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BIOSYNTHESIS OF THE ISOPRENE UNITS OF CHAMOMILE SESQUITERPENES

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Key Word Index—*Matricaria recutita*; Asteraceae; chamomile; biosynthesis; bisabololoxide A; chamazulene; isopentenyl diphosphate; ^{13}C-labeling.

Abstract—Chamomile sesquiterpenes were labelled with ^{13}C by injection of an [1-^{13}C]glucose solution into the anthodia of the plant. The sesquiterpenes bisabololoxide A and chamazulene were isolated from the hydrodistillate of the labelled flowers. Analysis of the labelling patterns and absolute ^{13}C abundances using quantitative ^{13}C NMR spectroscopy showed that two of the isoprene building blocks were predominantly formed via the new triose/pyruvate pathway, whereas the third unit was of mixed origin, being derived from both the mevalonic acid pathway and the triose/pyruvate pathway. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Terpenoids are the largest group of secondary natural products with over 29.000 known compounds, including the steroids [1]. All terpenoids are derived from the common precursor isopentenyl diphosphate (IPP). In the past, the mevalonic acid (MVA) pathway has been accepted as the sole and universal biosynthetic route leading to IPP. Recently, a second, totally different pathway of isopentenyl diphosphate biosynthesis has been discovered. The first conclusive report of such a mevalonic acid-independent pathway was deduced from the incorporation patterns of ^{13}C-labelled precursors into bacterial triterpenes of the hopane series [2]. Investigations on *Scenedesmus obliquus* [4], *Ginkgo biloba* [5], *Taxus chinensis* [6], *Lemna minor* [7], *Daucus carota* [7], *Mentha piperita* and *Thymus vulgaris* [8] have shown that this alternative pathway is also involved in the formation of isoprene [9], monoterpenes [8], diterpenes [4–7] and carotenoids [4, 7] of higher and lower plants.

In this alternative pathway, IPP is formed from pyruvate and glyceraldehyde-3-phosphate yielding, after condensation, 1-desoxyxylulose-5-phosphate, the putative first C_5 intermediate of this pathway [3]. Several subsequent steps, including an intramolecular rearrangement finally lead to IPP [9, 10] (Fig. 1).

So far it is hypothesized that this new triose/pyruvate pathway is located in the plastids and is involved in the formation of mono-, di- and tetraterpenoids, whereas sesquiterpenes and the biogenetically related sterols are formed in the cytoplasm via the classical mevalonic acid pathway [6, 7].

The IPP biosynthetic pathway that is followed in the formation of a terpenoid can be identified by incorporation of [1-^{13}C]glucose into the respective compounds. Depending on the pathway that is followed glucose is metabolized differently resulting in a specific ^{13}C labelling pattern of the isoprene units [2, 5–7]. The labelling pattern of the isoprene units can be determined by ^{13}C NMR spectroscopy, thus allowing a clear assignment to one of both pathways (Fig. 1).

Incorporation studies in the early seventies on chamomile flowers (*Matricaria recutita*) using ^{14}C-labelled acetate and mevalonate led to the assumption that chamomile sesquiterpenes are biosynthesized via the mevalonic acid pathway [11, 12]. The ability to specifically differentiate both IPP biosynthetic pathways using [1-^{13}C] glucose as precursor led us to reinvestigate the biosynthetic origin of the isoprene units of the chamomile sesquiterpenes.

RESULTS AND DISCUSSION

Labelling of chamomile sesquiterpenes was achieved by multiple injection of a solution of [1-^{13}C]glucose into the anthodia (Fig. 3). Labelled flowers were subjected to steam distillation. Subsequent HPLC separation of the hydrodistillate yielded the two labeled sesquiterpenes bisabololoxide A (**1**) and

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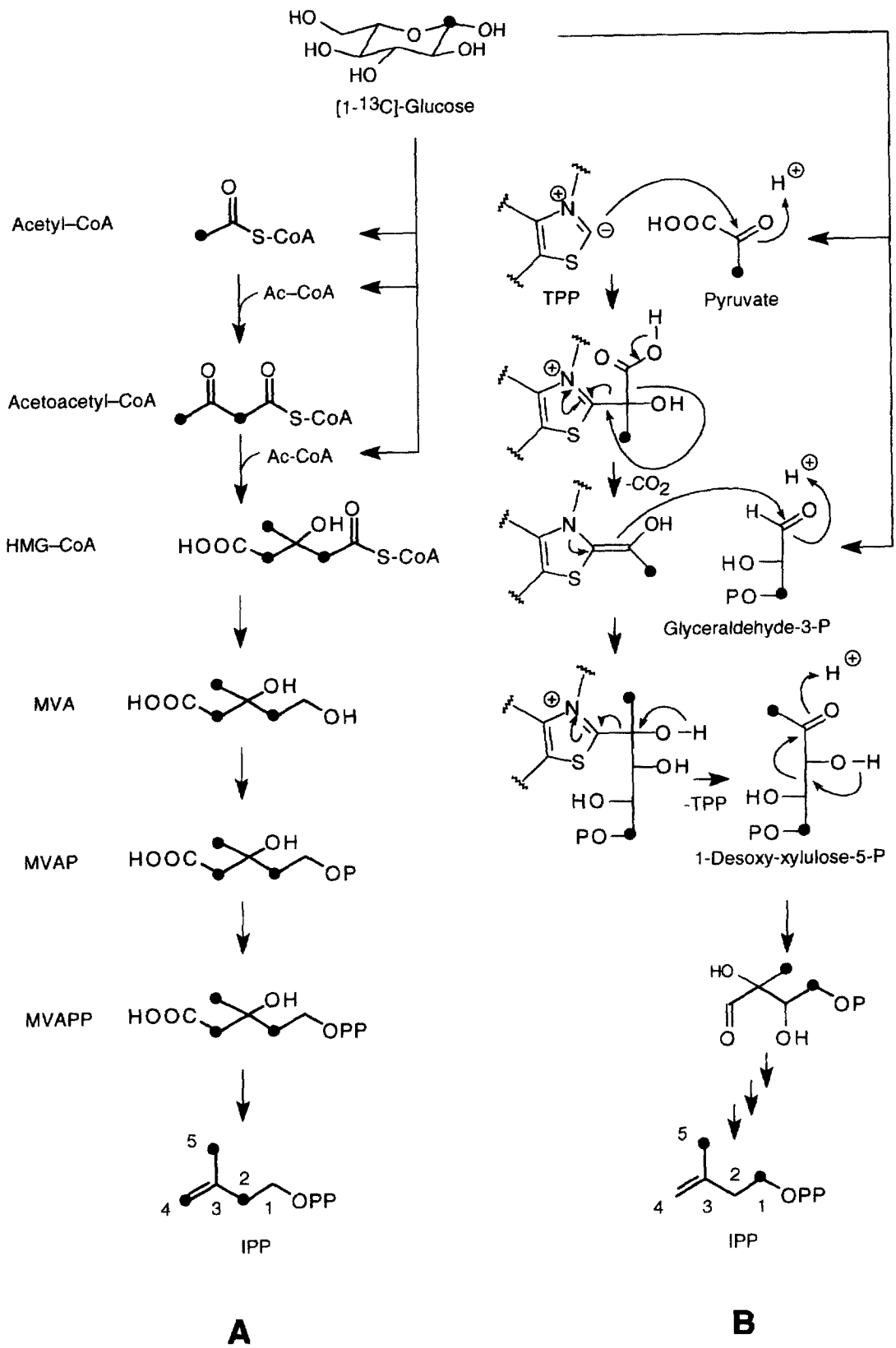

Fig. 1. Biosynthesis of isopentenyl diphosphate (IPP). A = Mevalonic acid pathway, B = Triose/pyruvate pathway, Ac-CoA = Acetyl coenzyme A, HMG-CoA = hydroxymethyl glutaryl coenzyme A, MVA = mevalonic acid, MVAP = mevalonic acid phosphate, MVAPP = mevalonic acid diphosphate, TPP = thiamine diphosphate, P = phosphate residue, PP = diphosphate residue, • = ^{13}C-labelled position.

chamazulene (**2**). Compound **2** is an artifact, formed during hydrodistillation from the guajane-type sesquiterpenelactone matricin (**3**). Unlabelled reference compounds were isolated from chamomile oil by following the same procedures.

In order to introduce an internal carbon standard with natural ^{13}C abundance (1.1%) unlabelled and labelled **1** were converted to the respective TMSi-ether (**1a**). The ^{13}C NMR signals of compounds **1a** and **2** were assigned by means of two-dimensional correlation experiments.

Quantitative ^{13}C NMR spectroscopy was used for the determination of the labelling patterns and to obtain exact data of the absolute ^{13}C abundances. The spectra were recorded with the inverse gated decoupling pulse sequence with $Cr(acac)_3$ in the sample as a relaxation reagent [13]. Recording conditions were optimized using synthetic linalool and unlabelled standards of **1a** and **2**. ^{13}C abundances of all carbons of the standard compounds (sample size 5 mg, each) were very similar as shown in the right columns of Tables 1 and 2. The method allows the precise and reproducible determination of ^{13}C abundances, and is especially suitable for the analysis of ^{13}C-labelled biosynthetic samples.

The absolute ^{13}C abundances of compounds **1a** and **2** were obtained from the analysis of well separated ^{13}C satellite signals in the ^1H NMR spectra. Based on the respective 12C/13C-proton signal ratio, ^{13}C abundances of the remaining carbon atoms were calculated from the integrals of their ^{13}C NMR signals (Tables 1 and 2). Additionally, the absolute ^{13}C abundances of the TMSi-derivative **1a** could be calculated from the integrals of the quantitative ^{13}C NMR spectrum, using the TMSi-signal as internal standard. Both quantitation methods are comparable since both methods led to similar data for **1a** (Table 1).

The absolute ^{13}C abundances of the biogenetically corresponding carbon atoms of **1a** and **2** were quite similar, and their labelling patterns were identical (Table 3, Fig. 2). Obviously, both sesquiterpenes were derived from the same biosynthetic pool. Since the branching point in chamomile sesquiterpene biosynthesis is the cyclization step of the universal sesquiterpene precursor farnesyl diphosphate (FPP) (**4**) leading to bisabolane- and guajane-type (matricin) compounds it can be assumed that **1a** and **2** are formed from the same FPP-pool. Thus, the labelling pattern can be discussed at the level of FPP (**4**, Fig. 2).

A significant ^{13}C enrichment in the positions 1 and 5 of isoprene unit 1 and unit 2 was observed (average absolute ^{13}C abundance of 3.07%). This labelling pattern is characteristic of the formation of these IPP units via the new triose/pyruvate pathway. In the case of the mevalonic acid pathway, the labelling should occur at positions 2, 4 and 5 of the isoprene units. Furthermore, a significant enrichment of the remaining carbons (positions 2, 3 and 4) was found (average absolute ^{13}C abundance of 1.60%). This general labelling might be due to complex metabolic turnover of [1-^{13}C] glucose with following statistical distribution

Table 1. ^{13}C NMR data and ^{13}C abundance of (**1a**) in benzene-d_6, 0.1 M $Cr(acac)_3$.

C	δ_C	% ^{13}C* labelled	% ^{13}C† labelled	% ^{13}C* unlabelled
1	23.7	3.25	3.48	1.12
2	74.6	1.63	1.74	1.16
3	29.4	1.78	1.89	1.10
4	25.0	2.99	3.19	1.19
5	73.3	1.67	1.78	1.15
6	75.5	1.63	1.75	1.16
7‡	25.5	3.12	3.34	1.14
8‡	29.6	1.42	1.52	1.10
1′	43.4	1.65	1.76	1.17
2′	27.5	2.19	2.34	1.19
3′	121.3	2.08	2.22	1.19
4′	133.8	1.86	1.98	1.14
5′	31.3	1.88	2.00	1.16
6′	23.5	2.86§	3.05§	1.11§
7′	23.5	2.86§	3.05§	1.11§
$-OSi(CH_3)_3$	0.15	1.1 each	1.29 each	3 × 1.10

* ^{13}C abundance calculated from integrals referenced to the TMSi signal as internal standard (3 × 1.10%).

† ^{13}C abundance obtained from ^{13}C satellite analysis of H-3′.

‡ Signals may be interchanged.

§ Signals overlapped, integrated together, indicated are mean values.

Table 2. ^{13}C NMR data and ^{13}C abundance of labelled and unlabelled (**2**) in benzene-d_6, 0.1 M $Cr(acac)_3$

C	δ_C	% ^{13}C* labelled	% ^{13}C† unlabelled
1	125.1	1.61‡	1.12‡
2	136.7	2.16	1.09
3	113.5	2.73	1.17
4	143.9	1.56	1.14
5	125.0	1.61‡	1.12‡
6	136.2	3.06	1.11
7	135.4	1.47	1.20
8	134.5	2.51	1.20
9	136.9	2.16	1.25
10	137.9	1.80	1.18
Me (C-1)	13.0	3.29	1.14
Me (C-4)	24.0	3.27	1.15
CH_2 (ethyl)	34.0	1.55	1.17
Me (ethyl)	17.4	3.25	1.10

* ^{13}C abundance obtained from ^{13}C satellite analysis of Me (ethyl).

† ^{13}C abundance calculated from ^{13}C signals, ^{13}C abundance of Me (ethyl) referenced to 1.10%.

‡ Signals overlapped, integrated together, indicated are mean values.

Table 3. Comparison of the ^{13}C abundances of biogenetically equivalent carbon atoms of **1a** and **2** (for numbering see **4**, Fig. 2).

C	% ^{13}C* (**1a**)	% ^{13}C† (**2**)	average % ^{13}C
Isoprene unit 1			
1	2.99 T	3.06 T	3.03
2	1.67	1.47	1.57
3	1.63	1.55	1.59
4	1.42	...	1.42
5	3.12 T	3.25 T	3.19
Isoprene unit 2			
1′	2.86 T	2.73 T	2.80
2′	1.65	1.80	1.73
3′	1.63	1.56	1.60
4′	1.78	1.61	1.70
5′	3.25 T	3.27 T	3.26
Isoprene unit 3			
1″	2.19 T	2.51 T	2.35
2″	2.08 M	2.16 M	2.18
3″	1.86	1.61	1.74
4″	1.88 M	2.16 M	2.02
C-5″	2.86 T/M	3.29 T/M	3.08

* ^{13}C abundances referenced to the TMSi signal as internal standard (3 × 1.10%).

† ^{13}C abundances obtained from ^{13}C satellite analysis of Me (ethyl).

T labeling from triose/pyruvate pathway.

M labeling from mevalonic acid pathway.

of the labelled carbon atoms in various positions of intermediates of carbohydrate metabolism. Enrichment in positions 1 and 5 was also observed in the remaining third isoprene unit (unit 3) but the enrichment in position 1 (average absolute ^{13}C abundance of 2.35%, Table 3) was significantly lower (50%) compared to the data of the respective positions of isoprene units 1 and 2. Additionally, isoprene unit 3, showed a significant labelling in positions 2 and 4 (average absolute ^{13}C abundance of 2.18% and 2.02% respectively, Table 3), indicating the involvement of the mevalonic acid pathway in the formation of this unit. Thus, the comparatively high enrichment in position 5 (average absolute ^{13}C abundance of 3.08%, Table 3) can be explained by labelling from both pathways. The involvement of the mevalonic acid pathway is also supported by the incorporation of ^{14}C-labelled MVA into chamomile sesquiterpenes [12].

These non-equivalent labelling patterns of the three isoprene units clearly indicate that the FPP molecules that are used in the biosynthesis of chamomile sesquiterpenes must be formed in a two step procedure, taking place in different cellular compartments. In the first step a C_{10} unit is formed consisting of two isoprene units that are predominantly derived from the triose/pyruvate pathway. In the second step, an isoprene unit of mixed biosynthetic origin is added. The involvement of different IPP pools, depending on the position of the isoprene unit, is only explainable when both steps occur in different cellular compartments or subcompartments.

Based on the current hypothesis of the cellular localization of the IPP biosynthetic pathways [5, 7] this two step procedure could occur as follows: The C_{10} building block geranyl diphosphate (GPP) is created in the plastid from triose/pyruvate pathway derived IPP. Then, GPP is transferred to another cellular compartment (or subcompartment) that has access to IPP formed via both pathways. In this compartment, GPP is extended by an isoprene unit of mixed origin, yielding FPP species consisting of only triose/pyruvate pathway derived isoprene units as well as chimeric FPP molecules with two triose/pyruvate pathway derived isoprene units and a terminal MVA derived isoprene unit.

The availability of both IPP species in the same compartment might be explained by either a leaking out of triose/pyruvate pathway-derived IPP from the plastid or an influx of MVA-derived IPP into the compartment where the terminal isoprene unit is added. This compartment could be either the cytoplasm itself or an additional compartment e.g. the intermembrane space of the plastid.

At the enzymatic level, this would imply the presence of two prenyltransferases: a geranyl diphosphate synthase, located in the plastid, and a farnesyl diphosphate synthase, located in the compartment of the terminal addition of IPP. Since it is a general property of farnesyl diphosphate synthases to build up FPP from dimethylallyl diphosphate (DMAPP) and IPP as well as GPP and IPP [14], it must be assumed, that this particular chamomile FPP synthase has a much higher affinity for GPP as substrate than for DMAPP. Otherwise, a significant amount of fully MVA-derived FPP should occur, detectable by a characteristic labelling pattern in isoprene units 1 and 2.

Additional labelling experiments with pathway specific precursors and investigations on the enzymatic level of sesquiterpene biosynthesis might provide more evidence for this hypothesis of a compartmentalized biosynthesis of FPP in chamomile flowers. So far we have identified a farnesyl diphosphate synthase and a bisabolol synthase in this plant. Further studies are under way.

Our results also show that the triose/pyruvate pathway of IPP biosynthesis is not restricted to mono- di- and tetraterpenes [5, 7], but also occurs in the biosynthesis of sesquiterpenes. However, in a similar [1-^{13}C]glucose incorporation experiment it was shown that the sesquiterpene ricciocarpin A, a constituent of the liverwort *Ricciocarpos natans* [15], is exclusively composed of MVA-derived IPP [16]. Obviously, plants have developed two different biosynthetic machineries for the formation of sesquiterpenes. Further experiments with other sesquiterpene-containing plants are necessary to assess the abundance and the distribution of both processes in the plant kingdom and might give some interesting insights in the evolution of terpenoid biosynthesis in plants.

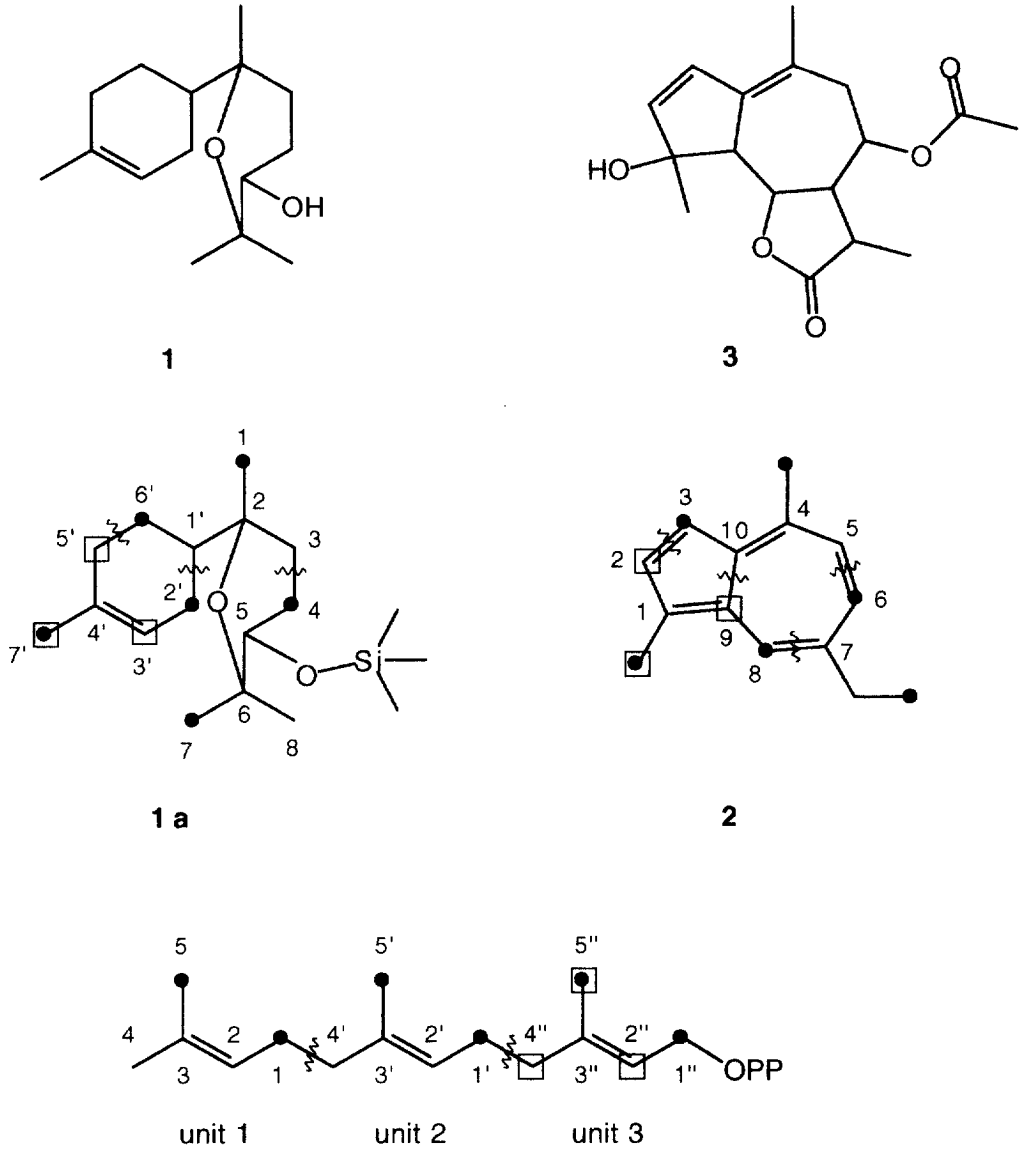

Fig. 2. Sesquiterpenes of chamomile flowers and their labelling patterns after [1-^{13}C]glucose incorporation. • = ^{13}C labelling according to the triose/pyruvate pathway, □ = ^{13}C labelling according to the mevalonic acid pathway.

EXPERIMENTAL

Spectroscopy

NMR: benzene-d_6 [^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz)] relative to benzene-d_6 at δ_H 7.15, δ_C 128.0. ^{13}C multiplicities were determined using the DEPT pulse sequence. 2D spectra were recorded as HSQC, HMBC and COSY. Quantitative ^{13}C NMR measurements were recorded with the inverse gated decoupling pulse sequence in the presence of 0.1 M Cr(acac)$_3$. For integration, the signal-to-noise ratio of the ^{13}C signals was at least 40:1 [13].

Reagents

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

Plant material

Chamomile plants were raised from seeds in the green house of the institute. Seeds were obtained from Garden City Seeds, Victor MT, 59875, U.S.A.

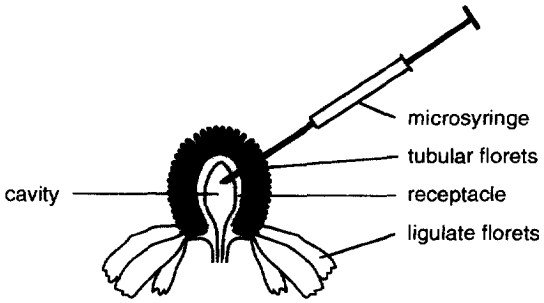

Fig. 3. Injection of the [1-^{13}C]glucose solution into the chamomile anthodium.

Labelling procedure

5 μl of an 20% soln of [1-^{13}C]glucose were injected in the cavity of the anthodia of 8-week-old plants using a microsyringe just before the onset of the flowering of the tube flowers (Fig. 3). The injection was repeated twice a day for four days. A total of 100 anthodia were subjected to the procedure. They were collected at the 7th day after the first injection.

Isolation

Labelled and unlabelled compounds **1** and **2** were obtained by hydrodistillation of the anthodia and subsequent HPLC of the hydrodistillate; hexane-EtOAc (16:1), 250 × 8 mm, Si 100 Lichrospher (Merck), 5 μm. Yield of labeled compounds: **1** (4 mg) and **2** (3 mg).

GC–MS

GC–MS was used to check the identity and purity of the different compounds and the respective TMSi derivatives; He at 1 ml min^{-1}, temp. programmed, 40° for 5 min, then at 5° min^{-1} to 200°, injector at 250°, mass detector at 280°, HP-5 column (30 m × 0.25 mm).

Derivatization

The TMSi derivative **1a** was obtained by addition of 0.3 ml bis(trimethylsilyl)acetamide (BSA) to labelled and unlabeled **1** (4 and 40 mg, respectively) and subsequent heating for 5 hr at 80°. HPLC (hexane-EtOAc (49:1), 250 × 8 mm, Si 100 Lichrospher (Merck), 5 μm) of the reaction mixture yielded 5 mg labelled and 42 mg unlabelled **1a**.

Bisabololoxide A-TMSI ether (**1a**)

^{1}H NMR (benzene-d_6) δ: 0.09 (9H, *s*, $-OSi(CH_3)_3$), 1.07 (1H, *s*, H-1), 1.11 (1H, *m*, H-3β), 1.24 (1H, *s*, H-8), 1.28 (1H, *s*, H-7), 1.30 (1H, *m*, H-6′α), 1.56 (1H, *m*, H-3), 1.63 (3H, *s*, H-7′), 1.66 (1H, *m*, H-4α), 1.77 (1H, *m*, H-2′α), 1.88 (1H, *m*, H-1′), 1.90, (2H, *m*, H-5′), 1.96 (1H, *m*, H-3α), 1.98 (1H, *m*, H-2′β), 2.18 (1H, *m*, H-6′β), 3.38 (1H, *dd*, J = 3.4, 7.1 Hz, H-5), 5.42 (1H, *s br*, H-3′); ^{13}C NMR (benzene-d_6) δ:0.12 (*q*, $-OSi(CH_3)_3$), 23.6 (*t*, C-6′†), 23.7 (*q*, C-7′†), 23.9 (*q*, C-1), 25.1 (*t*, C-4), 25.6 (*q*, C-7*), 27.7 (*t*, C-2′), 29.5 (*t*, C-3), 29.7 (*q*, C-8*), 31.4 (*t*, C-5′), 43.5 (*d*, C-1′), 73.5 (*d*, C-5), 74.8 (*s*, C-2), 75.7 (*s*, C-6), 121.5 (*d*, C-3′), 134.0 (*s*, C-4′); *,† signals may be interchanged.

Chamazulene (**2**)

^{1}H NMR (benzene-d_6) δ: 1.15 (3H, *t*, J = 7.8 Hz, Me (ethyl)), 2.56 (2H, *dd*, J = 7.5, 15.0 Hz, CH_2 (ethyl)), 2.59 (3H, *s*, Me (C-4)), 2.60 (3H, *s*, Me (C-1)), 6.73 (1H, *d*, J = 10.5 Hz, H-5), 7.12 (1H, *dd*, J = 1.2, 10.8 Hz, H-6), 7.27 (1H, *d*, J = 3.7 Hz, H-3), 7.65 (1H, *d*, J = 3.6 Hz, H-2), 8.11 (1H, *d*, J = 1.2 Hz, H-8); ^{13}C NMR (benzene-d_6) δ: 13.0 (*q*, Me (C-1)), 17.4 (*q*, Me (ethyl)), 24.0 (*q*, Me (C-4)), 34.0 (t, CH_2 (ethyl)), 113.6 (*d*, C-3), 125.1 (*d*, C-5), 125.3 (*s*, C-1), 134.5 (*d*, C-8), 135.6 (*s*, C-7), 136.2 (*d*, C-6), 136.8 (*d*, C-2), 137.1 (*s*, C-9), 138.1 (*s*, C-10), 144.0 (*s*, C-4).

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