Quantitative ^{2}H NMR spectroscopy
2.* "H/D-Isotope portraits" of cyclic monoterpenes and discrimination of their biosynthetic pathways**

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Site-specific deuterium distribution in molecules of the representative series of natural monoterpenes was studied by quantitative ^{2}H NMR spectroscopy. "H/D-isotope portraits" of these compounds have general characteristic features reflecting biosynthetic pathways. The data obtained suggest that monoterpenes in plants are formed through 1-deoxy-D-xylulose-5-phosphate (DXP pathway) rather than by the classical mevalonate scheme.

Key words: terpene, biosynthesis, 1-deoxy-D-xylulose-5-phosphate, deuterium, ^{2}H NMR spectroscopy.

^{2}H NMR spectroscopy is an efficient tool for quantitative determination of the total deuterium content in organic molecules. An important advantage of the method is a possibility to determine the selectivity of this isotope distribution over different structural fragments of a molecule in one direct experiment without labor-consuming consecutive degradation of the compound. The total content of deuterium in organic substances of different origin can differ significantly from the average natural content of this isotope (155.8 ppm in water of the world ocean[2]), and its content in different structural fragments of a molecule can vary substantially due to kinetic isotope effects[3] in chemical and biochemical processes. Therefore, the "H/D-isotope portrait" of an organic molecule is a unique characteristic dependent on its chemical pre-history. The first studies by ^{2}H NMR at the natural abundance level of the isotope already demonstrated convincingly wide challenges of using this method for analysis of mechanisms of chemical reactions and biosynthetic pathways and to determine sources of origin of natural organic compounds.[4—9]

Monoterpenes play a significant role in living nature as signal substances that transmit information between individuals of the same and different species and between organs of the same organism and perform protective functions.[10—13] The ^{2}H NMR spectra of some cyclic monoterpenes have previously been described in several works. The data on selective distribution of this isotope in molecules of (1*R*)-camphor,[7] (1*R*)- and (1*S*)-α-pinenes,[8,14] (1*S*)-β-pinene,[8,15] (*R*)-(+)-limonene,[16] and linalool[17] showed that the deuterium content in different structural fragments of molecules of these natural compounds differed substantially from the statistical value and depended on the plant source from which they were isolated.

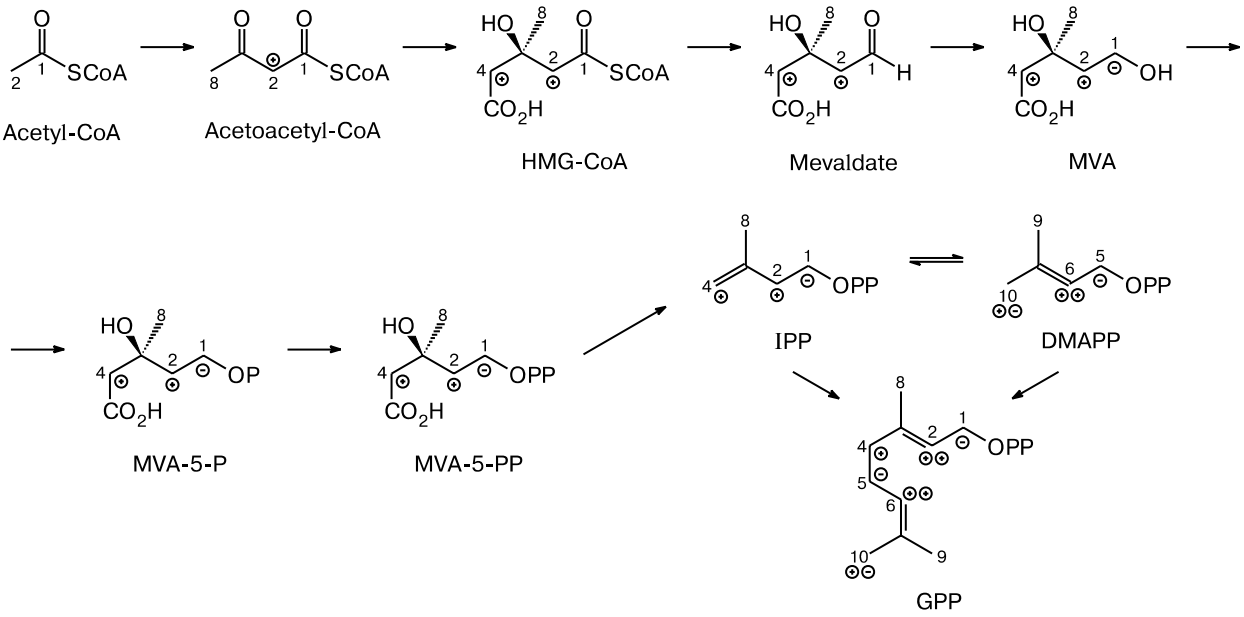
It is well known that the universal biosynthetic precursor of monoterpenes is geranyl diphosphate (GPP), which is formed, in turn, by the condensation of two isoprenic C_5 components, *viz.*, isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).[11—13,18,19] Up to recently researchers believed that IPP and DMAPP are formed by one general biosynthetic pathway through mevalonic acid (MVA)[13] (Scheme 1). DMAPP was also considered to be formed by IPP isomerization. It has been found only in recent years that terpene biosynthesis in plants can proceed also *via* an alternative scheme through 1-deoxy-D-xylulose-5-phosphate (DXP)[13,20—22] (Scheme 2). In this case, IPP and DMAPP can be formed from a common precursor but no direct isomerization of IPP to DMAPP occurs. This scheme is confirmed[10,23,24] in part by experiments using selectively ^{2}H- and ^{13}C-labeled DXP in biosynthesis. The mechanisms of terpene and terpenoid biosynthesis have been considered.[10]

* For Part 1, see Ref. 1.

** Dedicated to Academician N. K. Kochetkov on the occasion of his 90th birthday.

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 5, pp. 1222—1228, May, 2005.

Scheme 1



Hereinafter ⊙ and ⊖ are the increase and decrease, respectively, in the deuterium content

Thus, a new urgent problem appeared during identification of monoterpenes: *via* which of the two biosynthetic pathways (through MVA or DXP) is each specific substance of this class formed?

To compare at a qualitative level the concept of "H/D-isotope portraits" of the main precursors, let us consider the expected changes in the deuterium distribution at each step of the two biosynthetic pathways that are related to the formation or cleavage of C—H bonds. When based on the known fact that the kinetic H/D-isotope effects are positive in these reactions ($k_H/k_D > 1$), then it is evident that the formation of a C—H bond should be accompanied by a relative decrease in the deuterium content (an increase in the protium content) in the corresponding position compared to the initial value, and C—H bond cleavage should be accompanied by a relative increase in the deuterium content. Taking into account these considerations, one can suggest that the "H/D-isotope portraits" of IPP, DMAPP, and GPP formed *via* two alternative biosynthetic pathways would be qualitatively similar to those presented in Schemes 1 and 2. To retain the general approach, the numbering of atoms of the carbon framework in all molecules studied in this work will be the same as in geranyl diphosphate. Terpenes and terpenoids C_{10}, according to the commonly accepted views, are formed from geranyl diphosphate (Scheme 3): the allyl rearrangement occurs first to form linalyl diphosphate (LPP), and then the intramolecular cyclization in LPP affords an intermediate to which the α-terpenyl cation structure is conventionally ascribed. Although the existence of the α-terpenyl cation as a kinetically independent species in biological media is impossible, this conventionality makes it possible to consider all subsequent transformations of the framework (ring closures, hydride shifts, and the Wagner—Meerwein rearrangements) from the single point of view. These are the transformations that produce the whole variety of terpenes and terpenoids of the C_{10} series.

Thus, the "H/D-isotope portraits" of the α-terpenyl cations formed *via* two alternative biosynthetic pathways under consideration should differ substantially. Geranyl diphosphate formed *via* the classical mevalonate pathway and terpenes and terpenoids formed from the geranyl diphosphate have the following distinctive features (see Scheme 1).

1. Decreased (compared to the 8-Me group) content of deuterium in positions at the C(1) and C(5) atoms.

2. Increased (compared to the 8-Me group) content of deuterium in positions at the C(2) and C(6) atoms (to a great extent), as well as at the C(4) atom.

3. Weakly pronounced difference in deuterium content in the 9-Me and 10-Me groups.

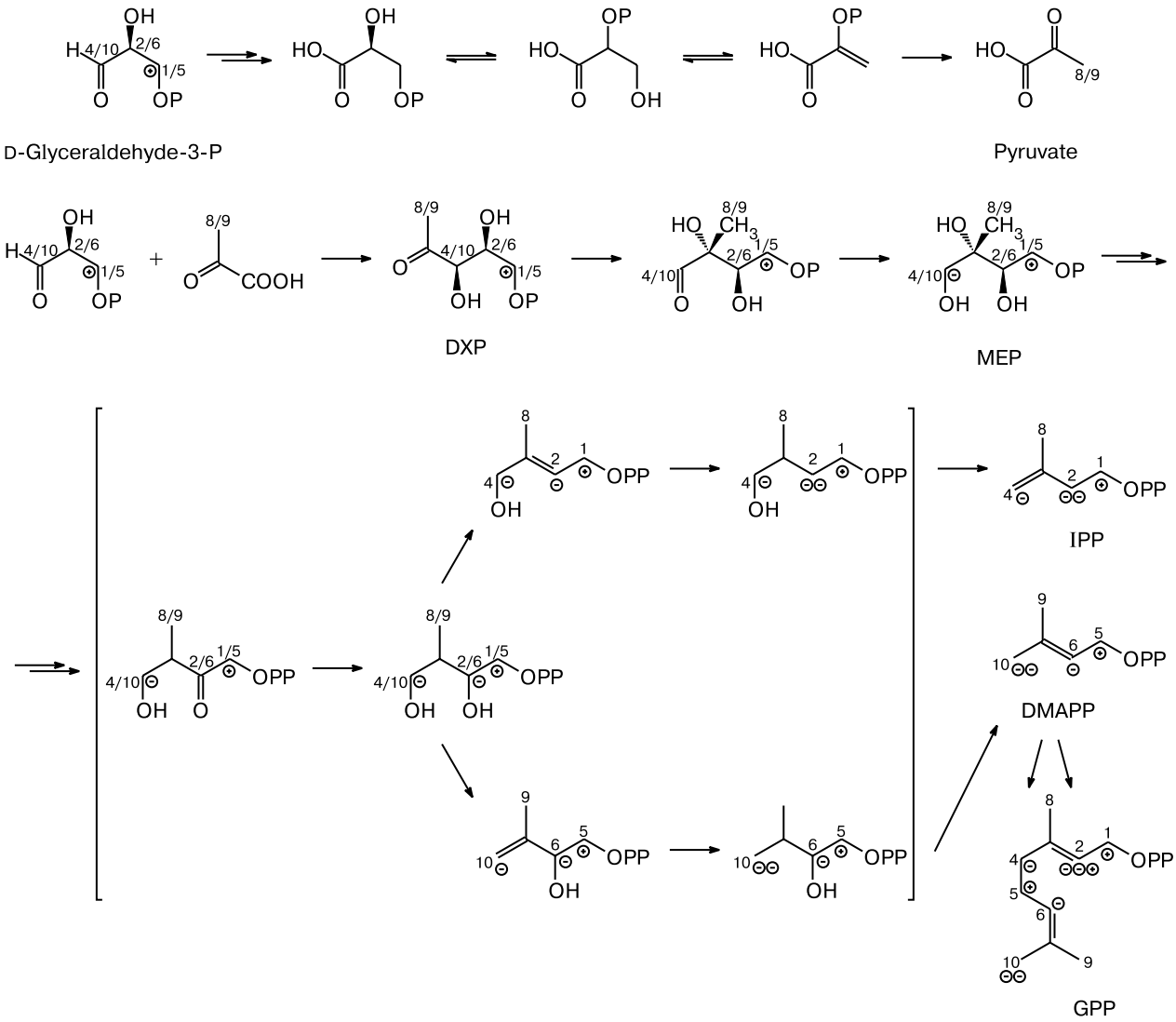
Geranyl diphosphate, terpenes, and terpenoids formed *via* the DXP pathway (see Scheme 2) have different isotope characteristics.

1. Increased (compared to the 8-Me group) content of deuterium in positions at the C(1) and C(5) atoms.

2. Decreased (compared to the 8-Me group) content of deuterium in positions at the C(2), C(4), C(6), and C(10) atoms.

3. Different deuterium contents in the 9-Me and 10-Me groups.

Scheme 2



The expected deuterium distribution in GPP for two cases considered is given in Fig. 1. It is significant that the kinetic isotope effects at different steps in each scheme can differ substantially and, hence, the "H/D-isotope por-

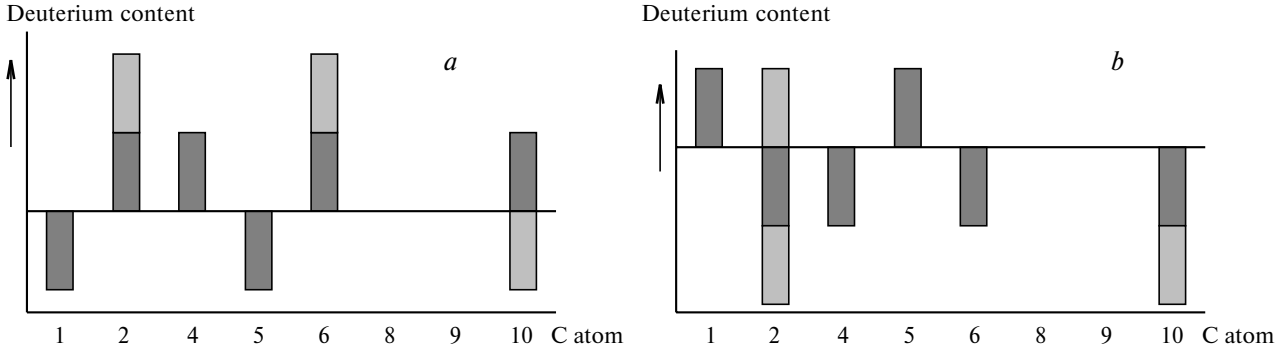
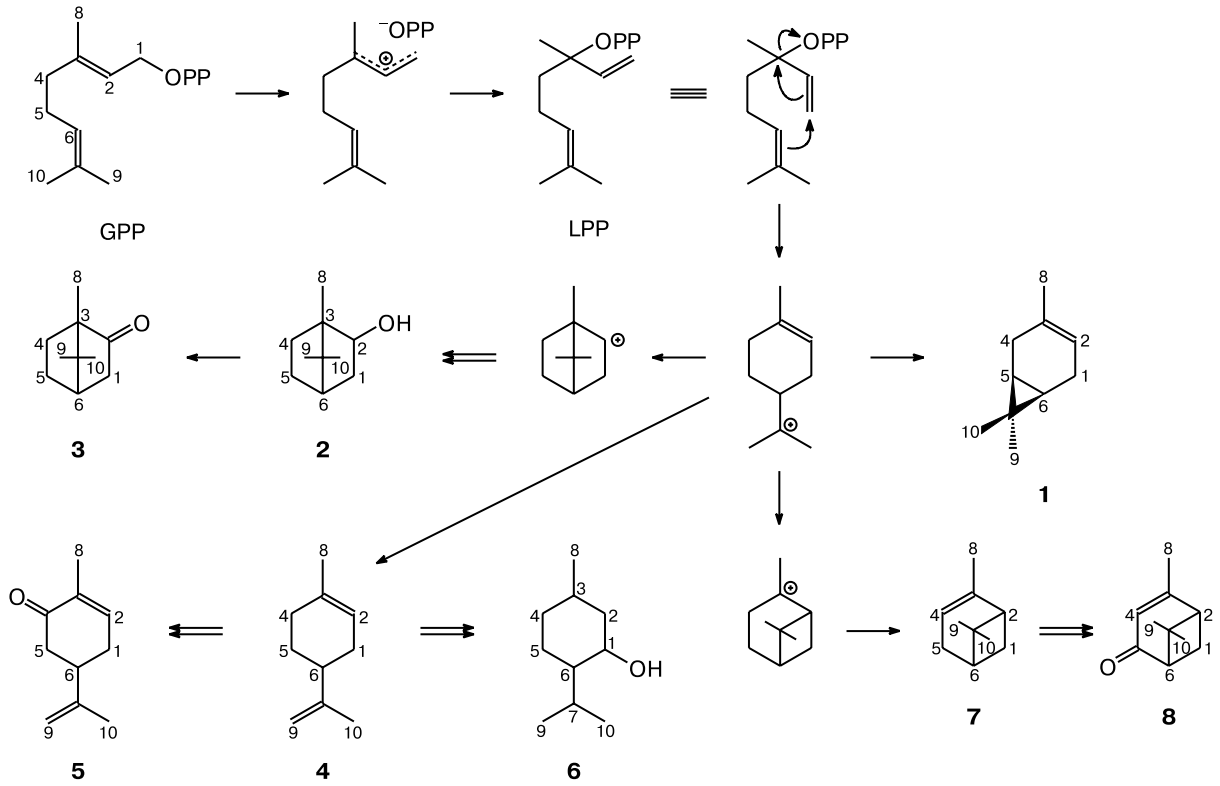


Fig. 1. Assumed deuterium distribution in GPP for the MVA (*a*) and DXP pathways (*b*) of terpenoid biosynthesis.

Scheme 3



traits" presented are qualitative and can reflect only general tendencies of deuterium distribution in terpenes and terpenoids.

In this work, we measured and analyzed the ^{2}H NMR spectra (see Ref. 1) for several earlier unstudied monoterpenes obtained from different sources. The general tendencies of deuterium distribution in molecules of these natural compounds were experimentally determined and compared to different biosynthetic pathways predicted on the basis of analysis.

Experimental

(1*S*)-(+)-Δ^3-Carene **1** (*ee* 88%, $[\alpha]_D$ +16.2) was isolated from the pine-wood turpentine oil (Medvezh´egorsk Wood-Chemical Industrial Complex, Karelia, Russia). (1*S*)-(−)-Borneol **2** ($[\alpha]_D$ −42.0) was prepared from bornyl acetate, which was isolated from the fir oil (Neivo-Rudyansk Wood-Chemical Plant, Sverdlovsk Region, Russia). (1*S*)-(−)-Camphor **3a** ($[\alpha]_D$ −43.5), (±)-camphor **3b**, and (1*R*)-(+)-α-pinene **7a** (*ee* 36%, $[\alpha]_D$ +19) were isolated from the turpentine oil (Neivo-Rudyansk Wood-Chemical Plant). (1*R*,2*S*,5*R*)-(−)-Menthol **6** ($[\alpha]_D$ −49.3) and (1*R*)-(+)-verbenone **8** (*ee* 40%, $[\alpha]_D$ +119) were pharmaceutical preparations. (*R*)-(+)-Limonene **4a** (Aldrich), (*S*)-(+)-carvone **5a** (Fluka), (*R*)-(−)-carvone **5b** (Aldrich), and (±)-α-pinene **7b** (Aldrich) were used as received.

Carvones, Δ^3-carene, menthol, limonene, α-pinene, and verbenone were studied in the pure state, and measurements for borneol and camphors were carried out for their saturated solutions in chloroform. ^{2}H NMR spectra (46.05 MHz) were recorded on a Bruker DPX-300 spectrometer equipped with a special deuterium probe. Stabilization of the resonance conditions was achieved using the ^{19}F signal. The duration of 90° pulse for the ^{2}H nuclei was 18 μs. The WALTZ-16 pulse sequence was employed for broad-band proton decoupling. The measurements were carried out in an NMR tube with an external diameter of 10 mm containing a coaxial cylindrical capillary (diameter 2 mm) filled with hexafluorobenzene. ^{2}H NMR signals were accumulated at delay times between pulses of at least $5T_1$ (4—6 s). The acquisition (AQ) time of the free induction signal was at least $4T_1$. The number of scans was chosen in such a way to achieve an optimum signal-to-noise ratio (the overall duration of the experiments on acquiring the ^{2}H NMR spectra was 12 h and more). All measurements were carried out at 30 °C. Integral intensities of signals were determined by an iteration analysis of the total line shape taking into account the residual field inhomogeneity and phase distortions using the INTSPECT2 program.[1] The linewidth in the spectra of carene, limonene, and pinene was 0.2—0.7 Hz. The width of the resonance signals of oxygen-containing monoterpenes was 0.7—1.5 Hz. For a partial overlap of lines, the integration of the spectra enabled us to achieve an accuracy not worse than 5% in all cases. In the spectrum of menthol, the linewidth reached 5 Hz; the lines overlapped strongly and, therefore, the deuterium content was reliably determined only for some fragments.

Spectral lines of deuterons of monoterpenes were assigned by the ^{1}H NMR spectra using differential NOE spectroscopy, 2D COSY ^{1}H—^{1}H and ^{1}H—^{13}C correlations, and the INADEQUATE method. ^{1}H (300.13 MHz) and ^{13}C (75.43 MHz) NMR spectra were measured on a Bruker DPX-300 spectrometer in 5-mm tubes using a standard broadband sensor.

Results and Discussion

The results of measuring integral intensities of the resonance lines in the ^{2}H NMR spectra of monoterpenes are presented in Tables 1—6. Borneol, Δ^3-carene, menthol, and verbenone were studied for the first time. Available literature data are given for camphors, limonenes, and α-pinenes. For convenience of comparison, the integral intensities of lines of deuterons of the Me groups were recalculated per one H atom. For all substances except menthol, the integral values (***I***) corresponding to

Table 1. Chemical shifts (δ) in the ^{2}H NMR spectrum of Δ^3-carene **1** and the relative intramolecular content (***I***) of deuterium normalized to C(8)

Atom	δ	***I***	Atom	δ	***I***
1_{exo}	1.95	1.39	5	0.70	1.42
1_{endo}	2.34	1.53	6	0.61	1.30
2	5.25	0.25	8*	1.59	1.00
4_{exo}	1.81	0.92	9*	1.04	0.99
4_{endo}	2.15	1.27	10*	0.82	0.77

* The ***I*** values are recalculated per one hydrogen atom.

Table 2. Chemical shifts (δ) in the ^{2}H NMR spectrum of borneol **2** and camphor **3** and the relative intramolecular content (***I***) of deuterium normalized to C(8)

Atom	Borneol		Camphor			
	δ	***I***	δ	***I***		
				3a	**3b**	**3c** [7]
1_{exo}	2.19	1.27	2.01	1.16	1.50	1.11
1_{endo}	1.02	1.30	1.51	1.20	1.50	1.17
2_{exo}	3.91	0.25	—	—	—	—
4_{exo}	2.13	0.85	1.41	0.76	0.98	0.99
4_{endo}	1.20	0.76	1.11	*	**	—
5_{exo}	1.69	1.19	1.71	1.30	1.10	1.09
5_{endo}	1.29	1.21	1.11	*	**	—
6	1.53	0.80	1.84	0.84	0.95	0.75
8***	0.86	1.00	0.61	1.00	1.00	1.00
9***	0.81	0.96	0.73	0.87	0.96	0.87
10***	0.78	0.82	0.58	0.70	0.86	0.82

* Deuterons 4_{endo} and 5_{endo} in camphor are equivalent, and the total ***I*** value is 2.35.
** The total ***I*** value is 2.14.
*** The ***I*** values are recalculated per one hydrogen atom.

Table 3. Chemical shifts (δ) in the ^{2}H NMR spectrum of limonenes **4** and the relative intramolecular content (***I***) of deuterium normalized to C(8)

Atom*	δ	***I***	
		4a	**4b** [13]
$1_{ax'}$	1.99	1.34	—
$1_{eq'}$	1.83	1.42	—
2	5.35	0.42	0.40
$4_{ax'}$	1.86	1.02	—
$4_{eq'}$	1.96	0.77	—
$5_{ax'}$	1.42	1.34	—
$5_{eq'}$	1.72	1.42	—
6	2.01	0.76	—
8**	1.57	1.00	1.00
9**	4.67	1.30	1.31
10**	1.65	0.83	0.75

* Hereinafter designations of atoms: ax′ is pseudo-axial, and eq′ is pseudo-equatorial.
** The ***I*** values are recalculated per one hydrogen atom.

Table 4. Chemical shifts (δ) in the ^{2}H NMR spectrum of carvone **5** and the relative intramolecular content (***I***) of deuterium normalized to C(8)

Atom	δ	***I***	
		5a	**5b**
$1_{ax'}$, $5_{ax'}$	2.30	3.16*	3.00*
$1_{eq'}$, $5_{eq'}$	2.16	3.50*	3.20*
2	6.67	0.31	1.32
6	2.51	1.14	0.85
8**	1.63	1.00	1.00
9**	4.86	1.58	1.46
10**	1.59	1.25	1.18

* The total ***I*** value for two isochronic atoms.
** The ***I*** values are recalculated per one hydrogen atom.

Table 5. Chemical shifts (δ) in the ^{2}H NMR spectrum of menthol **6** and the relative intramolecular content (***I***) of deuterium normalized relatively to the average value

Atom	δ	***I***
1	3.30	0.40
2_{ax}	1.96	0.42
3	1.25	0.52
4_{eq}, 5_{ax}	1.54	3.02*
7	2.32	1.63

* The total ***I*** value for two isochronic atoms.

Table 6. Chemical shifts (δ) in the ^{2}H NMR spectra of α-pinene **7** and verbenone **8** and the relative intramolecular content (***I***) of deuterium normalized to C(8)

Atom	α-Pinene						Verbenone	
	δ	***I***					δ	***I***
		7a	**7b**	**7c** [11]	**7d** [11]	**7e** [12]		
1_{exo}	1.41	1.26	1.25	1.29	1.27	—	2.55	1.24
1_{endo}	2.56	1.30	1.28	1.46	1.24	—	1.72	1.31
2	2.15	0.26	0.20	0.28	0.32	—	2.51	0.23
4	5.44	1.01	1.05	1.06	0.71	—	5.39	1.55
5_{exo}	2.45	1.38	1.32	1.57	1.26	—	—	—
5_{endo}	2.39	1.39	1.32	1.39	1.39	—	—	—
6	2.29	1.17	1.16	1.18	1.21	—	2.57	1.24
8*	1.87	1.00	1.00	1.00	1.00	1.00	1.76	1.00
9*	1.10	0.96	0.91	1.04	0.92	0.91	0.74	0.96
10*	1.51	0.74	0.80	0.84	0.75	—	1.25	0.72

* The ***I*** values are recalculated per one hydrogen atom.

the deuterium content were normalized relatively to the internal reference, 8-Me group, which is not involved potentially in processes associated with the formation and cleavage of C—H bonds during biosynthesis.

As already mentioned in literature, the deuterium distribution in the compounds under study is nonuniform, which is well illustrated by the spectrum of Δ^3-carene **1** (Fig. 2). The spectrum contains an especially pronounced low-intensity signal of the olefinic hydrogen at the C(2) atom (***I*** = 0.25), whereas the positions at C(1) (***I*** = 1.39 and 1.50) and C(5) (***I*** = 1.42) are substantially enriched in deuterium. The maximum difference in deuterium content in molecule **1** reaches a factor of 6. Similar variations in the deuterium distribution are observed for compounds **2—8**, and they are clearly presented in the diagram (Fig. 3). Although considerable differences in

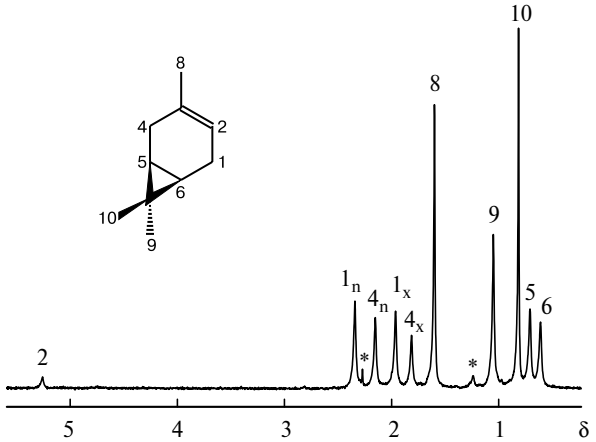


Fig. 2. ^{2}H NMR spectrum (46.05 MHz) at the natural abundance level of deuterium in Δ^3-carene **1** (x is *exo*, n is *endo*, symbol "*" denotes signals from admixtures).

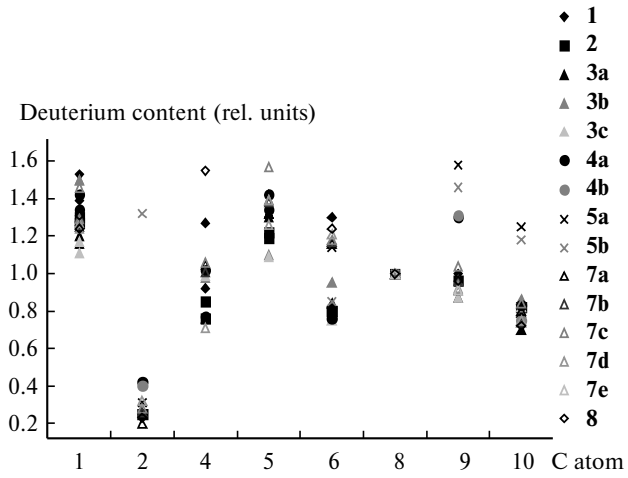


Fig. 3. Diagram of intramolecular deuterium distribution in monoterpenoids **1—8**.

"H/D-isotope portraits" are observed for all compounds, their similarity is also evident. All of them, except (*R*)-carvone, have properties characteristic of terpenes formed *via* the DXP pathway. This is rather easily seen when compared the patterns of ^{2}H isotope distribution presented in Figs 1 and 3. It is noteworthy that the deuterium contents in positions at the C(2) and C(6) atoms are different. During biosynthesis, these C atoms originate from different precursors: C(2) comes from IPP, and DMAPP gives rise for C(6). Thus, the ^{2}H NMR spectra additionally confirm that IPP and DMAPP are products of different biochemical processes and IPP does not isomerize to DMAPP. This conclusion agrees with the results obtained[23] for cineole biosynthesis using isotope-labeled DXP.

A rather high dispersion is observed in the contents of the heavy hydrogen isotope in related fragments of the studied compounds. In particular, for the position at the C(6) atom, the ***I*** values change from 0.75 to 1.30. In camphor, borneol, and limonene, this position is relatively depleted in deuterium, while it is enriched, on the contrary, in carene, pinene, (*S*)-carvone, and verbenone. At the same time, it should be mentioned that a tendency assumed for the DXP pathway obeys for the intramolecular deuterium distribution in monoterpenes. The deuterium content at the C(6) atom in terpene molecule is lower than that in the position at the C(1) and C(5) atoms, which are terminal in molecules of their precursors, IPP and DMAPP, respectively.

Taking into account available data, we cannot explain unambiguously the "isotope portrait" of (*R*)-carvone (Aldrich) in which the C(2) fragment is strongly enriched in deuterium (***I*** = 1.32) compared to other compounds **1—8**. For other fragments of this terpene, the deuterium distribution corresponds, as a whole, to the general pattern, *i.e.*, it has properties of the DXP path-

ways of biosynthesis. (*R*)-Carvones are usually isolated from the mint oil.[25,26] Therefore, it is of doubtless interest to study the isotope composition of other terpenes obtained from the same natural source.

For the Me groups in the series of bicyclic compounds **1—3**, **7**, and **8** of different origin, a relatively low dispersion of ***I*** values is observed. The range of changing the deuterium content in the 9-Me and 10-Me groups (for 8-Me, $I = 1$) does not exceed 17%. In this case, the situation when one of these groups has a lower isotope content is retained. This fact agrees rather well with the hypothetical "H/D-isotope portrait" for the DXP pathway (see Fig. 1). The different selective isotope distribution in this fragment, whose precursor is DMAPP, is typical of monocyclic limonenes and carvones. The formation of an isotope composition in the unsaturated fragment at the C(9) atom, as already mentioned previously,[16] is determined, to a great extent, by proton elimination on going from the α-terpenyl cation to limonene (see Scheme 3). The relative deuterium enrichment at the C(9) atom exceeds 30 and 50% in limonene and carvone, respectively. According to the earlier published results,[3] the kinetic isotope effects (k_H/k_D) can be determined from these data. For limonenes **4b** and **4a**, k_H/k_D are 3.35 [16] and 2.28, and those for carvones **5a** and **5b** are 1.88 and 1.35, respectively. It is noteworthy that the deuterium content is noticeably increased in the 10-Me group of carvone.

Terpene functionalization, if not related to a great extent to dramatic rearrangements of the carbon framework, does not substantially change the "H/D-isotope portrait" inherent in terpenes. The observed changes make it possible to monitor the character of structural transformations. In particular, the formal scheme of limonene transformation into menthol[11] includes its allyl oxidation at the C(1) position to form isopiperitenone, the reduction of the cyclic and terminal double bonds, and then keto group reduction. These processes are pronounced in a change in the isotope content in the C(1)—C(3) fragment of menthol. The addition of hydrogen atoms at the final steps of menthol formation is accompanied by the substantial depletion in deuterium of this fragment (see Table 5). Our estimation of the isotope effect k_H/k_D of reduction in the position at the C(3) atom is ~1.5.

Available data indicate that the "H/D-isotope portraits" of natural enantiomeric terpenes and terpenoids differ substantially. As shown earlier,[14] the isotope characteristics of enantiomeric α-pinenes (**7c** and **7d**) obtained from the same source differ. The considerable difference in ***I*** is observed for the positions at the C(1), C(4), and C(5) atoms, being 30% for the two latter. In the (1*R*)-enantiomer, these positions are enriched in deuterium compared to the (1*S*)-enantiomer (see Table 6). The "H/D-isotope portrait" of a mixture of enantiomers reflects its composition, which makes it possible to determine the optical purity of terpenes by their isotope distribution. It is of interest that the ***I*** values for mixture **7a** containing 68 and 32% of the (1*R*)- and (1*S*)-enantiomers, respectively, and racemic mixture **7b**, which are of different origin, lie within intervals for pure enantiomers **7c** and **7d**. The camphor enantiomers exhibit a strong difference in deuterium content in (1*S*)-enantiomer **3a** and in racemic mixture **3b** in the positions at the C(1) (34%) and C(6) (15%) atoms (see Table 2). However, data on the deuterium content for (1*R*)-camphor **3c** of different natural origin[7] cannot be predicted from the ***I*** values for **3a** and **3b**. It is most likely that this fact indicates possible differences in deuterium distribution in molecules of the same enantiomers originating from different plant sources. Unfortunately, rigid conclusions on this problem are presently impossible, because the selection of the compounds under study is not representative.

Thus, the results obtained in our work demonstrate a principal possibility to use NMR spectroscopy at the natural abundance level of the ^2H isotope for discrimination of biosynthetic pathways of monoterpenes. The results of measurements of the relative intramolecular distribution of the deuterium content for these substances and their comparison with the qualitative "H/D-isotope portraits" obtained by the analysis of the metabolism pathways confirm the conclusion that the biosynthesis of the studied monoterpenes proceeds in plants *via* the DXP pathway rather than by the classical scheme through mevalonic acid. Our data indicate that two C_5-isoprenic precursors of monoterpenes, *viz.*, isopentyl diphosphate IPP and dimethylallyl diphosphate DMAPP, are formed in plants due to different biochemical processes without their mutual isomerization. The extension of the scope of studied substances from different natural sources will provide new interesting results.

This work was financially supported by the Russian Foundation for Basic Research (Project No. 02-03-33030).

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Received December 24, 2004;
in revised form April 21, 2005