the pentalenene synthase to refined mechanistic views.[41] In this context it should be emphasised that no model of any reaction mechanism can ever gain absolute proof, but can only be supported by experimental data, until its revision may be required because of newly obtained disputing results. In this section a series of recent labelling experiments are presented that served not only in the unravelling of biosynthetic mechanisms to sesquiterpenes, but also in their structure elucidation.

4.1. Hypodoratoxide

Hypodoratoxide is a sesquiterpene ether from the fungus *Hypomyces odoratus* for which initially the structure of **16** was published (Scheme 5).[42] Feeding experiments with (2,3,4,5,6-



Scheme 5. Structure and biosynthesis of hypodoratoxide. (A) Originally proposed (**16**) and revised structure (**17**) for hypodoratoxide, (B) biosyntetic pathway from mevalonolactone (**18**). Black and blue dots represent ^{13}C-labelled carbon atoms.

$^{13}C_5$)mevalonolactone in which all carbon atoms that are incorporated into terpenes resulted in the isolation of completely ^{13}C-labelled hypodoratoxide, allowing for a structure elucidation by $^{13}C,^{13}C$-COSY NMR spectroscopy that resulted in the revised structure of **17**.[43] This NMR technique in combination with ^{13}C enrichment by labelled precursor feeding has recently also been used in the structure elucidation of a few other natural products and is particularly useful for compounds with a high C/H ratio.[44] The absolute configuration of **17** as shown in Scheme 5 was deduced from the absolute configuration of the biosynthetic side product *cis*-dihydroagarofuran (**22**) that pointed to the absolute configuration of the common pathway intermediate **21**. Feeding experiments with the labelled mevalonolactone isotopomers (3-^{13}C)-**18a** and (4,6-$^{13}C_2$)-**18b** supported the rearrangement steps from **21** to **23** and from **23** to **24** in the biosynthetic pathway towards **17**, because by the carbon skeleton rearrangements covalent bonds between two labelled carbon atoms are formed, resulting in intensive doublets in the ^{13}C NMR spectrum.[43]

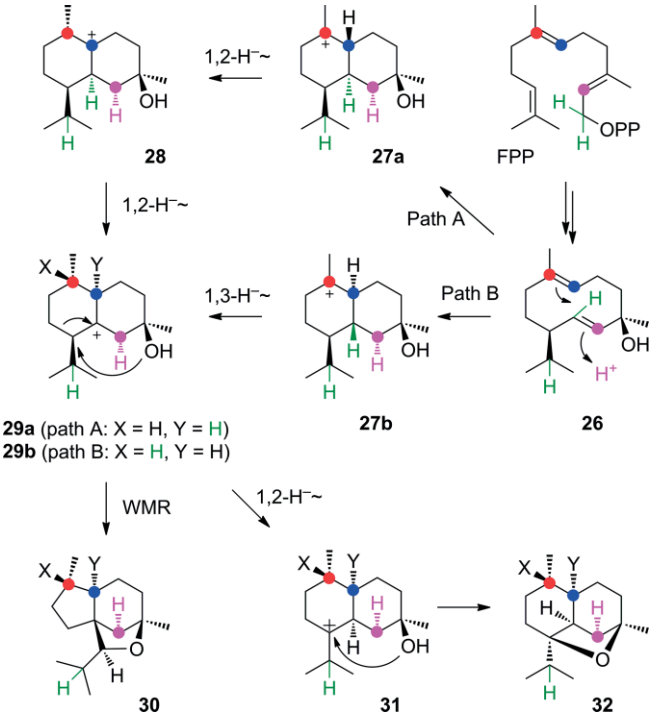
4.2. (1(10)*E*,4*E*,6*S*,7*R*)-Germacradien-6-ol

The product of a sesquiterpene cyclase from *Streptomyces pratensis* was recently identified as (1(10)*E*,4*E*,6*S*,7*R*)-germacradien-6-ol (**25**),[45] which is the opposite enantiomer as in the plant *Santolina rosmarinifolia*.[46] Germacrane sesquiterpenes frequently occur as mixtures of slowly interconverting conformers resulting in broadened signals in the NMR spectra (Scheme 6).[47] The spectra of **25** recorded at –50 °C showed two sets of sharp signals for the conformers **25a** and **25b**, but their unambiguous assignment to the two conformers was prevented by several signal overlaps.[45] An assignment of ^{13}C NMR signals to each individual carbon atom of the two conformers was possible by enzymatic conversion of fully labelled synthetic ($^{13}C_{15}$)FPP, followed by $^{13}C,^{13}C$-COSY NMR spectroscopy. The ^1H NMR signals for the hydrogen atoms attached to each carbon atom were identified by conversion of each of the 15 isotopomers of ($^{13}C_1$)FPP, yielding 15 products ($^{13}C_1$)-**25** with the ^{13}C labels in different positions, followed by HSQC spectroscopy. After full assignment of the NMR spectroscopic data, the two conformers **25a** and **25b** were identified. The ($^{13}C_1$)-**25** isotopomers were also used to study the EI-MS fragmentation mechanisms, a technique that was also applied in subsequent studies on *epi*-isozizaene,[48] the corvol ethers, *epi*-cubebol, and isodauc-8-en-11-ol.[49]

Scheme 6. The two main confomers of **25**. For their description the "up/down" nomenclature is used.[47] "U" indicates a methyl group that points up, "D" a methyl group pointing down.

4.3. Corvol Ethers

The corvol ethers A (**32**) and B (**30**) are two novel sesquiterpene ethers from *Kitasatospora setae* for which the sesquiterpene cyclase was recently identified (Scheme 7).[50] Their biosynthesis was extensively investigated by isotopic labelling experiments. The cyclisation mechanism from FPP proceeds by a first cyclisation to germacrene-D-4-ol (**26**). Its reprotonation initiates a second cyclisation either to **27a** that reacts in two sequential 1,2-hydride migrations via **28** to **29** (Path A), or to its stereoisomer **27b** from which a 1,3-hydride shift yields **29** (Path B). A ring contraction by Wagner–Meerwein rearrangement and intramolecular attack of the hydroxy function results in **30**, while **32** is formed by another 1,2-hydride migration and intramolecular attack of the alcohol function to the cationic centre.

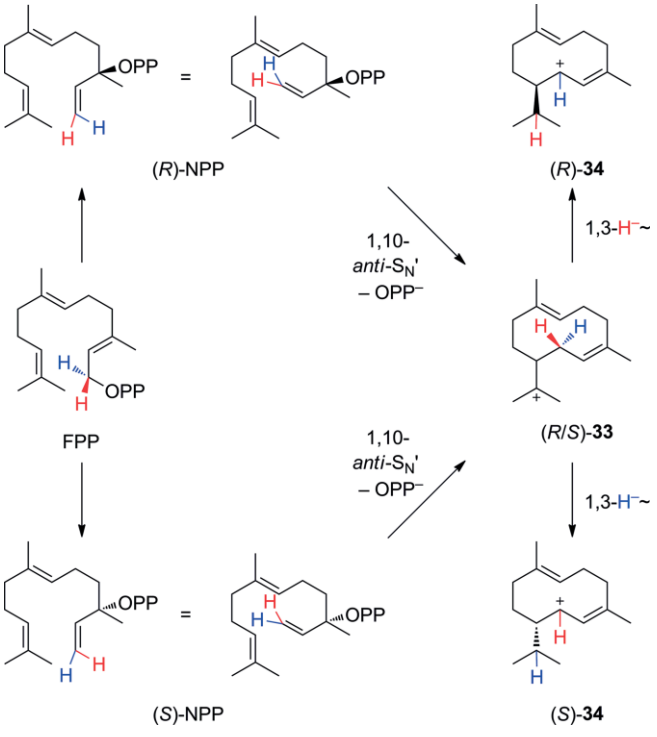


Scheme 7. Labelling experiments for investigations on the mechanism for the cyclisation of FPP to corvol ethers A (**32**) and B (**30**). Coloured dots and hydrogen atoms indicate ^{13}C and ^2H labellings, respectively.

The reprotonation step at **26** was evident from an incubation of (2-^{13}C)FPP in deuterium oxide (^{13}C and ^2H labellings are shown in purple), resulting in strong triplets for the labelled carbon atoms of **30** and **32** in the ^{13}C NMR spectrum due to ^{13}C-^2H spin coupling,[50] while the stereochemical course of the reprotonation was investigated by HSQC that revealed incorporation into the α-position.[51] The different options from **26** to **29** by Path A or Path B were distinguished by a double labelling strategy using the substrates (6-^{13}C,1,1-$^2H_2$)FPP and (7-^{13}C,1,1-$^2H_2$)FPP (^{13}C labels shown in blue and red, deuterium labelling shown in green). With these substrates the deuterium labels end up in different positions (**29a** vs. **29b**) depending on whether Path A or path B is used, that are either ^{13}C-labelled or not, which allowed for efficient product analysis by ^{13}C NMR spectroscopy. The experiments revealed the conversion of **26** into **29** only by Path A, which was also supported by quantum chemical calculations.[51]

The absolute configurations of the corvol ethers as shown in Scheme 7 were determined by use of stereospecifically labelled

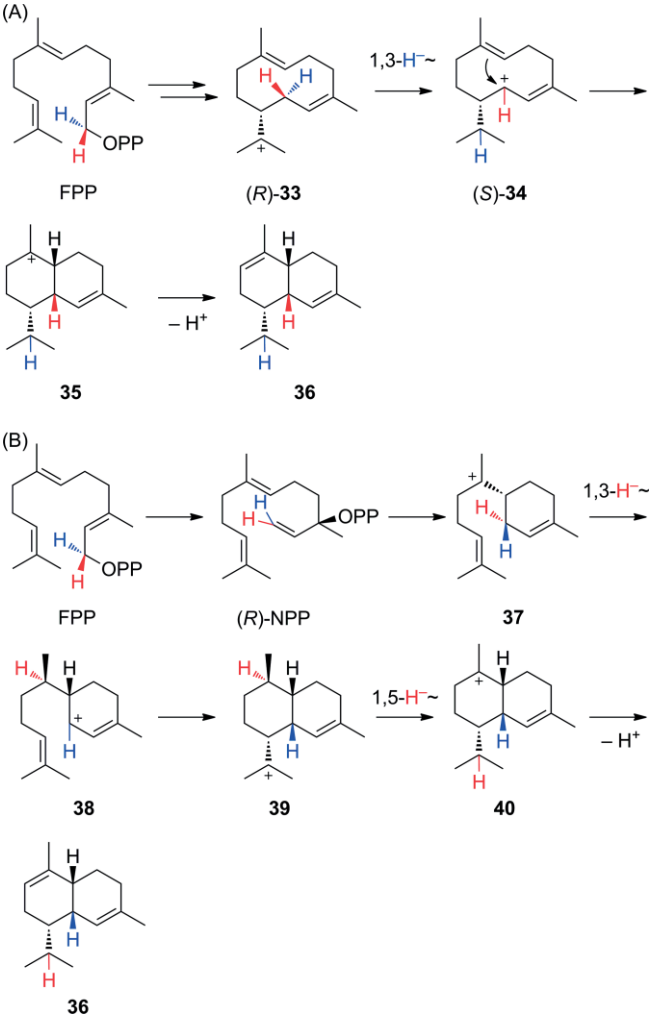
(*R*)- and (*S*)-(1-^{2}H)FPP (Scheme 8; blue or red hydrogen atom exchanged by a deuterium atom).[52] The reaction of FPP to **33** containing a *Z* double bond requires isomerisation to nerolidyl diphosphate (NPP) prior to cyclisation. This is possible either via (*R*)- or (*S*)-NPP by a *syn*-allylic transposition of diphosphate with different stereochemical consequences for the 1-*pro*-*R* and 1-*pro*-*S* hydrogen atoms of FPP. However, the subsequent 1,10-cyclisation by *anti*-S_N2' attack of C10 at C1 yields (*R*)- or (*S*)-**33** with the same absolute orientations of the hydrogen atoms at C1, independent of the absolute configuration of the intermediate NPP. If (*S*)-**33** is produced, the 1-*pro*-*S* hydrogen atom (red) is in close proximity to the cationic centre at C11 and will migrate to yield (*R*)-**34**, while in (*R*)-**33** the 1-*pro*-*R* hydrogen atom (blue) will shift to result in (*S*)-**34**. Conclusively, the absolute configurations of terpenes arising via **34** can be determined by investigating whether the 1-*pro*-*R* or the 1-*pro*-*S* hydrogen atom of FPP migrates to the isopropyl group. For the corvol ethers a stereospecific migration of the 1-*pro*-*S* hydrogen atom was shown by observing the EI-MS fragment ion that is formed by cleavage of the isopropyl group, pointing to the intermediate (*R*)-**34** that is the precursor of **26** (Scheme 7) by capture with water.



Scheme 8. Labelling experiments for the determination of the absolute configuration of the corvol ethers.

4.4. α-Amorphene

An interesting example for which the stereochemical implications of Scheme 8 do not fit is observed for the cyclisation mechanism of the α-amorphene synthase from *Streptomyces viridochromogenes*.[32a] The absolute configuration of the product **36** as shown in Scheme 9 was determined from its optical rotation and pointed to the opposite enantiomer as in the plant *Vetiver zizanioides*.[53] A possible cyclisation mechanism from FPP to **36** may proceed via (*R*)-**33** (Scheme 9A), but labelling experiments with stereospecifically deuterated (*R*)- and (*S*)-(1-^{2}H)FPP revealed migration of the 1-*pro*-*S* hydrogen atom (red) and not the 1-*pro*-*R* hydrogen atom (blue) to the isopropyl group of **36**,[52] which contradicted the otherwise generalisable model of Scheme 8. An alternative mechanism by initial 1,6-cyclisation has been suggested by Hong and Tantillo that proceeds by 1,6-cyclisation of (*R*)-NPP to the (*R*)-bisabolyl cation (**37**),[54] followed by a 1,3-hydride shift of the original 1-*pro*-*S* hydrogen atom of FPP to yield the allyl cation **38**. A second cyclisation to **39** is followed by another 1,5-hydride transfer that brings indeed the 1-*pro*-*S* hydrogen atom of FPP to the isopropyl group of **40**. A terminal deprotonation results in **36** with opposite fates for the enantiotopic protons at C1 of FPP as compared to the mechanism in Scheme 9A. While the work by Hong and Tantillo showed that the pathway of Scheme 9A is princi-

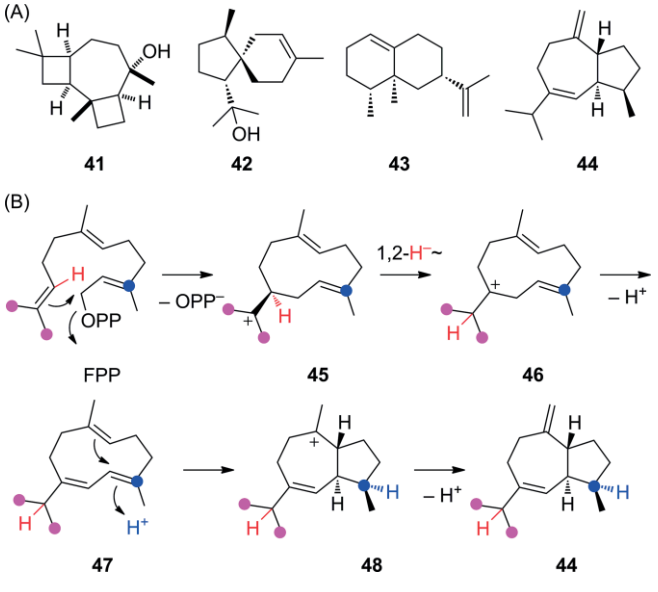


Scheme 9. Cyclisation mechanisms for α-amorphene (**36**). Mechanism (A) involves an initial 1,10-cyclisation, and mechanism (B) proceeds with initial 1,6-cyclisation. Mechanism (B) is favoured by quantum chemical calculations and labelling experiments in which the blue and red hydrogen atoms were replaced by deuterium.

pally feasible, their quantum chemical calculations were in favour of the pathway of Scheme 9B,[54] in agreement with the described labelling experiments.[52]

4.5. (1*R*,4*R*,5*S*)-Guaia-6,10(14)-diene

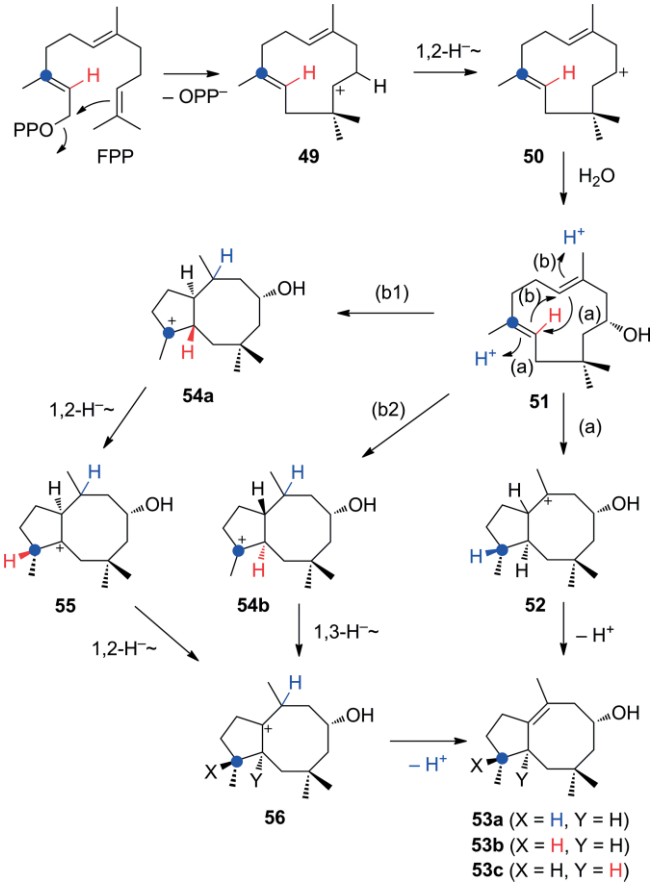
The rice pathogenic fungus *Fusarium fujikuroi* encodes nine sesquiterpene cyclase homologues in its genome of which two enzymes were known to produce the sesquiterpene alcohols (+)-koraiol (**41**) and (–)-α-acorenol (**42**; Scheme 10A).[55] Recently, two more enzymes were characterised for their products,[56] revealing their functions as cyclases for (+)-eremophilene (**43**), which is the opposite enantiomer as in corals,[57] but the same as in the myxobacterium *Sorangium cellulosum*,[58] and (1*R*,4*R*,5*S*)-guaia-6,10(14)-diene (**44**) for which the absolute configuration was established by total synthesis.[56] The cyclisation mechanism for the enzyme reaction to **44** starts with a 1,10-cyclisation of FPP to the (*E*,*E*)-germacradienyl cation (**45**), followed by a 1,2-hydride shift to **46** and deprotonation to germacrene C (**47**). Its reprotonation initiates a second cyclisation to **48** that yields **44** by loss of a proton. This mechanism was established by enzyme incubation of (10-^{2}H)FPP (red hydrogen atom exchanged by a deuterium atom) that gave evidence for the 1,2-hydride shift from **45** to **46** by product analysis through GC/MS (the base peak ion of **44** is formed by fragmentation of the isopropyl group). The reprotonation of the neutral intermediate **47** was evident from an enzymatic conversion of (3-^{13}C)FPP (blue carbon atom ^{13}C-labelled) in deuterium oxide, yielding a strong triplet in the ^{13}C NMR spectrum of the product. Notably, the (1*R*,4*R*,5*S*)-guaia-6,10(14)-diene synthase is one of the very few known terpene cyclases that causes a distribution of labelling from the terminal geminal methyl groups (purple) over the two corresponding diastereotopic methyl groups in **44**.



Scheme 10. Sesquiterpene cyclases from *Fusarium fujikuroi*. (A) Identified products from four sesquiterpene cyclases, (B) cyclisation mechanism for (1*R*,4*R*,5*S*)-guaia-6,10(14)-diene. Coloured dots and hydrogen atoms indicate positions of ^{13}C and ^{2}H labellings.

4.6. Pristinol

A recently characterised sesquiterpene cyclase from *Streptomyces pristinaespiralis* was shown to produce the sesquiterpene alcohol pristinol (**53**), a compound with a new carbon skeleton for which the absolute configuration was determined by X-ray diffraction analysis (Scheme 11).[59] The pathway from FPP to **53** starts with the cyclisation of FPP to the humulyl cation (**49**), followed by a 1,2-hydride shift to **50** and attack of water to (*R*)-hyemalol (**51**). Several possibilities were taken into consideration for the further cyclisation to pristinol. The most direct mechanism (a) via **52** was excluded, because incubation of (3-^{13}C)FPP (blue dots indicate labelling) in deuterium oxide yielded a product with a strong singlet for the labelled carbon atom in the ^{13}C NMR spectrum, which is not in agreement with the expected labelling pattern of **53a**. Two alternative suggestions (b1) and (b2) included reprotonation of **51** at C7 and further reactions either via **55a** and **55** or via **54b** to **56** that is the precursor of **53** by deprotonation. A similar labelling experiment with (7-^{13}C)FPP in deuterium oxide was not performed, because the deuterium atom that is introduced at C7 will for both mechanisms be lost in the last step. Consistent with both mechanisms was the finding that no deuterium uptake into **53** during incubation experiments in deuterium oxide could be detected by GC/MS. To distinguish between mechanisms (b1)

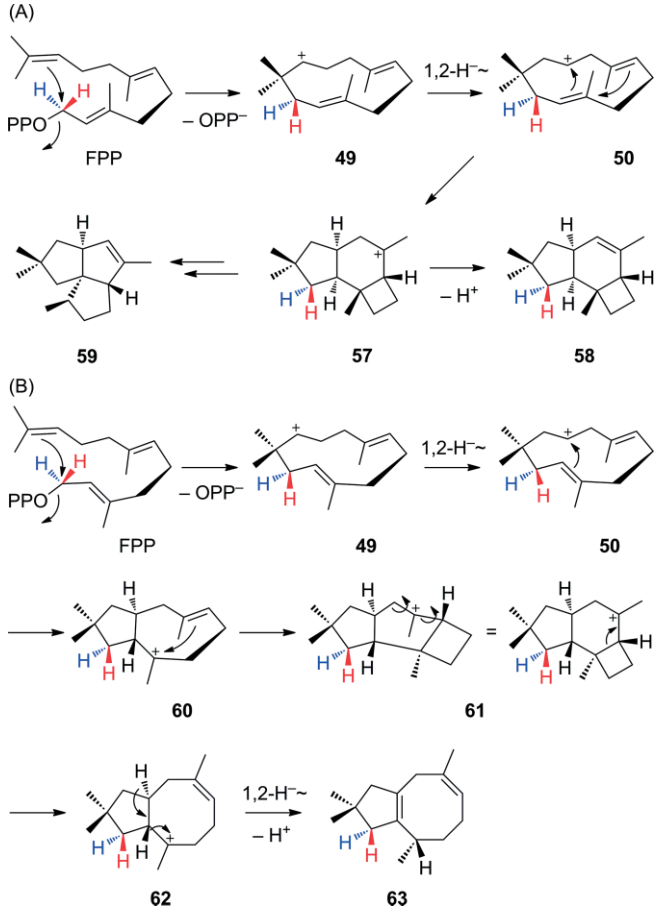


Scheme 11. Three possible mechanisms for the cyclisation of FPP to pristinol (**53**) by pristinol synthase. Coloured dots and hydrogen atoms indicate positions of ^{13}C and ^{2}H labellings.

and (b2), (3-^{13}C,2-^{2}H)FPP was enzymatically converted, yielding a triplet for the original C3 of FPP in the ^{13}C NMR spectrum of the product, which points to the labelling pattern of **53b** and not of **53c**. These data are in agreement with mechanism (b1).

4.7. Protoillud-7-ene and Asterisca-2(9),6-diene

Recently, the first terpene cyclases from social amoebae were reported from *Dictyostelium discoideum*.[60] This organism encodes nine full-length terpene cyclases in its genome, two of which have been characterised by in vitro experiments for their products and enzyme mechanisms using isotopically labelled precursors.[61] The first enzyme was identified as protoillud-7-ene (**58**) synthase. This compound is formed from FPP by 1,11-cyclisation to **49**, a 1,2-hydride shift to **50**, and cyclisation to the protoilludyl cation (**57**) that yields **58** by deprotonation (Scheme 12A). The cation **57** was proposed by Tantillo as an intermediate towards pentalenene (**59**)[41] which was reflected by the production of **59** as a trace component generated by the protoillud-7-ene synthase.



Scheme 12. Cyclisation mechanisms for (A) the protoillud-7-ene synthase and (B) the asterisca-2(9),6-diene synthase from *D. discoideum*. Blue and red hydrogen atoms were exchanged by deuterium atoms for determination of the absolute configurations of **58** and **63**.

The second enzyme produced asterisca-2(9),6-diene (**63**) from FPP via **49** and **50**, followed by cyclisation to **60** and **61**, which is a diastereomer of **57**. After a conformational rearrangement, the cation **61** can ring-open to **62**, giving an explanation for the formation of the 6*Z* double bond in **63** (Scheme 12B).[61] A similar mechanism for the formation of **63** via the *cis*-diastereomer of **62** has been suggested based on quantum chemical calculations.[41]

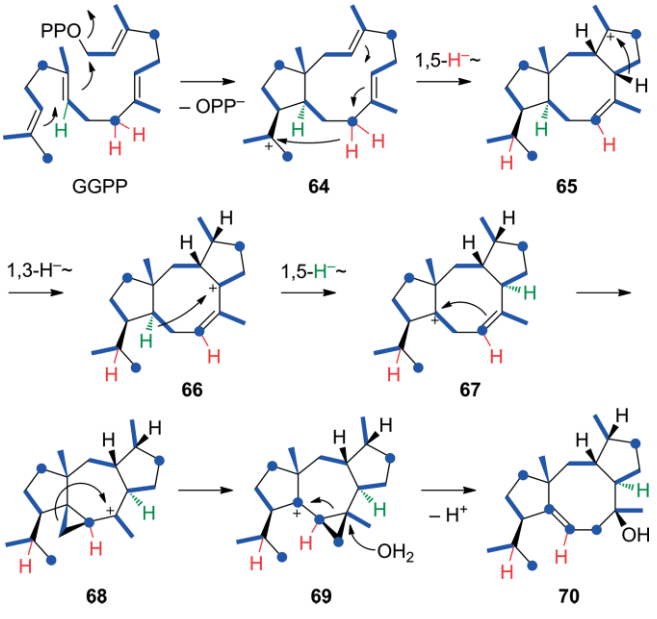
The absolute configurations of **58** and **63** were determined by enzymatic conversion of (*R*)- and (*S*)-(1-^{2}H)FPP. Since the 1,11-cyclisation of FPP proceeds with inversion of configuration,[62] the absolute configurations at the deuterated carbon atoms of the obtained product was known, which allowed for determination of the relative orientation of the other stereocentres to deduce the absolute configurations of **58** and **63**.[61]

5. Diterpene Cyclases

The first bacterial type I terpene cyclase (CotB2) was described from *Streptomyces melanosporofaciens* by Kuzuyama and coworkers and identified as cyclooctat-9-en-7-ol synthase.[63] Additional interesting enzymes have subsequently been reported from other streptomycetes and fungi, and extensive labelling experiments have been performed to study their mechanisms. The highlights of this recent work will be discussed here.

5.1. Cyclooctat-9-en-7-ol

After discovery of the cyclooctat-9-en-7-ol (**70**) synthase, a surprising mechanistic study based on isotopic labelling experiments has been published by the Kuzuyama group in 2015.[64] The cyclisation mechanism (Scheme 13) starts with a 1,11/10,14-cyclisation of GGPP to **64**, followed by a sequence of three hydride shifts via **65** and **66** to **67**. Its rearrangement via the cyclopropane intermediates **68** and **69**, followed by ring-opening with attack of water yields **70**. The interesting re-



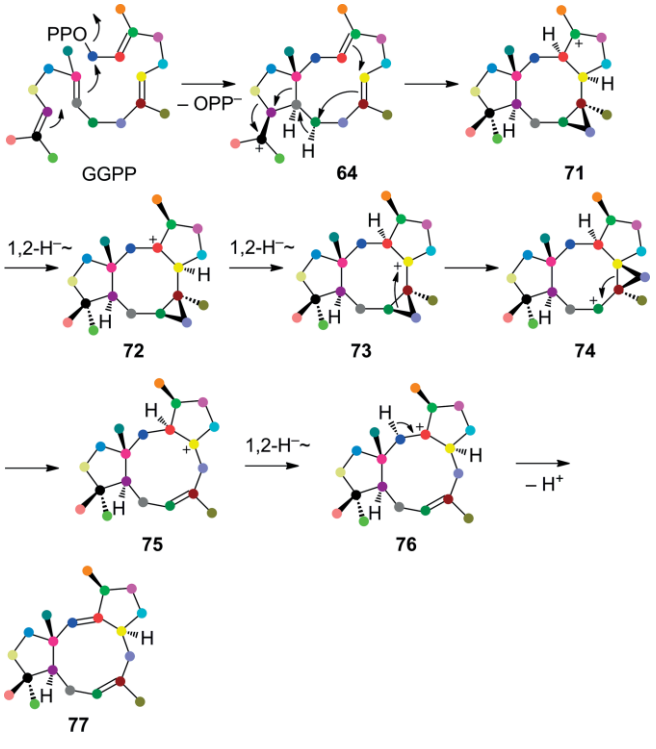
Scheme 13. Cyclisation mechanism for cyclooctat-9-en-7-ol synthase. Blue lines and dots indicate ^{13}C labels after feeding of fully ^{13}C-labelled glucose, red and green hydrogen atoms indicate deuterium labellings.

arrangement in this mechanism was supported by feeding of uniformly ^{13}C-labelled glucose, resulting in the expected labelling pattern for GGPP as indicated in Scheme 13. During the rearrangement step an intact C_2 unit (bold blue bonds) is disrupted, which could be monitored by ^{13}C NMR spectroscopy showing singlets for the corresponding carbon atoms, while doublets would be expected for directly connected ^{13}C-labelled carbon atoms from an unaffected C_2 unit.

Both 1,5-hydride shifts during the biosynthesis of **70** were investigated by incubation of regiospecifically deuterated (8,8-$^2H_2$)GGPP (red hydrogen atoms exchanged by deuterium atoms) and (10-^{2}H)GGPP (green hydrogen atom exchanged by deuterium atom), resulting in the labelling pattern for **70** as shown and thus supporting the proposed 1,5-hydride shifts.[64] The 1,3-hydride transfer from **65** to **66** could not be monitored by these experiments, but subsequent theoretical studies that supported the overall mechanism were in favour of two sequential 1,2-hydride migrations.[65]

5.2. Tsukubadiene

Ikeda and co-workers recently reported on a diterpene cyclase from *Streptomyces tsukubaensis* whose product was identified as tsukubadiene (**77**) by heterologous expression in an engineered *Streptomyces avermitilis* host.[66] The enzyme mechanism has subsequently been deeply studied in vitro by isotopic labelling experiments in which each of the individual carbon atoms of GGPP was specifically labelled in a series of twenty experiments (coloured dots in Scheme 14).[67] These experiments supported



Scheme 14. Cyclisation mechanism for tsukubadiene synthase. Dots of same colour show the result from one of twenty individual ^{13}C-labelling experiments.

a mechanism with a similar cyclopropane rearrangement as described for cyclooctat-9-en-7-ol, although the two enzymes for cyclooctat-9-en-7-ol and tsukubadiene are phylogenetically distant. The process starts with the same cyclisation of GGPP to **64**, followed by a ring expansion/contraction with a 1,2-hydride shift and two cyclisation events to yield **71**. If this process is understood as concerted, secondary cations can be avoided as intermediates. Two subsequent 1,2-hydride migrations via **72** to **73** are followed by the cyclopropylcarbinyl/cyclopropylcarbinyl rearrangement to **74** and ring opening to **75**. A 1,2-hydride shift to **76** and deprotonation yield **77**.

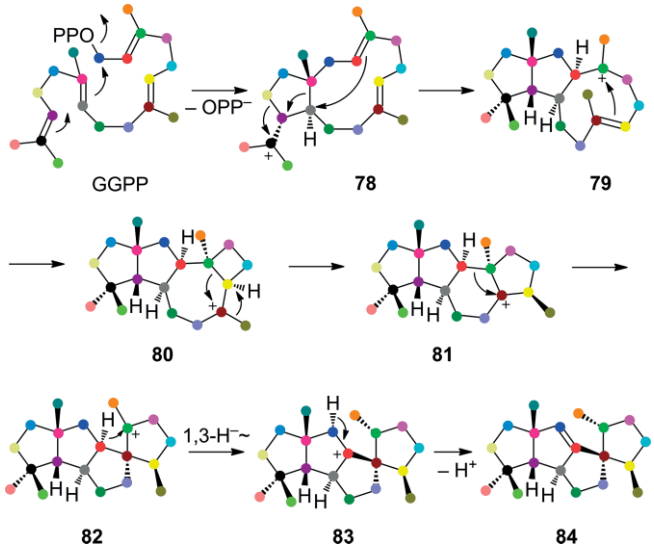
The absolute configuration of **77** was determined using essentially the same strategy as described above for protoillud-7-ene and asterisca-2(9),6-diene.[67] For **77**, the enantioselectively deuterated substrates (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)FPP were used that were elongated with IPP and the GGPP synthase from *Streptomyces cyaneofuscatus* to (*R*)- and (*S*)-(5-^{13}C,5-^{2}H)GGPP with inversion of configuration at C1 of FPP.[3a,68] The additional ^{13}C label in these probes was introduced to allow for a highly sensitive detection of deuterium incorporation into one of two possible diastereotopic hydrogen atoms of **77** by HSQC spectroscopy. Together with the known absolute configuration at the deuterated carbon atom it was possible to delineate the absolute configuration of **77** from the relative orientations of the substituents at the other stereocentres. The question which of the stereochemically distinct hydrogen atoms migrates in the reaction from **64** to **71**, or is lost during the last deprotonation step from **76**, was addressed using (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)GPP and (*R*)- and (*S*)-(1-^{2}H)GGPP, respectively. Finally, the enzymatic conversion of (3-^{13}C,^{2}H)GGPP with tsukubadiene synthase resulted in a product exhibiting an intensive triplet in the ^{13}C NMR spectrum, which supported the 1,2-hydride shift from **71** to **72**.[67]

5.3. Spiroviolene

During the course of the same study a diterpene hydrocarbon with a new spirocyclic backbone was identified as the product of a terpene cyclase from *Streptomyces violens*. The compound was named spiroviolene (**84**; Scheme 15) and the enzyme spiroviolene synthase that is phylogenetically closely related to the tsukubadiene synthase.[67]

The catalytic mechanism of spiroviolene synthase was investigated by the same labelling strategy as for tsukubadiene synthase, by ^{13}C labelling of each individual carbon atom in a series of twenty experiments.[67] The results pointed to a mechanism by GGPP cyclisation to **78**, a stereoisomer of **64**, followed by a similar ring expansion/contraction as for tsukubadiene. The downstream steps differed, showing for spiroviolene synthase two cyclisations via **79** to **80**, followed by a dyotropic rearrangement to **81** and ring contraction to yield the spirocyclic backbone in **82**. A terminal 1,3-hydride shift and deprotonation result in **84**.

The absolute configuration of **84** was determined using both enantiomers of stereospecifically deuterated (1-^{13}C,1-^{2}H)GPP and (1-^{13}C,1-^{2}H)FPP by the same strategy as outlined above,

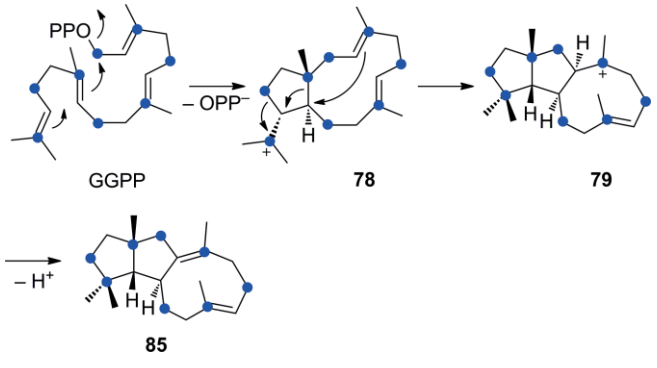


Scheme 15. Cyclisation mechanism for spiroviolene synthase. Dots of the same colour show the result from one of twenty individual ^{13}C-labelling experiments.

while the 1,3-hydride shift from **82** to **83** was established by enzymatic conversion of (3-^{13}C,2-^{2}H)GGPP to yield a triplet signal for the labelled carbon atom in the ^{13}C NMR spectrum, and the stereochemical course of the final deprotonation as shown in Scheme 15 was addressed using (*R*)- and (*S*)-(1-^{2}H)GGPP.

5.4. Variediene

A fungal terpene cyclase that is mechanistically related to the spiroviolene synthase from *Streptomyces violens* is the recently identified variediene synthase from *Emericella variecolor*.[69] Its product **85** has been obtained by efficient heterologous expression in *Aspergillus oryzae*.[70] Interestingly, the fungal diterpene **85** arises by simple deprotonation from **79**, which is a cationic intermediate along the spiroviolene cyclisation cascade. The mechanism shown in Scheme 16 was supported by feeding experiments with sodium (1-^{13}C)acetate to the *A. oryzae* transformant.



Scheme 16. Cyclisation mechanism for variediene synthase. Blue dots indicate positions of incorporation of ^{13}C labelling from sodium (1-^{13}C)acetate.

6. Conclusions

Isotopic labelling experiments continue to be an important method for biosynthetic investigations. As shown in this article, isotopes are particularly useful to investigate the mechanisms of enzymes involved in the biosynthesis of isoprenoid precursors and of terpene cyclases. An interesting other approach is offered by quantum chemical calculations. Both methods can stimulate each other to address further mechanistic problems of terpene biosynthesis in the future.

Keywords: Terpenoids · Isotopes · Enzymes · Reaction mechanisms · Cations · Biosynthesis

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