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Incorporation of [1-^{13}C]1-deoxy-D-xylulose into isoprenoids of the liverwort *Conocephalum conicum*

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Abstract

The incorporation of ^{13}C labeled 1-deoxy-D-xylulose into the monoterpene bornyl acetate, the sesquiterpene cubebanol, and the diterpene phytol has been studied in axenic cultures of the liverwort *Conocephalum conicum*. Quantitative ^{13}C NMR spectroscopic analysis of the labeling patterns of the sesquiterpene indicated a possible degradation of 1-deoxy-D-xylulose to acetate and subsequent incorporation via the mevalonic acid pathway. In bornyl acetate, the labeling occurred only in the acetate moiety whereas the isoprene units remained unlabelled. The isoprene units of the diterpene phytol showed incorporation of intact deoxy-D-xylulose. These results indicate the involvement of both IPP biosynthetic pathways and two independently operating compartments/cell types with MEP pathway machinery. One MEP compartment is presumably the plastid where phytol is formed; the second, involved in the build-up of the isoprene part of bornyl acetate, might be located in the oil cells. The acetylation of borneol to bornyl acetate in turn occurs in a cellular compartment that is not involved in the build-up of the isoprene units of borneol. © 2002 Published by Elsevier Science Ltd.

Keywords: Liverwort; *Conocephalum conicum*; Hepaticae; Isopentenyl diphosphate; Mevalonic acid pathway; Methylerythritol phosphate pathway; Bornyl acetate; Cubebanol; Phytol; [1-^{13}C]1-Deoxy-D-xylulose

1. Introduction

Terpenoids are the largest group of secondary natural products with over 30,000 known compounds, mainly of plant origin (Dictionary of Natural Products, 2000). For decades, it has been assumed that their common building block, the isoprene unit, is formed from isopentenyl diphosphate (IPP) solely via the mevalonic acid (MVA) pathway. Recently, an alternative biosynthetic route for the formation of IPP, the mevalonate-independent methylerythritol phosphate (MEP) pathway, was discovered. In the MEP pathway the first C_5-intermediate 1-deoxy-D-xylulose 5-phosphate, is formed in a transketolase mediated reaction from an activated C_2-unit (derived from pyruvate) and glyceraldehyde-3-phosphate (Lange et al., 1998; Lois et al., 1998). The involvement of a 1-deoxy-D-xylulose moiety has been shown by its specific incorporation in isoprenoids of bacteria and plants (Arigoni et al., 1997; Schwender et al., 1997; Zeidler et al., 1997; Putra et al., 1998; Adam et al., 1999). To date, the reaction sequence of this pathway is established up to the formation of the cyclic diphosphate intermediate 2-*C*-methylerythritol 2,4-cyclodiphosphate (Rohmer et al., 1993; Sprenger et al., 1997; Lois et al., 1998; Takahashi et al., 1998; Rohdich et al., 1999; Herz et al., 2000; Lüttgen et al., 2000); the subsequent steps to IPP are still uncharacterized.

This new pathway has received much attention as it has been found to play a major role in isoprenoid biosynthesis in bacteria and plants (Rohmer et al., 1993; Schwartz, 1994; for review, see Lichtenthaler et al., 1997a; Eisenreich et al., 1998; Rohmer, 1999; Lichtenthaler, 1999). Whereas vertebrates and archaebacteria appear to use only the mevalonate pathway for the build-up of isoprenoids, various eubacteria have been found to use either the mevalonate or the MEP pathway. In contrast, algae (with the exception of chlorophytes), bryophytes and higher plants use the MVA pathway for the biosynthesis of steroids and the MEP pathway for the formation of hemiterpenoids, monoterpenoids, carotenoids, phytol and other diterpenes (for review, see Lichtenthaler et al., 1997a; Eisenreich et

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al., 1998; Lichtenthaler, 1999; Rohmer, 1999). The MVA pathway appears to be operative in the cytoplasmic compartment of plant cells, whereas the MEP pathway seems to be located in the plastids (Lichtenthaler et al., 1997b; Rohdich et al., 1999).

Segregation of the two pathways, however, is not necessarily complete, and exchange of metabolites between the two pools has been observed in plants and plant cell cultures (Schwarz, 1994; Nabeta al., 1995; Arigoni et al., 1997; Adam and Zapp, 1998; Piel et al., 1998; Adam et al., 1999). Despite a significant amount of recent research in this area, our knowledge of the contribution of both pathways to the biosynthesis of the different plant terpenoid classes is still limited.

In previous studies of IPP biosynthesis in various bryophytes with the pathway independent precursor [1-^{13}C]glucose (Thiel et al., 1997; Adam et al., 1998; Eisenreich et al., 1999; Barlow et al., 2001; Hertewich et al., 2001) we demonstrated the different origin of IPP units in several classes of isoprenoids (hemi-, mono-, sesqui-, diterpenes and sterols). In these studies axenic cultures of the liverwort *Conocephalum conicum* have proven to be a particularly useful experimental system for the comparative analysis of the biosynthesis of isoprenoids from different classes. We could show that in *C. conicum* cultures the monoterpene bornyl acetate (**1**) as well as the phytol (**3**) side chain of chlorophyll were derived from the MEP pathway whereas the sesquiterpene cubebanol (**2**) and the sterol sitosterol originated from the MVA pathway (Thiel et al., 1997; Adam et al., 1998).

In order to obtain more detailed information of the presence and localization of different MEP compartments within the plant we tested the incorporation of the labeled, pathway-specific, precursor [1-^{13}C]1-deoxy-D-xylulose into different isoprenoids of *C. conicum*. In this experiment we investigated whether the bornyl acetate (**1**) is biosynthesized in the chloroplasts like phytol (**3**) or in a different plastidial compartment. The formation of both compounds in the chloroplast should be indicated by a similar ^{13}C enrichment in both terpenoids whereas the build-up in different plastid types/organelles should result in quantitative differences in the ^{13}C enrichment of both compounds.

In this paper, we present our results of the incorporation of [1-^{13}C]1-deoxy-D-xylulose into bornyl acetate, phytol and cubebanol in the liverwort *C. conicum*.

2. Results and discussion

The incorporation of the pathway specific precursor [1-^{13}C]1-deoxy-D-xylulose can be expected to be quite different from the incorporation of [1-^{13}C]glucose. It must be assumed that—compared to the non-specific precursor glucose—the incorporation levels of a specific precursor are to a much greater extent dependent upon the availability of the compound (or its metabolites) in the respective cellular compartments. The availability is influenced by several factors such as the permeability of the biomembranes that separate the different compartments and the rates of the degradation of the precursor in each compartment. Furthermore, the utilization of 1-deoxy-D-xylulose in the plastidial IPP production is dependent upon the activity of kinases that convert 1-deoxy-D-xylulose to 1-deoxy-D-xylulose phosphate, the true intermediate of the MEP pathway. Recently, such a D-xylulokinase has been reported from *Escherichia coli* (Wungsintaweekul et al., 2001). The theoretical fate of the label in case of an intact incorporation of [1-^{13}C]1-deoxy-D-xylulose into isopentenyl diphosphate is illustrated in Fig. 1.

In our experiment, [1-^{13}C]1-deoxy-D-xylulose was administered to aseptic cultures of the plant via the culture medium. After a 20-fold fresh weight increase, the plant material was harvested and the monoterpene bornyl acetate (**1**), the sesquiterpene cubebanol (**2**) and the diterpene *trans*-phytol (**3**) were isolated with standard chromatographic procedures. *Trans*-phytol (**3**) served as a plastidial marker compound since it was obtained by alkaline cleavage from chlorophyll. Assignments of ^{13}C NMR spectroscopic signals of the purified compounds were obtained from the literature [Bohlmann and Zeisberg, 1975 (**1**); De Rosa et al., 1994 (**2**); Arigoni et al., 1997 (**3**)] and 2D NMR experiments. Labeling patterns and absolute ^{13}C enrichments of the isoprenoids were determined by quantitative ^{13}C NMR spectroscopy using $Cr(acac)_3$ as a relaxation reagent (Braun et al., 1996). In order to introduce an internal ^{13}C reference with the natural ^{13}C abundance of 1.11%, phytol (**3**) was acetylated to the 1-*O*-acetate (**3a**) and cubebanol (**2**) was converted to its 4-*O*-TMSi-derivative (**2a**). For the non-derivatized bornyl acetate (**1**), the absolute ^{13}C enrichment was obtained from ^{13}C satellite analysis of C-2 in the ^{1}H NMR spectrum.

From the ^{13}C-glucose feeding study it was known that the sesquiterpene cubebanol is formed exclusively via the MVA pathway (Adam et al., 1998). Therefore, we did not expect a labeling of the sesquiterpene in this incorporation experiment. However, cubebanol showed ^{13}C enrichment of the positions 2,4 and 5 of all three isoprene units (1.87 ± 0.08% ^{13}C, Fig. 2, Table 1). This labeling pattern can be explained by the degradation of [1-^{13}C]1-deoxy-D-xylulose to [2-^{13}C]acetyl CoA and subsequent incorporation into IPP via the MVA pathway (Fig. 3). The background labeling was in the same range as in phytol and bornyl acetate (1.20±0.03% ^{13}C, Table 1).

The quantitative ^{13}C NMR spectroscopic analysis of phytol and its acetate displayed four enriched carbons (2.96 ± 0.01% ^{13}C, Fig. 2, Table 2) each in position 5 of the corresponding isoprene unit. This incorporation of

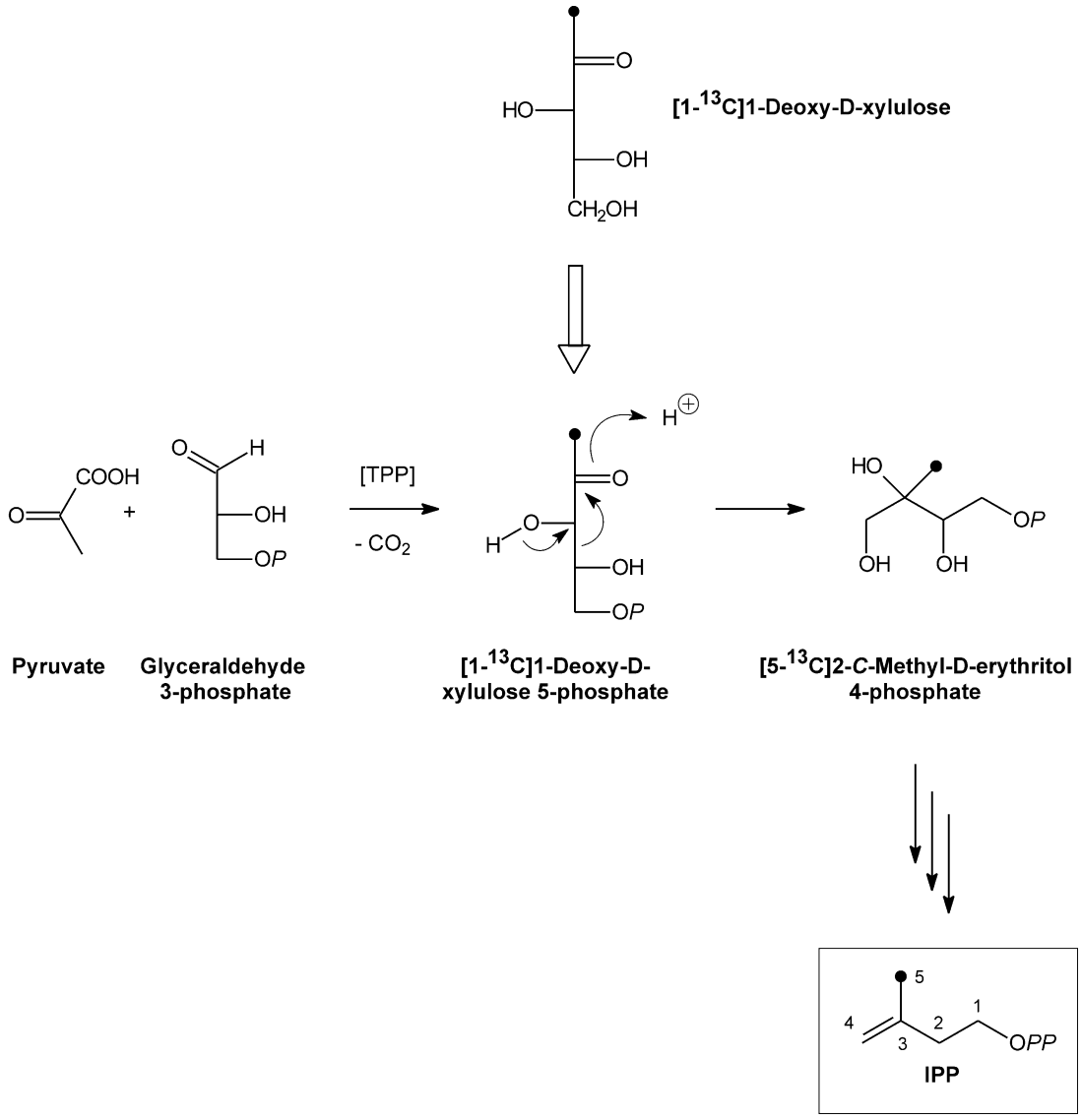

Fig. 1. Incorporation of [1-^{13}C]1-deoxy-D-xylulose into isopentenyl diphosphate. TPP = thiamine diphosphate, *P* = phosphate residue, *PP* = diphosphate residue, ●: ^{13}C labeled position.

[1-^{13}C]1-deoxy-D-xylulose proves that phytol is biosynthesized via the MEP pathway. Furthermore, it is remarkable that the background labeling with 1.20± 0.09% ^{13}C is slightly higher than the natural abundance of 1.11% ^{13}C. This fact indicates some degradation of [1-^{13}C]1-deoxy-D-xylulose.

In contrast with the result of phytol, incorporation of [1-^{13}C]1-deoxy-D-xylulose in the corresponding positions (C-8 und C-10) of the isoprenoid moiety of bornyl acetate (**1**) could not be detected (Fig. 2). However, the methyl group of the acetate residue was significantly labeled (3.96% ^{13}C, Table 3). An explanation of the lack of incorporation into the isoprene units of bornyl acetate could be that the monoterpene is biosynthesized to a much greater extent than phytol (estimated 8–10 fold) and that IPP is formed to a much higher extent from plastidial, non-labeled primary metabolites than from labeled 1-deoxy-D-xylulose that has entered the plastid. As a consequence, only a small fraction of the IPP units is ^{13}C labeled, too small to be detected NMR spectroscopically. The high enrichment of the acetate methyl group and the slightly increased background labeling demonstrate again that [1-^{13}C]1-deoxy-D-xylulose is metabolized and presumably degraded to [2-^{13}C]-labelled acetyl-CoA (Fig. 2). A second explanation for the missing incorporation of [1-^{13}C]1-deoxy-D-xylulose into the isoprenoidal part of bornyl acetate may be derived from the discrepancy between background labeling and the enrichment in the acetate unit: Increased [1-^{13}C]1-deoxy-D-xylulose metabolism in the cytoplasm could prevent the uptake of intact [1-^{13}C]1-deoxy-D-xylulose (phosphate) by the plastid. Possibly in the cytoplasm [1-^{13}C]1-deoxy-D-xylulose is significantly degraded to [2-^{13}C]acetate which in turn is utilized for the acetyla-

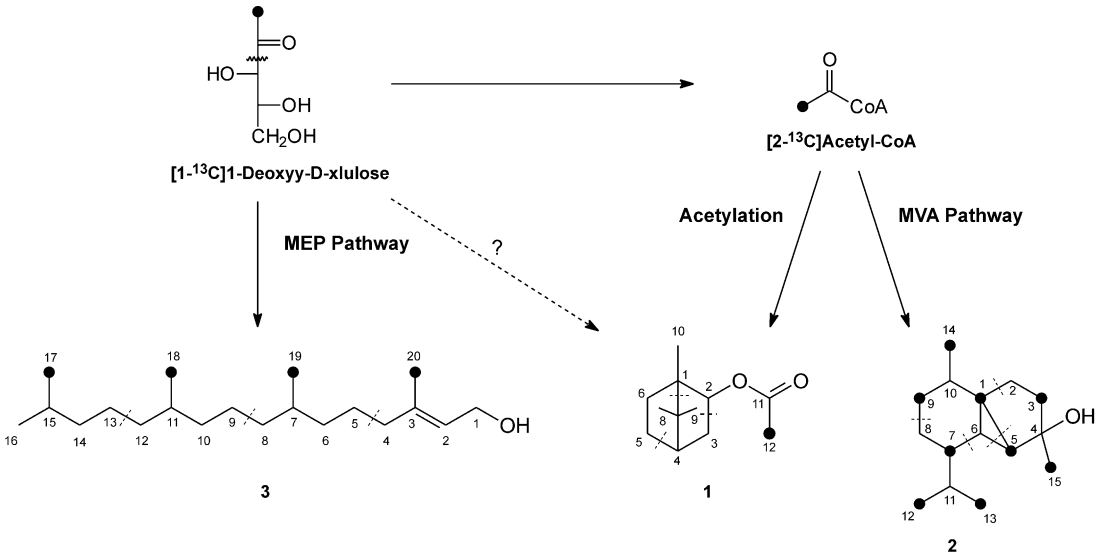

Fig. 2. Labeling patterns of bornyl acetate (**1**), cubebanol (**2**) and phytol (**3**) in *C. conicum* after incorporation of [1-^{13}C]1-deoxy-D-xylulose; ●: ^{13}C labeled position; CoA = Coenzyme A.

Table 1
^{13}C abundances of labeled cubebanol-TMSi (**2a**)

Carbon atom in IPP-units[c]	Carbon atom	δ_C	% ^{13}C[a]
1^{I}	8	25.9	1.17
2^{I}	7	43.4	**1.87**[b]
3^{I}	11	33.3	1.18
4^{I}	13	19.1	**1.93**
5^{I}	12	18.3	**1.96**
1^{II}	2	28.4	1.20
2^{II}	1	32.9	**1.86**
3^{II}	10	30.6	1.24
4^{II}	9	31.2	**1.86**
5^{II}	14	19.8	**1.93**
1^{III}	6	22.7	1.20
2^{III}	5	38.4	**1.72**
3^{III}	4	82.1	1.22
4^{III}	15	28.9	**1.93**
5^{III}	3	36.6	**1.80**
3 x TMSi-Methyl	[b]	1.8	3.32

[a] ^{13}C abundances referenced to the TMSi-signal (3×TMSi-methyl = 3.33% ^{13}C).
[b] **Bold type**: labeled positions.
[c] I, II, III: denote individual C_5 units.

Table 2
^{13}C abundances of acetylated phytol (**3a**)

Carbon atom in IPP-units[d]	Carbon atom	δ_C	% ^{13}C[a]
1^{I}	13	24.2	1.29
2^{I}	14	38.8	1.23
3^{I}	15	27.4	1.24
4^{I}	16	22.1	1.10
5^{I}	17	22.2	**2.96**[c]
1^{II}	9	23.9	1.21
2^{II}	10	36.8	1.16b[b]
3^{II}	11	32.1	1.16c
4^{II}	12	36.7	1.16b
5^{II}	18	19.2	**2.95**d
1^{III}	5	24.5	1.24
2^{III}	6	36.1	1.08
3^{III}	7	32.2	1.16c
4^{III}	8	36.9	1.16b
5^{III}	19	19.2	**2.95**d
1^{IV}	1	60.9	1.20
2^{IV}	2	117.5	1.12
3^{IV}	3	142.1	1.47
4^{IV}	4	39.3	1.22
5^{IV}	20	15.8	**2.98**
COO (acetyl)	21	170.5	1.11
CH_3 (acetyl)	22	20.5	1.13

[a] ^{13}C abundances obtained from the ^{13}C signal of C-21 referenced to 1.11% (natural abundance of the acetate residue).
[b] b, c, d: averaged values due to signal overlapping.
[c] **Bold type**: labeled positions.
[d] I, II, III, IV: denote individual C_5 units.

tion of plastidially formed borneol. It could also be possible, that the absence of an active kinase that phosphorylates 1-deoxy-D-xylulose to the true substrate prevents the incorporation of [1-^{13}C] 1-deoxy-D-xylulose. However, which of the possible processes or conditions contributes to the observed labeling pattern or to what extent, cannot be deduced from the data.

The fact that phytol and bornyl acetate are formed via the MEP pathway (as shown in the ^{13}C glucose labelling study) and a significant incorporation of [1-^{13}C]1-deoxy-D-xylulose was observed only in phytol implies the presence of two independent compartments with MEP pathway in *C. conicum*. Since the phytol is part of chlorophyll it can be assumed that its biosynthesis is localized in the chloroplasts of the green cells of the thallus. Borneol however, must be formed in a different compartment. Taking into account the morphological structure of the *C. conicum* thallus, consisting of green cells, oil body cells and rhizoids, it could be

Table 3
^{13}C abundances of labeled bornyl acetate (**1**)

Carbon atom in IPP-units[c]	Carbon atom	δ_C	% ^{13}C[a]
1^I	5	27.2	1.11
2^I	4	44.1	1.23
3^I	7	46.9	1.23
4^I	9	18.0	1.08
5^I	8	18.9	1.26
1^{II}	3	35.9	1.11
2^{II}	2	79.0	1.14
3^{II}	1	47.8	1.26
4^{II}	6	26.2	1.13
5^{II}	10	12.6	1.28
COO (acetyl)	11	170.5	1.19
CH_3 (acetyl)	12	20.5	**3.96**[b]

[a] ^{13}C abundances obtained from ^{13}C satellite analysis of C-2.
[b] **Bold type**: labeled positions.
[c] I, II: denote individual C_5 units.

hypothesized that the formation of monoterpenes occurs in the oil cells. Possibly the MEP pathway is associated with oil bodies or leucoplasts since chloroplasts are not present in *C. conicum* oil cells. An involvement of oil bodies in isoprenoid biosynthesis has been recently demonstrated for the liverwort *Marchantia polymorpha* (Suire et al., 2000). However, the intracellular localization within the oil cells cannot be derived from the incorporation data.

The quantitative differences between the labeled positions of cubebanol and the acetate residue of bornyl acetate imply that different acetyl CoA pools are utilized for the acetylation of borneol and the IPP biosynthesis of cubebanol via the MVA pathway. Therefore, it can be assumed that these two reactions occur most probably in different cellular compartments or cell types of the plant. On the assumption that bornyl acetate biosynthesis takes place in the oil cells it can be deduced that the sesquiterpene is formed in the cytoplasm of the green cells.

3. Experimental

3.1. Spectroscopy

NMR spectra were recorded in $CDCl_3$ (^1H NMR (500 MHz), ^{13}C NMR (125 MHz)) relative to $CDCl_3$ at δ_H 7.26; δ_C 77.0. ^{13}C multiplicities were determined using the DEPT pulse sequence. 2D spectra were recorded as COSY, HSQC and HMBC. Quantitative ^{13}C NMR measurements were recorded with the inverse gated decoupling pulse sequence in 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.

3.2. Plant material

The axenic culture of *C. conicum* was initiated from surface disinfected gametophytes (Adam, 1996) obtained from a native site near Clarkia, ID, USA. The cultures were grown in 250 ml Erlenmeyer flasks under continuous incandescent light (60 μmol photons m^{-2} s^{-1} photosynthetically active radiation) at 22 °C in 75 ml of modified Gamborg B5 medium (Gamborg et al., 1968) containing 10 g/l glucose. The medium was solidified with 9 g/l agar.

3.3. Labeling procedure

In labeling experiments, the culture medium contained 0.4 g/l [1-^{13}C]1-deoxy-D-xylulose and the cultures were grown for 3 months. Synthesis of [1-^{13}C]1-deoxy-D-xylulose was performed according to Kennedy et al. (1995). The ^{13}C label at position 1 was introduced by using ^{13}C labeled iodomethane in the Grignard reaction of the corresponding synthetic step.

3.4. Extraction, isolation and derivatization

Fresh plant material (460 g) was homogenized and extracted (4×) with CH_2Cl_2. After removal of the solvent, the crude extract (1.2 g) was chromatographed on silica gel (vacuum liquid chromatography, VLC) employing a hexane–EtOAc gradient. The VLC fraction containing crude **1** (2.5% EtOAc) was further purified via HPLC (250×4 mm, Si 60 Lichrospher (Merck), 5 μm, hexane–EtOAc, 50:1), yielding 80 mg of bornyl acetate **1**. HPLC separation of the VLC fraction obtained with 6.5% EtOAc yielded 11 mg of **2** (250×4 mm, Si 60 Lichrospher (Merck), 5 μm, hexane–EtOAc, 11:2). The corresponding 4-*O*-TMSi derivative **2a** was obtained by addition of 0.3 ml bis(trimethylsilyl)acet amide (BSA) to 11 mg of **2** and subsequent heating for 5 h at 80 °C. HPLC [hexane–EtOAc (98:2), 250×8 mm, Si 100 Lichrospher (Merck), 5 μm] of the reaction mixture yielded 14 mg of **2a**. For the isolation of **3**, the chlorophyll-containing fractions (>13% EtOAc) were saponified overnight at room temperature in a 6% (w/v) solution of KOH in methanol. After hydrolysis, 2 vol. of water were added and the reaction mixture was extracted 5× with CH_2Cl_2. After removal of the solvent, the mixture was separated on silica gel (VLC) employing a hexane–EtOAc gradient. The obtained *trans*-phytol **3** was acetylated overnight at room temperature (acetic anhydride–pyridine–toluene, 1:1:2, by vol.). VLC of the reaction mixture on silica gel (hexane–EtOAc 99:1) yielded 14 mg of the 1-*O* acetyl derivative **3a**.

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