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Biosynthesis of the sesquiterpene germacrene D in *Solidago canadensis*: ^{13}C and ^{2}H labeling studies

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Abstract

The biogenetic origin of the isoprenoid building blocks of the sesquiterpene germacrene D was studied in *Solidago canadensis*. Feeding experiments were carried out with 1-[5,5-D_2]deoxy-D-xylulose-5-phosphate (D_2-DOXP), [5-^{13}C]mevalonolactone (^{13}C-MVL) and [1-^{13}C]-D-glucose. The hydrodistillate of a cut shoot fed with D_2-DOXP was investigated by enantio-MDGC–MS and the volatile fraction of a shoot supplied with ^{13}C-MVL was examined by GC–C–IRMS. The incorporation of [1-^{13}C]-D-glucose was analyzed by quantitative ^{13}C NMR spectroscopy after isolation of germacrene D from the essential oil. Our labeling studies revealed that the biosynthesis of the C-15 skeleton of sesquiterpene germacrene D in *Solidago canadensis* proceeds predominantly via the methylerythritol phosphate pathway.

Keywords: *Solidago canadensis*; Asteraceae; Biosynthesis; Methylerythritol phosphate pathway; Sesquiterpenes; Germacrene D; Labeling studies

1. Introduction

The chiral hydrocarbon germacrene D is a commonly found plant constituent and is considered to be a key intermediate in the biosynthesis of many sesquiterpenes (Yoshihara et al., 1969; Bülow and König, 2000). Furthermore it is recognized as a sex stimulant of the male American cockroach (*Periplanata americana* L.) (Kitamura et al., 1976). (*S*)-(−)-germacrene D [Fig. 1, (**2**)] mainly occurs in higher plants, whereas the (*R*)-(+)-enantiomer (**1**) is mostly found in lower plants like liverworts (Bülow, 1998; König et al., 1996). As an exceptional case, the higher plant *Solidago canadensis* generates both optical antipodes of this compound by enzymatic cyclization of farnesyl diphosphate using two different enantiospecific synthases (Schmidt et al., 1998, 1999). The enantiomeric ratio of germacrene D in *Solidago canadensis* can vary from individual to individual (Bülow, 1998).

Farnesyl diphosphate is the common precursor of all sesquiterpenes. In plants, it is assembled from isoprene units that are biosynthesized generally via the mevalonate pathway; in contrast to mono- or diterpenes which are built up from isoprene units that are formed exclusively or at least predominantly via the methylerythritol phosphate route (Arigoni et al., 1997; Lichtenthaler et al., 1997a,b; Eisenreich et al., 1996; Adam et al., 1998; Eichinger et al., 1999). However, recent studies have demonstrated that in plants the methylerythritol phosphate pathway can also be involved in the formation of sesquiterpenes (Adam and Zapp, 1998; Adam et al., 1999). Obviously, biosynthesis of sesquiterpenes does not proceed uniformly in the plant kingdom.

In order to obtain further information on the occurrence of the methylerythritol phosphate pathway in sesquiterpenes biosynthesis we investigated the origin of the isoprenoid building blocks of the sesquiterpene germacrene D in *Solidago canadensis*. This paper reports on in vivo feeding experiments with intact plant material using the two pathway specific precursors 1-[5,5-D_2]deoxy-D-xylulose-5-phosphate (D_2-DOXP) and [5-^{13}C]mevalonolactone (^{13}C-MVL) and the non specific precursor [1-^{13}C]-D-glucose. Incorporation and analysis of labeling patterns by different techniques

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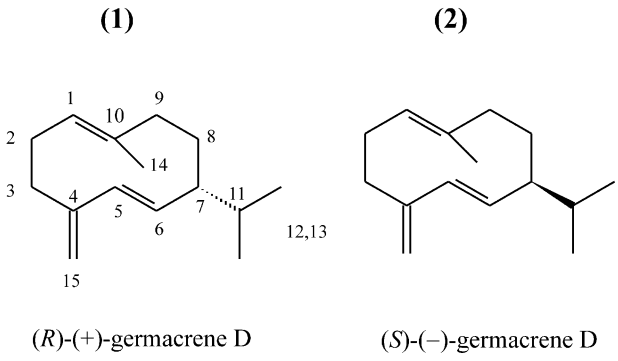


Fig. 1. Enantiomers of germacrene D.

(enantioselective multidimensional gas chromatography-mass spectrometry (enantio-MDGC–MS), gas chromatography-combustion-isotope ratio mass spectrometry (GC–C–IRMS), quantitative ^{13}C NMR spectroscopy) are described.

2. Results and discussion

Fig. 2 shows the hypothetical metabolic conversion of D_2-DOXP into D_6-farnesyl diphosphate via the methylerythritol phosphate pathway and the following cyclization to [2,2,6,7,8,8-D_6]-(*R*)- (**1a**) and [2,2,6,8,8,11-D_6]-

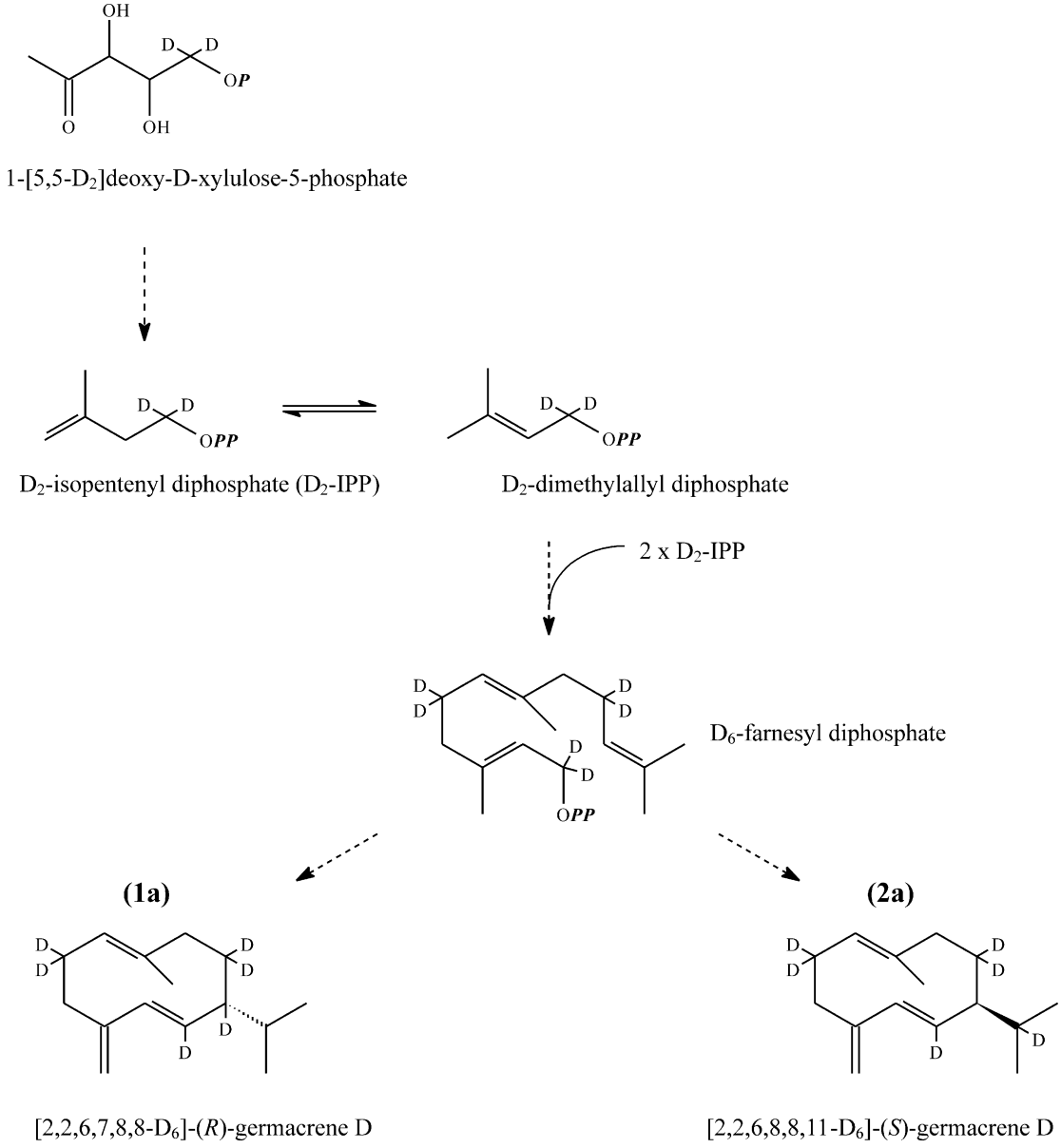
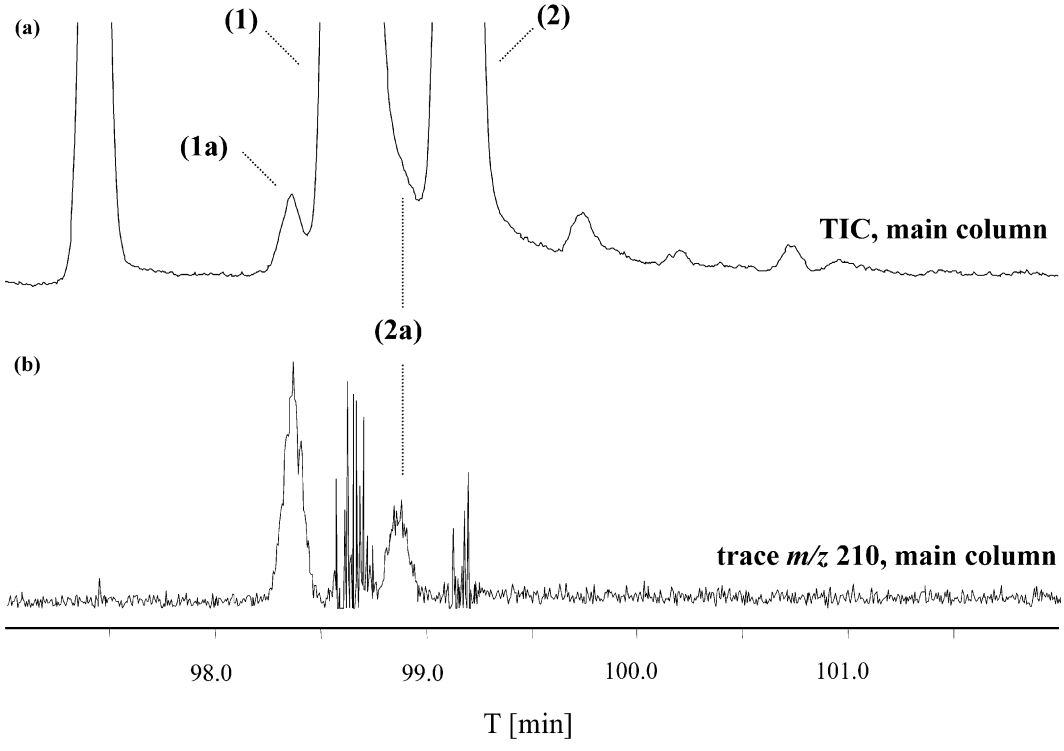


Fig. 2. Metabolic conversion of D_2-DOXP into D_6-farnesyl diphosphate via the methylerythritol phosphate pathway and subsequent formation of [2,2,6,7,8,8-D_6]-(*R*)- and [2,2,6,8,8,11-D_6]-(*S*)-germacrene D by cyclization according to the mechanisms proposed by Schmidt et al. (1999).

(*S*)-germacrene D (**2a**) according to the mechanisms proposed by Schmidt et al. (1999). The two isotopomers differ in the position of one deuterium label and, hence, can be distinguished by their mass spectra. The most abundant signal of the mass spectrum of unlabeled germacrene D appears at *m*/*z* 161 and is generated by the loss of an isopropyl group from the molecular ion (*m*/*z* 204) (Schmidt et al., 1999). Therefore, the mass spectrum of [2,2,6,7,8,8-D_6]-(*R*)-germacrene D must show its base peak at *m*/*z* 167 and its molecular ion at *m*/*z* 210, whereas the spectrum of [2,2,6,8,8,11-D_6]-(*S*)-germacrene D must display the corresponding signals at *m*/*z* 166 and 210.

Figs. 3 and 4 show the results of the enantio-MDGC–MS analysis of the hydrodistillate obtained from a shoot of S. *canadensis* fed with D_2-DOXP. Two D_6-isotopomers of germacrene D (**1a, 2a**) were detected. As a result of the inverse isotope effect in gas chromatography (Matucha et al., 1991), both D_6-isotopomers eluted shortly before their unlabeled analogues. Furthermore, the observed ratio of genuine (*R*)- to genuine (*S*)-germacrene D (around 3 : 1) was comparable to the ratio of the D_6-analogues. The mass spectrum of the first isotopomer contains the characteristic signals at *m*/*z* 167 and 210 which implies that it belongs to [2,2,6,7,8,8-D_6]-(*R*)-germacrene D (Fig. 4B). The mass spectrum of the second isotopomer (base peak: *m*/*z* 166, molecular ion: *m*/*z* 210) can be assigned to [2,2,6,8,8,11-D_6]-(*S*)-germacrene D (Fig. 4C). These results clearly indicate that the precursor was incorporated into the enantiomers of germacrene D via the methylerythritol phosphate pathway.

In a second experiment, ^{13}C-MVL was administered to a shoot and the volatile fraction was analyzed by GC–C–IRMS. This coupling technique enables the determination of $^{13}C/^{12}C$ ratios after chromatographic separation. $^{13}C/^{12}C$ isotope ratios are expressed in δ ^{13}C-values versus V-PDB standard [‰] given by:



(1a) : [2,2,6,7,8,8-D_6]-(*R*)-germacrene D

(1) : (*R*)-(+)-germacrene D

(2a) : [2,2,6,8,8,11-D_6]-(*S*)-germacrene D

(2) : (*S*)-(−)-germacrene D

Fig. 3. Enantio-MDGC–MS analysis of the essential oil obtained from a *Solidago canadensis* shoot fed with D_2-DOXP: (a) TIC, (b) trace *m*/*z* 210.

$$\delta^{13}C_{V\text{-}PDB} = \frac{(^{13}C/^{12}C)_{Sample} - (^{13}C/^{12}C)_{V\text{-}PDB}}{(^{13}C/^{12}C)_{V\text{-}PDB}} x1000‰$$

$(^{13}C/^{12}C)_{V\text{-}PDB} = 0.0112372$ (V-PDB: Vienna Pee Dee Belemnite carbonate).

Plant constituents display usually $\delta^{13}C_{V\text{-}PDB}$-values between −10 and −40‰ due to ^{13}C-depletion by kinetic isotope effects during biosynthesis (Smith and Epstein, 1971; Schmidt and Winkler, 1979; Bernreuther et al., 1990). Table 1 shows a $\delta^{13}C_{V\text{-}PDB}$-value of non labeled germacrene D and of germacrene D that was obtained

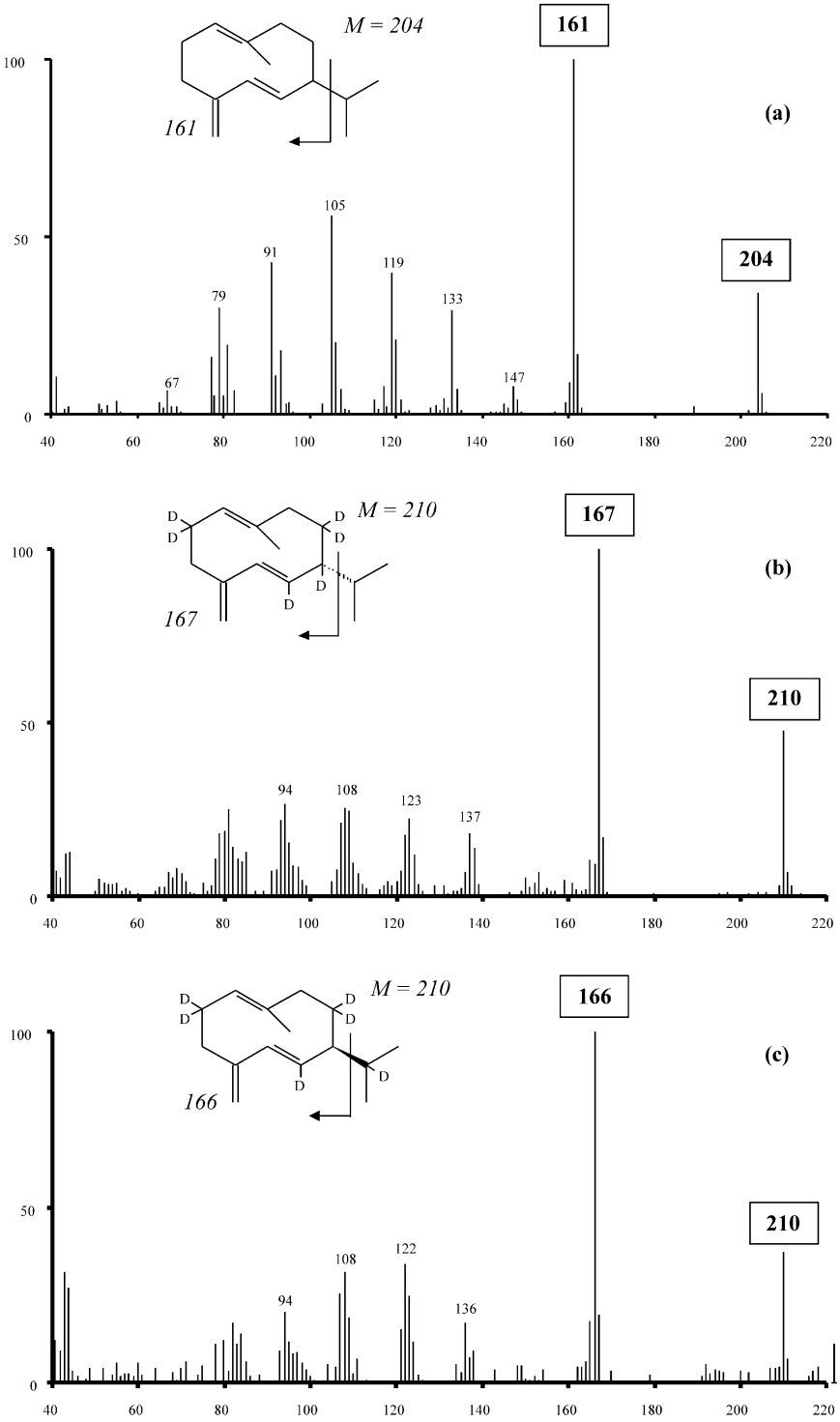


Fig. 4. (a) Mass spectrum of genuine germacrene D. (b) Mass spectrum of [2,2,6,7,8,8-D_6]-(*R*)-germacrene D obtained from a *Solidago canadensis* shoot fed with D_2-DOXP. (c) Mass spectrum of [2,2,6,8,8,11-D_6]-(*S*)-germacrene D obtained from a *Solidago canadensis* shoot fed with D_2-DOXP.

from the ^{13}C-MVL experiment. Our data prove unequivocal ^{13}C-incorporation into the target compound and demonstrate that the plant was able to utilize the precursor.

Additionally, [1-^{13}C]-D-glucose was chosen as a general precursor in order to determine the metabolic mainstream leading to the formation of germacrene D. The methylerythritol phosphate pathway can be distinguished from the mevalonate route on the basis of different labeling patterns of the isoprenoid skeleton resulting from the incorporation of [1-^{13}C]-D-glucose (Fig. 5) (Rohmer et al., 1993). After isolation of the sesquiterpene by steam distillation and subsequent column chromatography the labeling pattern was investigated by quantitative ^{13}C-NMR spectroscopy. The spectra of ^{13}C labeled and non labeled germacrene D were recorded under identical conditions. Relative ^{13}C-abundances were calculated for both and are listed in Table 2. A significant ^{13}C-enrichment was observed in six positions (Table 2). The resulting labeling pattern

Table 1
$\delta(^{13}C)_{V\text{-}PDB}$-Value of genuine germacrene D and of germacrene D derived from a *Solidago canadensis* shoot supplied with ^{13}C-MVL

	$\delta^{13}C_{V\text{-}PDB}$-value (‰)
Blank experiment	−22
Feeding experiment with ^{13}C-MVL	+ 317

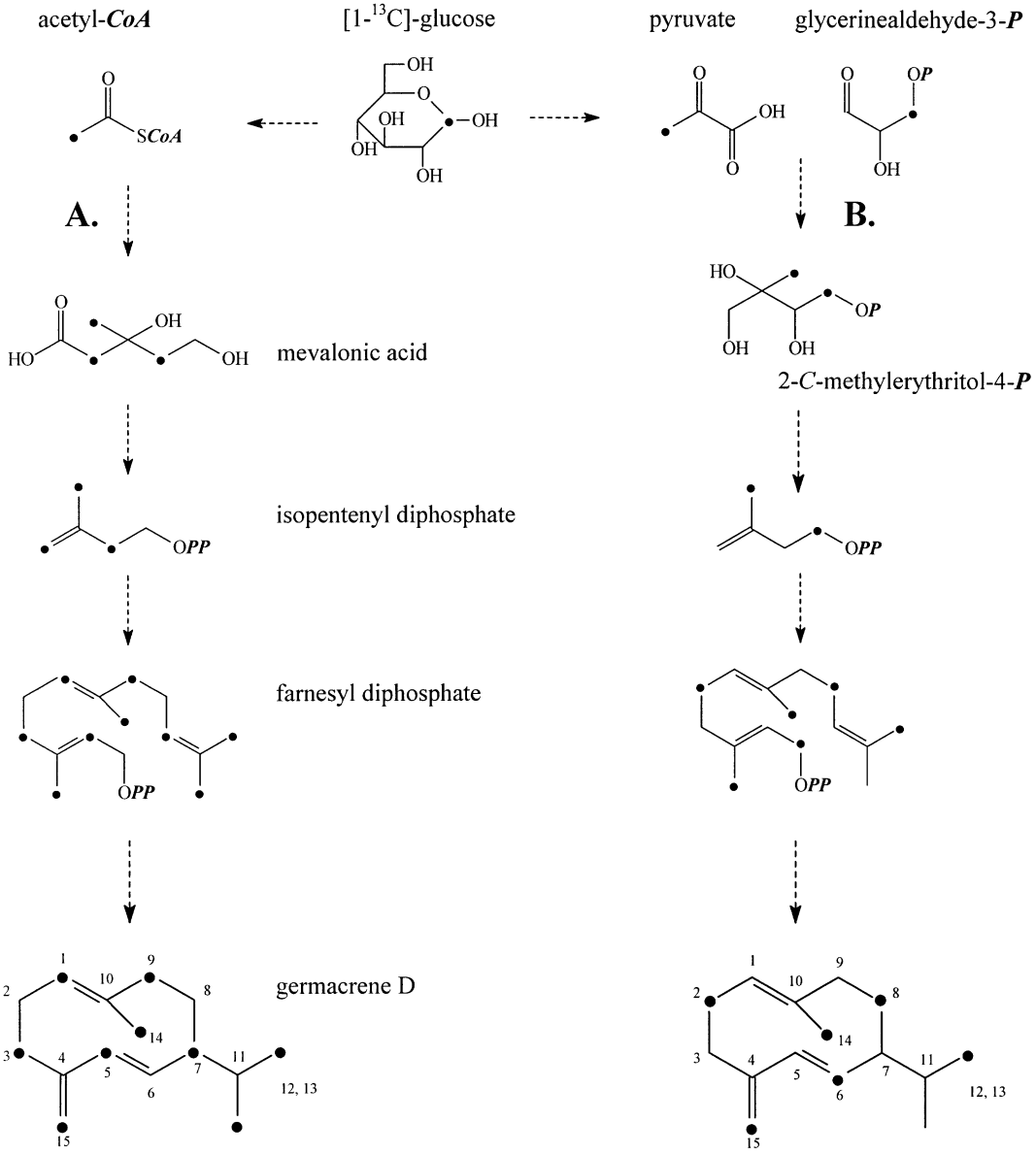


Fig. 5. Conceivable labeling patterns of germacrene D after incorporation of [1-^{13}C]-D-glucose: A. biosynthesis via the mevalonate pathway, B. biosynthesis via the methylerythritol phosphate pathway.

Table 2
Analysis of unlabeled germacrene D and of germacrene D isolated from *Solidago canadensis* shoots fed with [1-^{13}C]-D-glucose by quantitative ^{13}C NMR spectroscopy

C-position	Chem. shift δ (ppm)[a]	Unlabeled rel. ^{13}C (%)[b]	Labelled rel. ^{13}C (%)[b]
4	149.0	1.24±0.04	1.29±0.09
5	136.8	1.08±0.03	1.12±0.04
10	133.7	1.17±0.04	1.21±0.09
6	133.2	**1.10±0.06**	**1.75±0.06**
1	130.0	1.35±0.04	1.30±0.07
15	109.4	**1.05±0.04**	**1.73±0.07**
7	53.2	1.10±0.01	1.06±0.04
9	41.0	1.11±0.03	1.14±0.04
3	34.9	1.12±0.06	1.17±0.08
11	33.0	1.10	1.10
2	29.6	**1.17±0.05**	**1.72±0.09**
8	26.8	**1.11±0.04**	**1.76±0.07**
13, 12	21.0	**1.21±0.09**	**1.73±0.08**
	19.5	1.10±0.06	1.14±0.08
14	16.0	**1.00±0.04**	**1.71±0.07**

[a] Signal assignments according to Randriamiharisoa et al. (1968).
[b] Relative ^{13}C-abundances were calculated as follows:

$$\text{rel } {}^{13}C_x(\%) = \frac{\text{Integral}(C_x)}{\text{Integral}(C_{11})} \times 1.1\%;\ x: \text{position of carbon atom.}$$

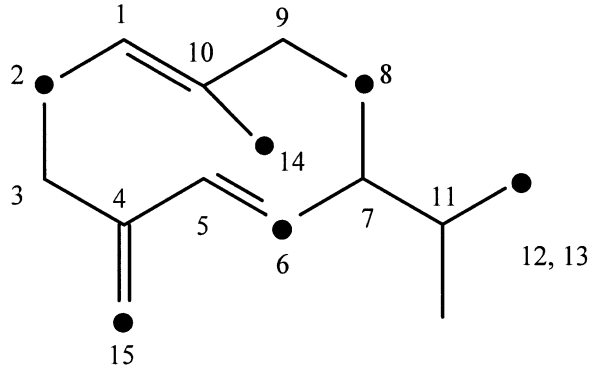


Fig. 6. Observed labeling pattern of germacrene D after incorporation of [1-^{13}C]-D-glucose.

(Fig. 6) is in full agreement with the pattern predicted for the formation of the IPP units via the methylerythritol phosphate route.

Obviously, the bulk amount of sesquiterpene germacrene D in *Solidago canadensis* is assembled from isoprene units formed via the methylerythritol phosphate pathway. However, the observed ^{13}C-incorporation after application of ^{13}C-MVL still raises the question about the role of the mevalonate pathway in the biosynthesis of germacrene D. Further investigations are necessary for a detailed assessment of the processes that are involved in the formation of germacrene D in *Solidago canadensis*.

In conclusion, it appears that the involvement of the methylerythritol phosphate pathway in sesquiterpene biosynthesis, is more common than originally postulated.

3. Experimental

3.1. Plant material, feeding experiments and isolation

Shoots (5–7 leaves, approximate length 7 cm) were cut from plants of *Solidago canadensis* grown in a greenhouse. One shoot was supplied with aqueous solution of 1-[5,5-D_2]deoxy-D-xylulose-5-phosphate (1 mg/ml, incubation time 5 days), a second one was fed with a solution of [5-^{13}C]mevalonolactone (1 mg/ml, incubation time 5 days). A blank experiment was carried out with tap water.

The respective essential oils were obtained by steam distillation using pentane as collector solvent.

Additionally, 27 cut shoots were supplied with aqueous solution of [1-^{13}C]-D-glucose (5 mg/ml, incubation time 28 days). After hydrodistillation of the plant material, germacrene D was isolated from the essential oil by column chromatography on $AgNO_3$-impregnated Al_2O_3 ($AgNO_3$:Al_2O_3 = 1:9) (Niwa et al., 1980) using a diethylether-hexane gradient.

3.2. Enantio-MDGC–MS

Enantio-MDGC–MS investigation of the hydrodistillate obtained from the D_2-DOXP feeding experiment was performed with a Siemens SiChromat 2–8 apparatus, equipped with two independent temperature controls and a live-T-switching device. The main column was coupled to a GCQ mass spectrometer (Finnigan MAT).

GC conditions: pre column: fused silica capillary (30 m×0.25 mm i.d.) coated with SE 52 (film thickness 0.25 μm); carrier gas: helium 200 kPa; injector temperature: 230 °C; FID temperature: 250 °C; oven temperature 60 °C, then 2 °C/min to 230 °C (40 min isothermal); cut time: 44.0–44.9 min; main column: fused silica capillary (30 m×0.25 mm i.d.) coated with 20% heptakis-(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in BGB 15 (film thickness 0.25 μm); carrier gas: helium 130 kPa; oven temperature 60 °C (45 min isothermal), then 1.5 °C/min to 180 °C; detector: GCQ mass spectrometer; transfer line 250 °C; helium sweeping flow 1 ml/min; ion source 170 °C; EI: 70 eV.

3.3. GC–C–IRMS

GC–C–IRMS analysis was performed with a Siemens SiChromat 2–8 double-oven instrument connected to a Finnigan MAT isotope mass spectrometer delta S via a modified combustion interface.

GC conditions: pre column: fused silica capillary (30 m×0.25 mm i.d.) coated with SE 52; carrier gas: helium 130 kPa; injector temperature: 220 °C; FID temperature: 250 °C; oven temperature 60 °C, then 2 °C/min to 230 °C (20 min isothermal); cut time: 43.0- 44.5 min; main column: fused silica capillary (30 m×0.32 mm i.d.) coated with 50% octakis-(2,3-di-*O*-butyryl-6-*O*-*tert*-butyldimethylsilyl)-γ-cyclodextrin in OV 1701; carrier gas: helium 70 kPa; oven temperature 60 °C (45 min isothermal), then 2 °C/min to 180 °C.

3.4. NMR spectroscopy

Quantitative ^{13}C NMR spectra were recorded at 125.8 MHz with the inverse gated decoupling pulse sequence in the presence of 0.1 M Cr(III)-acetylacetonate (Cr(acac)$_3$) as relaxation reagent using a Bruker AMX 500 spectrometer. The solvent was D_6-benzene. Chemical shifts were referenced to solvent signal. Signal assignments were obtained from literature (Randriamiharisoa et al., 1968).

3.5. Chemicals

[1-^{13}C]-D-Glucose was purchased from Deutero, Germany, [5-^{13}C]mevalonolactone was obtained from Isotec Inc., USA. 1-[5,5-D_2]deoxy-D-xylulose-5-phosphate was synthesized as described by Thiel and Adam (1999).

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