On the Early Steps of Cineol Biosynthesis in *Eucalyptus globulus*

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Dedicated, on the occasion of his 75th birthday, to *Albert Eschenmoser*, from whom I learned, among many other things, that one plus one gives much more than two, if the ones communicate.

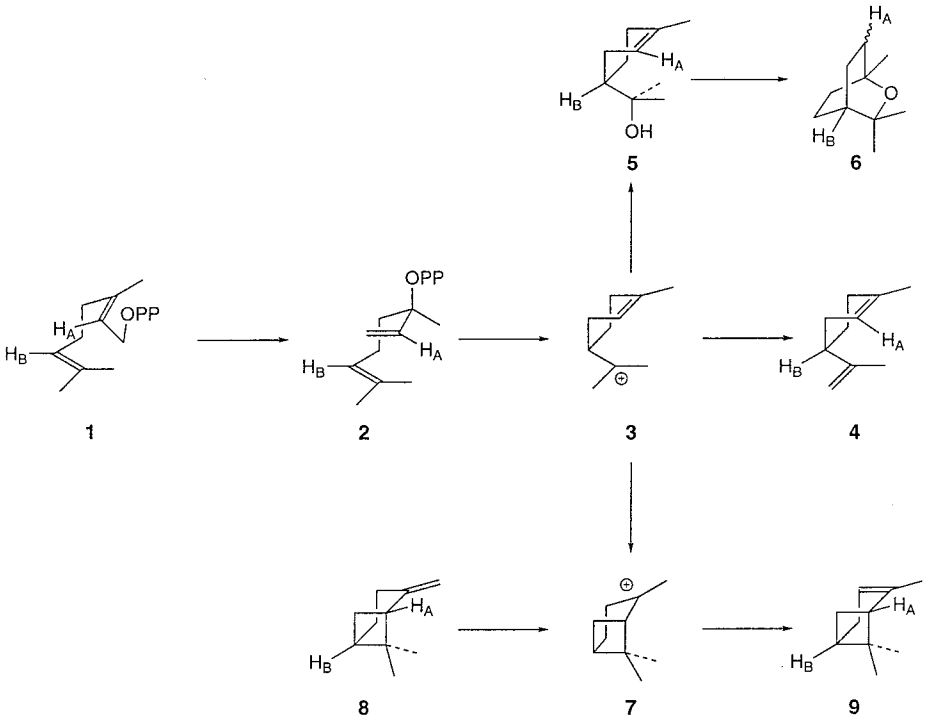
Samples of [4-$^2H_1$]-1-deoxyxylulose (**17a**) and [2-^{13}C, 4-$^2H_1$]-1-deoxyxylulose (**17b**), have been prepared by modification of known procedures and fed in aqueous solution to twiglets of *Eucalyptus globulus*. The probes of cineol (**6**) isolated from these experiments were analyzed by GC/MS, ^2H- and ^{13}C-NMR techniques. In the experiments with **17b**, the formation of five isotopomers of **6** could be detected. Their structure and relative abundance demonstrate that the ^{13}C-label is incorporated to the same extent into the two C_5-units of **6**, and that the ^2H label is retained to an extent of 57% in the starter dimethylallyl-diphosphate unit (DMAPP; **12**), but completely or almost completely lost in the unit derived from isopentenyl diphosphate (IPP; **11**), in the elongation step which leads to geranyl diphosphate (GPP; **1**). These results confirm that the recently discovered mevalonate-independent pathway to IPP and DMAPP is operative in the biosynthesis of cineol, and indicate, together with previous finding, that, within this pathway, formation of IPP and DMAPP occurs in independent rather than in sequential steps. In addition, the demonstration of different metabolic origins for the olefinic H-atoms of GPP (**1**), the aliphatic C_{10}-precursor of **6**, paves the way for a realistic interpretation of the strikingly consistent but hitherto unexplained anomalies detected in the natural-abundance ^2H-NMR spectra of (+)- and (−)-α-pinene and of (+)-limonene.

Introduction. – Extensive studies carried out by different groups in the last 25 years have established that the biosynthesis of a large variety of cyclic monoterpenes proceeds from the universal aliphatic precursor geranyldiphosphate (GPP; **1**), according to the mechanistic route outlined in *Scheme 1*[1]). Anionotropic rearrangement of **1**, followed by rotation about the newly created single bond, leads to the boat-like conformation of (3*R*)-linaloyl diphosphate (**2**). Subsequent ionization of **2** with concomital participation of the distal double bond generates the (4*S*)-α-terpinyl cation **3**, which can dismiss its charge by proton extrusion to provide (+)-limonene (**4**), capture a molecule of H_2O to give (+)-α-terpineol (**5**), or suffer an additional cyclization to generate **7**, the immediate precursor of (+)-α-pinene (**9**), and (−)-β-pinene (**8**). An equivalent set of reactions proceeding through the (3*S*)-enantiomer of **2** leads to the mirror images of all chiral cationic intermediates and substances indicated in *Scheme 1*.

Some years ago, three groups reported independently on natural-abundance ^2H-NMR as a tool for investigating the biosynthesis of cyclic monoterpenes [2–4]. In the paper by *Martin et al.* [2], it was pointed out specifically that the ^2H content at the position labelled with H_A in the formula of (+)-α-pinene (**9**) was markedly depleted

[1]) For a recent detailed review, see [1].

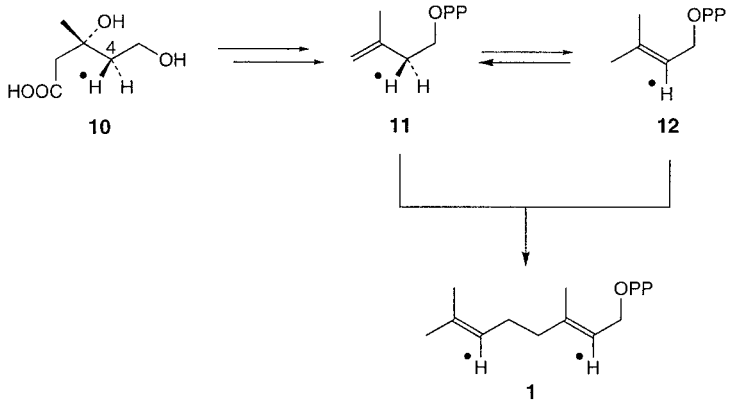
Scheme 1



(by a factor of 4) with respect to the value corresponding to H_B in the same compound, and that a similar depletion was also evident in the ^{2}H-NMR spectrum of (−)-α-pinene. A critical examination of the data published by *Pascal et al.* on (−)-α-pinene [3] has confirmed this anomalous ^{2}H distribution [5]. In addition, inspection of the natural-abundance ^{2}H-NMR of (+)-limonene (**4**) has revealed once again a significant depletion (by a factor of 2.5) in the ^{2}H content of the H_A-atom of **4** [5]. No explanation was offered in any of the three reports for this consistent anomalies. In subsequent work triggered by these striking findings, it could be shown that, with a set of cyclases from *Salvia officinalis*, the H_A-atom of the aliphatic precursor **1** is completely retained during formation of a variety of cyclic monoterpenes including *inter alia* **4**, **8**, and their enantiomers, as well as the achiral cineol (**6**), and it was concluded that the anomalies previously detected had to be connected with a pre-existing differential labelling of the two olefinic H-atoms H_A and H_B in the aliphatic precursor **1** [5].

The notion of an aberrant alteration in the natural ^{2}H content at the C(2)-atom of **1** rests, of course, on the preconceived and ill-supported conviction that **1** is assembled as illustrated in *Scheme 2* from the two building blocks isopentenyl diphosphate (IPP; **11**) and dimethylallyl diphosphate (DMAPP; **12**), generated sequentially from the 'universal' terpene precursor mevalonic acid (**10**). Since in this scheme the same H-atom of IPP (**11**) is lost in the formation of DMAPP (**12**) and in the elongation step which leads to GPP (**1**) [6], the two olefinic H-atoms of the latter share a common

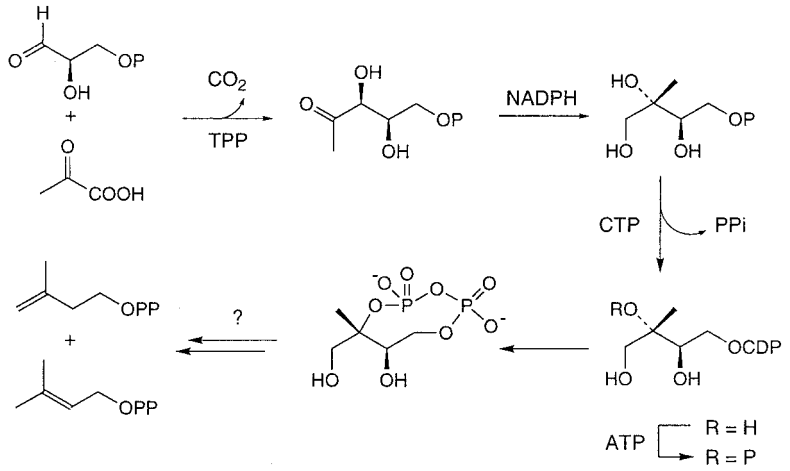
Scheme 2



source, the dotted H at C(4) of the mevalonic-acid precursor **10**, and are, accordingly, expected to display identical values for their levels of natural ^2H abundance.

For a long time, experimental evidence in support of a mevalonate route for the biosynthesis of monoterpenes in higher plants has been at best scanty and altogether less than convincing [7]. A dramatic turn of events has been brought about in recent years by the discovery of a new mevalonate-independent metabolic pathway to IPP (**11**) and DMAPP (**12**), which by now is known to be widespread in eubacteria (both *Gram*-positive and *Gram*-negative), green and red algae, as well as higher plants [8]. An updated version covering the latest developments [9] in our still incomplete knowledge of the new pathway is presented in *Scheme 3*. The specific demonstration that the new pathway plays a major, if not exclusive, role in the formation of the two C_5-units required for the biosynthesis of monoterpenes in higher plants [10] provided a welcome platform for tackling anew the problem of the suspected difference in the

Scheme 3

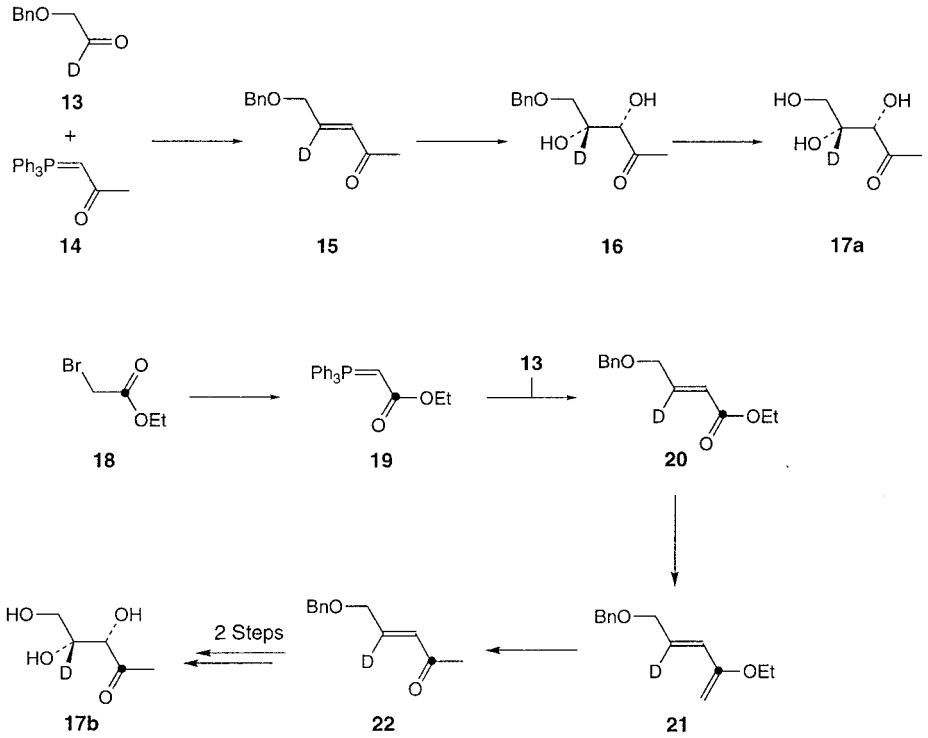


metabolic origin of the olefinic H_A- and H_B-atoms in the GPP intermediate **1**. For practical reasons, we decided to concentrate our efforts on the formation of cineol (**6**), the preponderant monoterpene in leaves of *Eucalyptus globulus*.

In a set of incisive studies carried out with a specific synthetase from *Salvia officinalis*, *Croteau et al.* succeeded in showing that the structure of **6** is elaborated from **1** *via* the intermediacy of **2** and **5** (*cf. Scheme 1*), and that the ether O-atom as well as the proton that triggers off the last cyclization step are derived from molecules of H_2O [11]. To date, no evidence is available on the cryptic stereochemical course of the sequence **3** → **5** → **6**.

Results and Discussion. – The feasibility of the selected approach was first tested in exploratory experiments with a specimen of 1-deoxyxylulose labelled with deuterium at C(4) (*cf.* **17a** in *Scheme 4*), and the preliminary results were then refined in a second set of experiments involving a probe of the doubly labelled [2-^{13}C,4-$^{2}H_1$]-1-deoxyxylulose (**17b**).

Scheme 4



The synthesis of **17a** was achieved according to known procedures [12][13], involving a *Wittig* reaction of the deuterated aldehyde **13** with **14** to provide the (*E*)-enone **15**, followed by asymmetric *Sharpless* dihydroxylation of the latter to **16** and hydrogenolytic removal of the protecting Bn group. The required deuterated aldehyde **13** was conveniently prepared by *Swern* oxidation of the [1-$^{2}H_2$]alcohol, which is easily

accessible from commercially available 2-(benzyloxy)acetyl chloride in two steps involving methanolysis and subsequent reduction of the resulting methyl ester with $LiAl(^2H_4)$. The ^2H content of all the intermediates and of the final compound **17a** was found by ^1H-NMR analysis to be larger than 98%.

The same deuterated aldehyde **13** could be used for the synthesis of the doubly labelled precursor **17b** ($\bullet = {}^{13}C$). Condensation of **13** with the *Wittig* reagent prepared with Ph_3P from ethyl 2-bromo[1-^{13}C]acetate (**18**) gave a 3:1 mixture of the (*E*)-ester **20** and of its (*Z*)-counterpart. After chromatographic separation of the two isomers, the pure (*E*)-compound was converted by a *Tebbe* olefination and subsequent hydrolysis of the resulting enol ether with AcOH in toluene to the (*E*)-enone **22**, and the synthesis of the desired material **17b** was then completed as described before. Once again, ^1H-NMR monitoring of the doubly labelled compounds ensured that the deuterium content was $>98\%$.

With these tools in hand, we could now proceed with the critical biological experiments. For this purpose, twiglets of *E. globulus* were allowed to absorb an aqueous solution of the labelled substrates over a period of 5 days under normal illumination conditions. The volatile fraction from the leaves was isolated by distillation and found by GC/MS analysis to consist of essentially pure cineol (**6**).

The mass spectrum of the cineol specimen from the feeding experiment with **17a** indicated a slight increase of *ca.* 1% in the intensity of the $[M+1]^+$ ion. The ^2H-NMR spectrum of this probe displayed a single enriched signal at 1.38 ppm, corresponding to the ^2H-atom at C(4) of cineol (for the numbering of cineol, see *Fig. 2*). Using the background signals of molecules with natural abundance (more specifically, the signal at 1.20 ppm, corresponding to the two enantiotopic Me groups) as a calibration, a 61-fold intensity increase was estimated for the enriched position, corresponding to a specific ^2H incorporation of *ca.* 0.9%.

To clarify the reason for the lack of ^2H incorporation in the second isopentane unit of cineol, a second feeding experiment was carried out with the doubly labelled substrate **17b**, in which the ^{13}C-label was expected to act as an internal standard for following the fate of the ^2H-atom. The mass spectrum of the resulting cineol suggested the presence of a small amount of molecules ($<1\%$) with up to three additional mass units, and once again the ^2H-NMR spectrum of the probe displayed a single recognizably enriched signal at 1.38 ppm with a relative intensity corresponding to a specific incorporation of *ca.* 0.45%.

The ^{13}C-NMR spectrum of the same material proved to be rewardingly rich in information. A comparison of the relative intensities of its lines with those of a sample with natural ^{13}C-abundance isolated from the same source (see *Table 1*) revealed an identical (or nearly identical) increase of *ca.* 57% in the ^{13}C-content of both C(1) and C(8). This corresponds to a specific incorporation of 0.61% at both sites. An expanded version of the signals corresponding to these two C-atoms is presented in *Fig. 1* and uncovers the presence of different satellites. From the relevant data collected in *Table 2*, the presence and relative abundance of the five isotopomers of cineol (**6**) depicted in *Fig. 2* can be inferred. Capital letters are used in *Table 2* and *Fig. 2* to correlate each isotopomer with the signals for which it is responsible; note that **A** and **B** refer to molecules generated *de novo* from the proffered precursor, whereas $\mathbf{A_0}$ and $\mathbf{B_0}$ correspond to molecules with normal background abundance. The relative intensity of

A was estimated by correcting the value of **C** with the ratio of the values of **E** and **D**, and the value of **B** was approximated by the sum of the values for **A** and **E**. The positive value of the δ shift caused in isotopomer **D** by the presence of a ^{2}H-atom at C(4) on the ^{13}C-signal of C(1) may, at first sight, seem unusual but is in fact well-precedented by a similar value of 13 ppb detected in a bicyclo[2.2.2]octane derivative [15].

Table 1. *Relative Intensities of the ^{13}C-NMR Signals in Samples of Cineol from* E. globulus (A: reference sample with natural abundance; B: sample from the experiment with **17b**)

Position[a])	δ Values[b]) (CD_2Cl_2)	Relative intensities[c])	
		A	B
C(8)	73.76	23.5	37
C(1)	69.92	23.5	37
C(4)	33.35	100	100
C(2/6)	31.91	217	231
C(9/10)	29.09	223	238
C(7)	27.71	114	116
C(3/5)	23.22	217	222

[a]) Numbering as in *Fig. 2*. [b]) For signal assignment, see [14]. [c]) Normalized for the intensity of the C(4) signal.

Table 2. *Selected Data for the ^{13}C-NMR Signals* (CD_2Cl_2) *of C(1) and C(8) in the Cineol Sample from the Feeding Experiment with* **17b**

Position	Isotopomer[a])	δ Value [ppm]	Relative intensities	J(C,C) [Hz]	Isotopic shift [ppb]
C(8)	$\mathbf{A}_0$	73.766	128		
	A	73.766	5.5		
	C	73.763	19.5	1.74	β-(C–C) = –3.38
	E	73.716	8		
	D	73.712	30	1.74	β-(D–C) = –51.2
C(1)	**D**	69.934	30	1.76	δ-(D–C) = +11.6
	$\mathbf{B}_0$	69.923	124		
	B	69.923	13		
	C	69.921	23	1.76	β-(C–C) = –2.6

[a]) *Cf. Fig. 2*.

Among the *de novo* synthesized labelled material, isotopomers **C** and **D** doubly labelled with ^{13}C have a preponderance of 67%; this shows that the starting material, which was essentially isotopically pure, was diluted only to an extent of *ca.* 20% by endogenous material, and, accordingly, the relatively low value of the specific incorporation is caused mainly by a large pool of cineol already present at the beginning of the feeding experiment.

A comparison of the relative abundancies of species **A**, **C**, **D**, and **E** reveals that 57% of all the molecules labelled with ^{13}C at C(8) have retained a ^{2}H companion at C(4); in sharp contrast, all (or nearly so; *cf.* below) of the ^{2}H originally associated in the precursor with the ^{13}C-label eventually detected at C(1) of cineol has been lost during formation of the monoterpene. This is tantamount to the statement that the ^{2}H label of the precursor **17b** is largely retained in the C_5-unit of cineol derived from DMAPP (**12**)

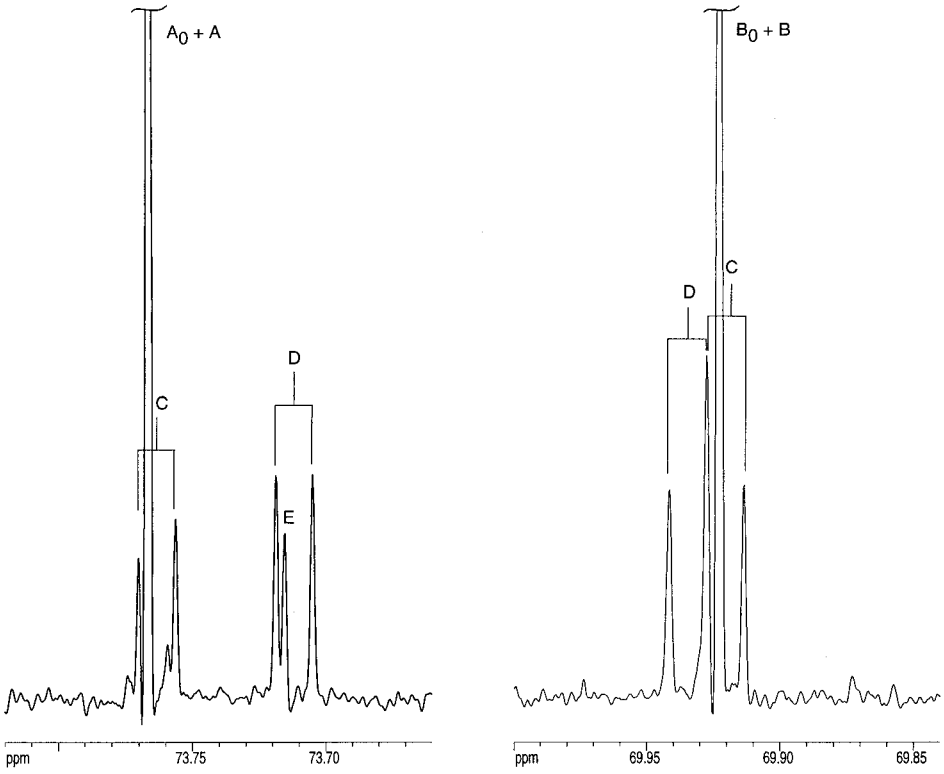


Fig. 1. *Expansions of the ^{13}C-NMR spectrum of the cineol sample from the feeding experiment with* **17b** *showing the signals of C(8)* (left) *and C(1)* (right). Conditions: 125 MHz, {^{1}H}-broad-band-decoupled, inverse-gated; processed with a gaussian filter function.

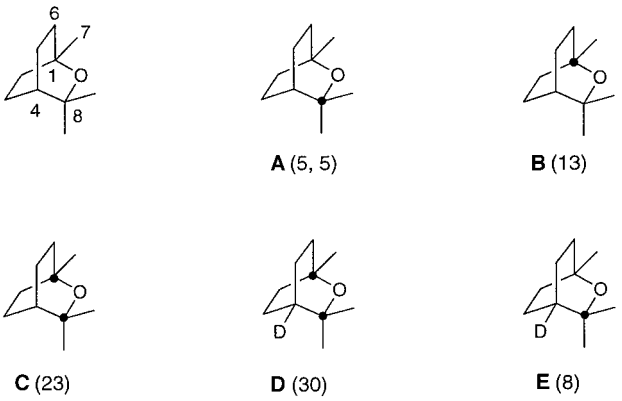
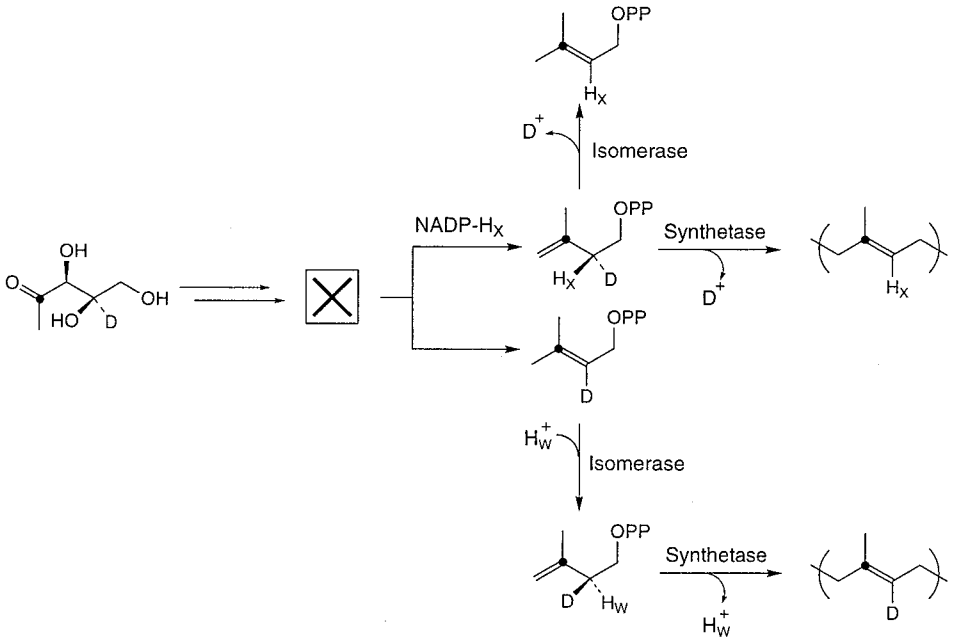


Fig. 2. *Cineol numbering and structure of the isotopomers responsible for the ^{13}C-NMR signals displayed in* Fig. 1 (• = ^{13}C)

and completely or almost completely lost in the C_5-unit of cineol derived from IPP (**11**) in the elongation process. Since it is firmly established that H_A of GGP (**1**) is fully retained during cineol biosynthesis [5], it follows that, as suspected, the H_A- and H_B-atoms of the GGP (**1**) precursor of cineol (**6**) have different metabolic origins. This, in turn, provides a long-sought new starting point for a realistic interpretation of the unexplained spectroscopic results discussed in detail in the *Introduction.*

The findings reported here match closely the ones previously reported for the mevalonate-independent formation of the octaprenyl side chain of ubiquinone in *Escherichia coli* [13]. In the meantime, work carried out in the *Poulter* group [16] has demonstrated that in *E. coli*, as in all eukariotes, the same H-atom is eliminated from C(2) of IPP (**11**) in the isomerization and elongation steps depicted in *Scheme 5*. From the bulk of the evidence, one is forced to conclude that in the new pathway IPP (**11**) cannot be a committed precursor of DMAPP (**12**), and that the two compounds must be formed independently from a late and yet unidentified intermediate **X**. Results recently secured in *Rohmer*'s group for the biosynthesis of bacteriohopanes in *Zymomonas mobilis* suggest that the H-atom of IPP (**11**) retained in the main elongation step of *Scheme 5* has been introduced from NADPH in one of the late and still unknown steps of the pathway [17]. The operation of a similar step during monoterpene biosynthesis in higher plants would be eminently well-suited for efficient isotopic discrimination (both in the formation of NADPH and in the subsequent hydride transfer) and might well represent the immediate cause for the marked depletion in natural ^2H consistently observed at the H_A-position of monocyclic terpenes [2–4].

Scheme 5



A final comment is required for the apparent loss of 43% of the ^2H label of **17b** detected in this work during formation of the DMAPP unit involved in cineol biosynthesis. Lack of information concerning the nature of the last two reductive steps leading to DMAPP does not allow to rule out a true partial loss associated with the mechanistic details of such steps. Alternatively, one can assume that the ^2H label of **17b** is fully retained in DMAPP molecules generated without the intermediacy of IPP and that ^2H-free DMAPP molecules stem from the subsequent isomerization of independently formed IPP labelled as indicated in *Scheme 5*. One might, of course, object that by the same token action of the isomerase on the originally formed DMAPP should generate another form of IPP, which, in contrast to the original one is expected to retain its ^2H in the elongation step; it is, however, important to note that in the early stages of the (incomplete) isomerization process the relative contribution of such a species to the pool of labelled IPP molecules will be critically dependent on the relative size of the pools of IPP and DMAPP; in other words, if the size of the original IPP pool is much larger than the original size of the DMAPP pool, the relative contribution of IPP molecules with a different location of the label emerging from the isomerization process will be mitigated accordingly. In this context, it may be relevant to point out that a closer look at the traces of *Fig. 1* uncovers the presence of what looks like a set of two low-intensity *doublets* centered at *ca.* 69.865 and 73.764 ppm and displaying an apparent *J* value of *ca.* 1.75 Hz. While the significance of these signals remains questionable, it is interesting to note that, both in terms of their spacing and of their estimated shifts from the main peaks, they would be compatible with the presence of minor amounts (less than 10% of **C**) of yet another isotopomer of cineol, generated from IPP derived from the isomerization of DMAPP as indicated in *Scheme 5* and labelled with ^{13}C at both C(1) and C(8) and with ^2H at C(6).

We are indebted to Prof. *P. Chen* for allowing us to use his GC/MS facilities, to Mrs. *P. Enz* and *R. Stalder* of the Zürich botanical garden for providing the biological material, and Prof. *H.-J. Borschberg* for technical assistance in the preparation of the manuscript. Financial support by *Novartis AG*, Basel, is gratefully acknowledged.

Experimental Part

General. GC/MS Measurements were carried out with a *Fisons MD 800* instrument equiped with a *DB-5MS* capillary columm. ^2H-NMR: *Bruker AMX-400* (61.42 MHz); in CH_2Cl_2 with a $CFCl_3$ lock. ^{13}C-NMR: *Bruker AMX-400* (100 MHz) and *Bruker DRX-500* (125 MHz), in CD_2Cl_2.

Feeding Experiments and Isolation of Cineol (**6**). *a*) The stems of two 20-cm long twiglets of *E. globulus*, carrying *ca.* 20 leaves each, were cut under running water with a sharp razor blade at an angle of *ca.* 30° and placed in a narrow 50-ml beaker containing 20 ml of H_2O. The labelled precursor **17a** (400 mg) was dissolved in a mixture of 8 ml of H_2O and 4 ml of EtOH; approximately half of this soln. was administered to the twiglets at the beginning of the experiment, and the rest was added in aliquots at regular intervals together with enough H_2O to make up for the amount lost through evaporation. To facilitate absorption, the immersed tips of the twiglets were cut anew twice a day under running water. The experiment was stopped on the fifth day after 150 ml of H_2O had been absorbed, and the detached leaves powered under liq. N_2. The powder (6.30 g) was suspended in 50 ml of H_2O, and the volatile components were distilled at r.t./1 Torr and trapped in a flask cooled with liquid N_2. Following addition of H_2O to the residue, the distillation was repeated three times, and the collected distillates were extracted with CH_2Cl_2 (4×100 ml). The org. phase was dried ($MgSO_4$) and the solvent removed at 40°/300 Torr to give 92 mg of an oil, shown by GC/MS analysis to consist essentially of pure cineol.

b) From a similar experiment with 390 mg of **17b** and a total absorption of 235 ml of H_2O, a sample of 427 mg of cineol was isolated.

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