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Phytochemistry 65 (2004) 2463–2470
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Biosynthesis of the irregular monoterpene artemisia ketone, the sesquiterpene germacrene D and other isoprenoids in *Tanacetum vulgare* L. (Asteraceae)

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Received 19 March 2004; received in revised form 22 June 2004

Abstract

The incorporation of [1-^{13}C]-labeled glucose into the irregular monoterpene artemisia ketone, the regular monoterpenes camphor and β-thujone, the sesquiterpene germacrene D, the diterpene *trans*-phytol and β-sitosterol and isofucosterol has been studied in axenic cultures of *Tanacetum vulgare* L. (Asteraceae).

Quantitative ^{13}C NMR spectroscopic analysis of the resulting labeling patterns showed that the isoprene units of the monoterpenes and the diterpene are formed via the methylerythritol phosphate (MEP) pathway, whereas the isoprene building blocks of the sesquiterpene and the sterols originate from the mevalonic acid (MVA) pathway.

Keywords: *Tanacetum vulgare*; Compositae; Methylerythritol phosphate pathway; Mevalonic acid pathway; Artemisia ketone; Camphor; β-Thujone; Germacrene D; *trans*-Phytol; β-sitosterol; Quantitative ^{13}C NMR spectroscopy

1. Introduction

Isoprenoids are the largest group of secondary metabolites with over 30,000 known compounds, including the steroids (Dictionary of Natural Products, 2000). Their common building block, the isoprene unit, is derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The biosynthesis of IPP can proceed via two different pathways, the long known mevalonic acid (MVA) pathway and the methylerythritol (MEP) pathway whose reaction sequence has been characterized in recent years (Rohmer et al., 1993; Sprenger et al., 1997; Lange et al., 1998; Lois et al., 1998; Takahashi et al., 1998; Herz et al., 2000; Lüttgen et al., 2000; Tagaki et al., 2000; Rohdich et al., 1999, 2002; Kollas et al., 2002).

In plants, the alternative MEP pathway appears to be generally involved in the formation of hemiterpenoids, monoterpenoids, diterpenoids including *trans*-phytol and carotenoids (for review, see Rohmer, 1999; Kuzuama and Seto, 2003). Sesquiterpene biosynthesis seems to be more complex since a formation via either pathway (MVA: e.g., Thiel et al., 1997; Adam et al., 1998; MEP: Steliopoulos et al., 2002) or a combination of both (Adam and Zapp, 1998; Piel et al., 1998) has been reported. However, despite a significant amount of recent research in this area (for review, see Rohmer, 1999; Kuzuama and Seto, 2003), our knowledge of the contribution of both pathways to the biosynthesis of the different plant isoprenoid classes is still limited.

In continuation of our previous work on IPP biosynthesis in plant isoprenoids (Thiel et al., 1997; Adam

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doi:10.1016/j.phytochem.2004.08.019

et al., 1998, 1999; Adam and Zapp, 1998; Barlow et al., 2001; Hertewich et al., 2001; Thiel and Adam, 2002), we investigated the biosynthetic origin of the isoprene units of the *irregular* monoterpene artemisia ketone (**1**) in common tansy (*Tanacetum vulgare*, Asteraceae). Since artemisia ketone (**1**) does not follow the "isoprene rule" with the typical head to tail linkage between its two isoprene units (Fig. 1) it is an interesting study topic for expanding the knowledge on the biosynthetic origin of isoprene units in plant isoprenoids. A second objective of this study was to investigate the biosynthesis of the sesquiterpene germacrene D (**4**) in another Asteraceae, since recently germacrene D (**4**) was found to be formed via the MEP pathway in the Asteraceae *Solidago canadensis* (Steliopoulos et al., 2002).

The formation of isoprene units via either pathway (MVA or MEP) can be determined by ^{13}C NMR spectroscopy on the basis of different ^{13}C labeling patterns after incorporation of [1-^{13}C]glucose into isoprenoids (Fig. 2) (Rohmer et al., 1993; Schwarz, 1994; Schwender et al., 1996). This methodology also allows the detection of a mixed biosynthesis with a contribution from both pathways (Adam and Zapp, 1998).

For the ^{13}C labeling study of artemisia ketone (**1**) we selected a chemotype of *T. vulgare*, containing artemisia ketone (**1**) as the major constituent of the essential oil fraction (Scheu, 1966; Forsén and von Schantz, 1971; Tétényi et al., 1975) along with the regular monoterpenes camphor (**2**), β-thujone (**3**) and the sesquiterpene germacrene D (**4**). For comparison we also investigated the biosynthesis of the plastidial diterpene *trans*-phytol (**5**) and the phytosterols β-sitosterol (**6**) and isofucosterol (**7**).

2. Results and discussion

T. vulgare seedlings were grown aseptically on culture medium containing two different [1-^{13}C]glucose concentrations. After six and eight weeks of growth, the plant material was harvested and compounds were isolated using standard chromatographic procedures. The first set of plants (experiment I) yielded artemisia ketone (**1**), camphor (**2**) and *trans*-phytol (**5**) and from the second set (experiment II), we isolated β-thujone (**3**), germacrene D (**4**), β-sitosterol (**6**) and isofucosterol (**7**). Assignments of the ^{13}C NMR signals of the purified compounds were obtained from the literature (Héthelyi et al., 1981; (**1**); Bohlmann et al., 1975; (**2**) Rees and Whittaker, 1981 (**3**); Steliopoulos et al., 2002 (**4**), Arigoni et al., 1997 (**5**, **6**), McInnes et al., 1980 (**7**)) and confirmed by 2D NMR-experiments. Labeling patterns and absolute ^{13}C enrichments of the compounds were determined by quantitative ^{13}C NMR spectroscopy using Cr(III)–acetyl acetonate as a relaxation reagent for the carbon nuclei (Braun et al., 1996). The absolute enrichments were calculated from the integrals of ^{13}C NMR signals on the basis of ^{13}C satellite analysis of well-separated proton signals in the respective ^{1}H NMR spectra.

As expected, the ^{13}C incorporation was lower in the plants that were raised on culture medium with the lower [1-^{13}C]glucose concentration and the longer incubation time.

The quantitative ^{13}C NMR analysis of artemisia ketone (**1**) showed an enrichment of four carbon atoms with an average^{13}C abundance of 9.19 ± 0.34% in incubation experiment I and 5.63 ± 0.10% in experiment II (Tables 1 and 7). The labeling positions in both isoprene

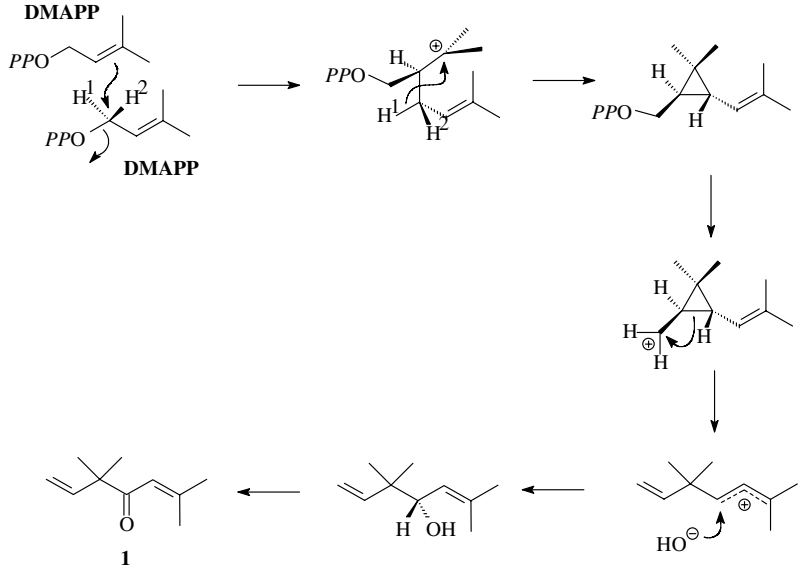

Fig. 1. Proposed biosynthesis of the irregular monoterpene artemisia ketone (**1**) (Seigler, 1995).

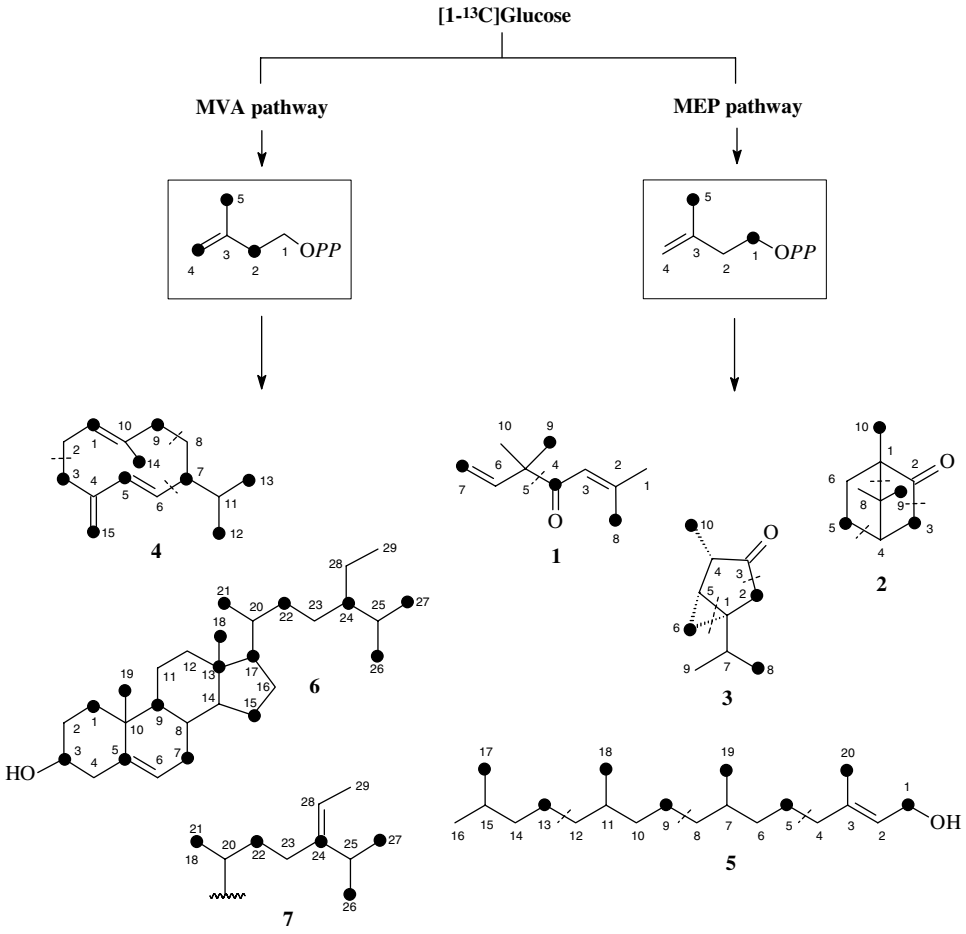

Fig. 2. Incorporation of [1-^{13}C]glucose into IPP via MVA pathway and MEP pathway and the resulting labeling patterns of compounds **1, 2, 3, 4, 5, 6** and **7**. PP, diphosphate residue, ●, ^{13}C labeled position.

Table 1
^{13}C abundances of labeled artemisia ketone (**1**)

Carbon atom in IPP-units	Carbon atom	δ_C (ppm)	% ^{13}C[a] (Experiment I)	% ^{13}C[a] (Experiment II)
1^{I}	1	20.4	**9.10**	**5.66**
2^{I}	2	155.1	7.26	4.47
3^{I}	3	120.0	5.61	3.68
4^{I}	8	27.5	6.58	3.85
5^{I}	4	200.8	**9.68**	**5.66**
1^{II}	9(10)	21.8	**8.96**[b]	**5.72**[b]
2^{II}	5	49.8	7.16	4.36
3^{II}	6	142.7	5.52	3.96
4^{II}	10(9)	21.8	6.22[b]	4.13[b]
5^{II}	7	113.2	**9.00**	**5.49**

Bold type: labeled positions.
I, II: denote individual C_5 units.
[a] ^{13}C abundances obtained from ^{13}C satellite analysis of H-1.
[b] Averaged values due to overlapping signals.

units correspond to the MEP pathway (Fig. 2). The remaining carbon atoms also displayed an increased ^{13}C abundance (6.39 ± 0.75%) compared to the natural abundance of 1.11% (Tables 1 and 7). This general background labeling might be explained by the complex metabolic turnover of [1-^{13}C]glucose during the growth period with the subsequent statistical distribution of the labeled carbon atoms in various positions of intermediates of the carbohydrate metabolism (e.g., metabolism of glucose to carbon dioxide and subsequent

assimilation via the Calvin cycle). The overall strong ^{13}C labeling is due to the long duration of the labeling experiments and the relatively high concentration of [1-^{13}C]glucose in the medium.

Camphor (**2**) showed also an enrichment pattern typical of the alternative MEP pathway, with an average ^{13}C abundance of 8.93 ± 0.17% (experiment I) and 5.75 ± 0.15% (experiment II) (Fig. 2, Tables 2 and 7). Again, a ^{13}C enrichment (6.32 ± 0.31% and 4.25 ± 0.20%) of the unlabeled positions could be observed as already discussed above (Tables 2 and 7).

β-Thujone (**3**), also displayed the enrichment pattern of the alternative MEP pathway, with an average ^{13}C abundance of 5.64 ± 0.09% (Fig. 2, Tables 3 and 7). As in the other monoterpenes a ^{13}C enrichment (4.32 ± 0.08%) of the unlabeled positions was also found (Tables 3 and 7).

In case of the sesquiterpene germacrene D (**4**), nine carbon atoms were labeled with an average ^{13}C abundance of 5.67 ± 0.14%, indicating the MVA origin of the isoprene units (Fig. 2, Tables 4 and 7). Furthermore, a significant enrichment of the remaining carbons was also found (average absolute ^{13}C abundance of 4.32 ± 0.23%).

The quantitative ^{13}C NMR analysis of β-sitosterol (**6**) and isofucosterol (**7**) showed an enrichment pattern typical of the mevalonic acid pathway. The isolated compound also showed a ^{13}C enrichment of the unlabeled positions as already discussed above for the monoterpenes (Fig. 2, Tables 6 and 7).

Remarkably, the C-24 C_2 side chain (carbons 28 and 29) of both sterols did not display an increased ^{13}C enrichment as is usually found in [1-^{13}C]glucose incorporation experiments (Arigoni et al., 1997; Adam et al., 1998; Barlow et al., 2001). Carbons 28 and 29 originate from S-adenosyl methionine and are added to the sterol precursor through subsequent methylation (Benveniste, 2002). Since glyceraldehyde (via serine) is the precursor of the *S*-adenosyl methionine *S*-methyl group, the lack of the methyl labels indicates that *S*-adenosyl methionine originates predominantly from photosynthethically de novo formed glyceraldehyde rather than [1-^{13}C]glucose metabolism.

Trans-phytol (**3**) was obtained from chlorophyll through hydrolytic cleavage. Its labeling pattern proves the formation of its isoprene units via the MEP pathway (average abundance of ^{13}C: 9.37 ± 0.12%, Fig. 2, Tables 5 and 7). Again, an increased ^{13}C background labeling could also be found (6.92 ± 0.42%, Tables 5 and 7).

The analysis of monoterpenes, sesquiterpenes, diterpenes and sterols of *T. vulgare* representing the basic isoprenoid building blocks C_{10}, C_{15} (C_{30}) and C_{20} gives a broad overview of the biosynthetic origin of isoprenoids in a single organism. The labeling patterns show the formation of the monoterpenes and the plastidial diterpene *trans*-phytol via the MEP pathway. Average ^{13}C abundances of labeled and background-labeled positions of the irregular artemisia ketone appear to be very similar to the other three regular monoterpenes. Therefore, despite the different linkage of the isoprene units all *T. vulgare* monoterpenes seem to originate from the same IPP/DMAPP pool.

The sesquiterpene germacrene D (**4**) and the two sterols in turn are derived from the MVA pathway. A significant mixing of both IPP/DMAPP biosynthesis pathways in any of the compounds could not be deduced from the data: a distinctive labeling pattern of the individual isoprene units indicative of a MVA pathway contribution could not be detected. However, a smaller incorporation (≈ up to 20%) of a second IPP/DMAPP species in an isoprene unit could not be excluded, since the respective labeling could have been masked by the variation of the ^{13}C abundance data.

The origin of the sesquiterpene germacrene D (**4**) from the MVA pathway is quite remarkable. In a similar

Table 2
^{13}C Abundances of labeled camphor (**2**)

Carbon atom in IPP-units	Carbon atom	δ_C (ppm)	% ^{13}C[a] (Experiment I)	% ^{13}C[b] (Experiment II)
1^I	5	26.5	**9.02**	**5.91**
2^I	4	42.5	6.18[c]	4.11[c]
3^I	7	46.2	6.54	4.49
4^I	9	19.2	5.93	4.03
5^I	8	18.6	**8.84**	**5.66**
1^{II}	3	42.7	**8.74**[c]	**5.58**[c]
2^{II}	2	218.8	6.19	4.19
3^{II}	1	57.06	6.81	4.49
4^{II}	6	29.4	6.28	4.19
5^{II}	10	8.7	**9.10**	**5.83**

Bold type: labeled positions.
I, II: denote individual C_5 units.
[a] ^{13}C abundances obtained from ^{13}C satellite analysis of H-10.
[b] ^{13}C abundances obtained from ^{13}C satellite analysis of H-9.
[c] Averaged values due to signal overlapping.

Table 3
^{13}C Abundances of labeled β-thujone (**3**) from experiment II

Carbon atom in IPP-units	Carbon atom	δ_C (ppm)	% ^{13}C[a]
1^I	2	40.9	**5.57**
2^I	1	26.6	4.20
3^I	7	31.8	4.33
4^I	9	18.9	4.38[b]
5^I	8	19.0	**5.75**[b]
1^{II}	6	13.9	**5.57**
2^{II}	5	23.8	4.29
3^{II}	4	44.6	4.29
4^{II}	3	217.5	4.42
5^{II}	10	11.7	**5.66**

Bold type: labeled positions.
I, II: denote individual C_5 units.
[a] ^{13}C abundances obtained from ^{13}C satellite analysis of H-6a.
[b] Averaged values due to overlapping signals.

Table 4
^{13}C Abundances of labeled germacrene D (**3**) from experiment II

Carbon atom in IPP-units	Carbon atom	δ_C (ppm)	% ^{13}C[a]
1^I	6	132.6	4.19
2^I	5	134.6	**5.66**
3^I	4	147.9	4.07
4^I	3	33.6	**5.70**
5^I	15	108.2	**5.46**
1^{II}	2	28.4	4.19
2^{II}	1	128.7	5.54
3^{II}	10	133.0	4.28
4^{II}	9	39.8	**5.50**
5^{II}	14	15.0	**5.74**
1^{III}	8	25.6	4.64
2^{III}	7	52.0	5.78
3^{III}	11	31.8	4.56
4^{III}	12	18.5	**5.78**
5^{III}	13	19.9	**5.86**

Bold type: labeled positions.
I, II: denote individual C_5 units.
[a] ^{13}C abundances obtained from ^{13}C satellite analysis of H-5.

[1-^{13}C]glucose incorporation study with the Asteraceae *Soligago canadensis* it has been demonstrated that germacrene D is formed predominantly via the MEP pathway (Steliopoulos et al., 2002). However, the *T. volgare* results are in line with the properties of a germacrene D synthase from *Populus trichocarpa* that has been recently characterized as a typical cytosolic enzyme, which matches with the localization of the MVA pathway (Arimura et al., 2004). The fact that also a mixed sesquiterpene biosynthesis with contribution of both pathways has been found in the Asteracee *Matricaria recutita* (nonequivalent labeling with approx. 50% of both pathways in one isoprene unit) (Adam et al., 1999), demonstrates that even within the same plant family sesquiterpene biosynthesis appears to be more variable than mono-, di-terpene and sterol biosynthesis. So far, the IPP/DMAPP biosynthesis of only a few plant species has been analyzed. For a detailed knowledge

Table 5
^{13}C Abundances of labeled *trans*-phytol (**3**) from experiment I

Carbon atom in IPP-units	Carbon atom	δ_C (ppm)	% ^{13}C[a]
1^I	13	25.1	**9.40**[d]
2^I	14	39.9	6.83
3^I	15	28.8	6.73
4^I	16	23.0	6.61[e]
5^I	17	23.1	**9.30**[e]
1^{II}	9	24.8	**9.40**[d]
2^{II}	10	37.8	7.37[b]
3^{II}	11	33.1	7.37[c]
4^{II}	12	37.6	7.37[b]
5^{II}	18	20.1	**9.42**[f]
1^{III}	5	25.5	**9.40**[d]
2^{III}	6	37.1	6.73
3^{III}	7	33.2	7.37[c]
4^{III}	8	37.7	7.37[b]
5^{III}	19	20.1	**9.42**[f]
1^{III}	1	59.8	**9.10**
2^{IV}	2	123.4	6.41
3^{IV}	3	140.7	6.28
4^{IV}	4	40.2	6.64
5^{IV}	20	16.5	**9.47**

Bold type: labeled positions.
I, II, III, IV: denote individual C_5 units.
[a] ^{13}C abundances obtained from ^{13}C satellite analysis of H-1.
[b–f] Averaged values due to overlapping signals.

about the quantitative role of both IPP/DMAPP biosynthesis pathways in the formation of the different isoprenoid classes a larger selection of plants needs to be investigated. From such studies, including incorporation experiments with pathway specific precursors and the investigation of key enzymes (prenyltranferases, cyclases) new insights into transport phenomena of intermediates and subcellular localization of plant isoprenoid biosynthesis should be expected.

3. Experimental

Spectroscopy and Spectrometry. NMR spectra were recorded in $CDCl_3$ [^1H NMR (500 MHz), and ^{13}C NMR (125 MHz) relative to $CDCl_3$ at δ_H 7.26; δ_C 77.36.] ^{13}C multiplicities were determined using the DEPT pulse sequence. 2D Spectra were recorded as COSY, HSQC and HMBC experiments. Quantitative ^{13}C NMR measurements were recorded with the inverse gated decoupling pulse sequence in the presence of 0.1 M $Cr(acac)_3$ (Braun et al., 1996). For integration, the signal-to-noise ratio of the ^{13}C-signals was at least 40:1.

Reagents. [1-^{13}C]-glucose was purchased from Deutero (Herresbach, Germany). All other chemicals were obtained from Sigma–Aldrich.

Plant material, screening and labeling procedure. Seeds were collected from an artemisia ketone (**1**) forming *T. vulgare* plant at a natural site near Nunkirchen, Saarland, Germany.

Table 6
^{13}C abundances of labeled β-sitosterol (**5**) and isofucosterol (**6**) from experiment II

Carbon atom in IPP-units	Carbon atom	β-Sitosterol (**5**)		Isofucosterol (**6**)	
		δ_C (ppm)	% ^{13}C[a]	δ_C (ppm)	% ^{13}C[a]
1^{I}	2	31.0	4.07[d]	31.0	3.95[d]
2^{I}	3	71.0	**5.19**	71.1	**5.10**
3^{I}	4	41.6	4.12[b]	41.7	3.95[b]
1^{II}	6	120.8	3.85	120.8	3.85
2^{II}	5	139.9	**5.63**	140.0	**5.15**
3^{II}	10	35.6	3.96[c]	35.6	3.95[c]
4^{II}	1	36.4	**4.91**	36.5	**4.91**
5^{II}	19	18.6	**5.08**[e]	18.6	**4.86**
1^{III}	11	20.2	4.07	20.2	3.76[f]
2^{III}	9	49.2	**5.35**	49.2	**4.86**
3^{III}	8	31.0	4.07[d]	31.0	3.95[d]
4^{III}	7	31.0	**5.36**[d]	31.0	**5.18**[d]
1^{IV}	12	38.9	3.85	38.9	3.90
2^{IV}	13	41.4	**5.41**[b]	41.4	**5.18**[b]
3^{IV}	14	55.9	4.01	55.9	3.75
4^{IV}	15	23.4	**4.96**	23.5	**5.10**
5^{IV}	18	11.0	**5.19**[f]	11.0	**4.91**
1^{V}	16	27.4	3.79	27.4	3.90[e]
2^{V}	17	55.2	**5.02**	55.1	**4.91**
3^{V}	20	35.2	3.96[c]	35.3	3.95[e]
4^{V}	22	33.0	**5.07**	35.1	**5.18**[e]
5^{V}	21	17.9	**5.08**[e]	18.0	**5.01**
1^{VI}	23	25.2	3.90	27.0	3.90[e]
2^{VI}	24	44.9	**5.30**	145.0	**4.76**
3^{VI}	25	28.3	3.74	27.7	3.90[e]
4^{VI}	26	19.0	**5.08**[e]	20.2	**4.94**[f]
5^{VI}	27	18.2	**5.08**[e]	20.2	4.94[f]
[g]	28	22.2	4.01	115.6	3.66
[g]	29	11.2	3.96[f]	12.0	3.66

Bold type: labeled positions.
I, II, III, IV: denote individual C_5 units.
[a] ^{13}C abundances obtained from ^{13}C satellite analysis of H-6.
[b–f] Averaged values due to overlapping signals.
[g] Non-isoprenoid origin.

After surface disinfection in 4% NaOCl solution, containing 2% Tween 80, seeds were rinsed with sterile water and transferred to 100 ml culture tubes. Seedlings were grown aseptically under continuous incandescent light (60 μmol photons m^{-2} s^{-1} photosynthetically active radiation) at 22 °C on 25 ml of modified Gamborg B5 medium (Gamborg et al., 1968) solidified with 0.9% agar and containing 10 g/l glucose.

For the administration experiment I, seedlings were screened for artemisia ketone formation by analyzing small explants of one-week old seedlings. Each explant was extracted with 1 ml of ethyl acetate and the solution

Table 7
Comparison of ^{13}C enrichments in isoprenoids from *T. vulgare* (% $^{13}C \pm SD$)

Compound	% ^{13}C labeled carbons	% ^{13}C background-labeled carbons	Ratio labeled/background
Experiment I			
Artemisia ketone (**1**)	9.19 ± 0.34	6.39 ± 0.75	1.44
Camphor (**2**)	8.93 ± 0.17	6.32 ± 0.31	1.41
trans-Phytol (**5**)	9.37 ± 0.12	6.92 ± 0.42	1.35
Experiment II			
Artemisia ketone (**1**)	5.63 ± 0.10	4.08 ± 0.30	1.38
Camphor (**2**)	5.75 ± 0.15	4.25 ± 0.20	1.35
β-Thujone (**3**)	5.64 ± 0.09	4.32 ± 0.08	1.31
Germacrene D (**4**)	5.67 ± 0.14	4.32 ± 0.23	1.31
Sitosterol (**6**)	5.18 ± 0.20	3.95 ± 0.12	1.31
Isofocusterol (**7**)	5.00 ± 0.14	3.86 ± 0.11	1.30

analyzed by GC–MS (He at 1 ml min^{-1}), temp. programmed, 40 °C for 5 min, then at 5 °C min^{-1} to 150 °C, then 25 °C min^{-1} to 200 °C, injector at 250 °C, mass detector at 280 °C, HP-5 column (30 m × 0.25 mm). Artemisia ketone producing plantlets were transferred to fresh tubes with medium containing 1% [1-^{13}C]glucose. The cultures were grown for an additional six weeks. In the administration experiment II, seedlings were not screened and one week old seedlings were grown for eight weeks on medium containing 0.5% [1-^{13}C]glucose.

Extraction and isolation. Administration of [1-^{13}C]glucose in experiment I yielded C-13 enriched artemisia ketone (**1**), camphor (**2**) and *trans*-phytol (**5**). The larger adminestation of [1-^{13}C]glucose in experiment II allowed the isolation of C-13 enriched artemisia ketone (**1**), camphor (**2**), β-thujone (**3**), germacrene D (**4**), β-sitosterol (**6**) and isofucosterol (**7**).

The essential oil containing artemisia ketone (**1**), camphor (**2**), β-thujone (**3**) and germacrene D (**4**) was obtained by hydro distillation of ^{13}C labeled plant material (experiment I: 29.5 g; experiment II: 135 g (fresh weight)) for 50 min and was collected in 2 ml pentane. After removal of the solvent, the essential oil was separated by HPLC (250 × 4 mm, LiChrospher 100 Diol, 5 μm, Merck, pentane) yielding 7/10 mg (experiment I/II) of (**1**), 3/25 mg (experiment I/II) of (**2**), 6 mg of (**3**) and a mixture of (**4**) with monoterpene hydrocarbons. This mixture was further purified by HPLC (Nucleosil 100-5 SA, 5 μm, 200 × 4.0 mm, Machery–Nagel), Ag^+ coated (van Beek and Subrtova, 1995), MeCN–Et_2O (1:99), to yield 2 mg of (**4**).

The remaining plant material was dried, ground and extracted in a Soxhlet apparatus with CH_2Cl_2. After evaporation of the solvent, the crude extract of experiment I was fractionated by size exclusion chromatography on Sephadex LH-20. The chlorophyll containing fractions were combined and saponified overnight at room temperature with 6% (w/v) KOH in MeOH. Following hydrolysis, the mixture was extracted with CH_2Cl_2. After removal of the solvent, the mixture was separated by vacuum liquid chromatography on silica gel employing a *n*-hexane–EtOAc gradient and further purified (3.8% EtOAc fraction) by HPLC (250 × 4 mm, Si 60 Lichrospher, 5 μm, Merck, *n*-hexane-EtOAc 93:7) yielding 2 mg of *trans*-phytol (**3**).

The crude CH_2Cl_2 extract from experiment II was saponified overnight at room temperature with 6% (w/v) KOH in MeOH. After hydrolysis, the mixture was extracted with Et_2O. After removal of the solvent, the mixture was separated by vacuum liquid chromatography on silica gel employing a *n*-hexane–EtOAc gradient. The crude sterol fraction (11–12% EtOAc) was further purified by HPLC (250 × 4 mm, Aqua C-18, 5 μm, Phenomenex, MeOH–MeCN–isopropanol 2:2:1) yielding 5 mg of β-sitosterol (**6**) and 3 mg of isofucosterol (**7**).

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