PHYSIOLOGY AND BIOCHEMISTRY

K. von Schwartzenberg · W. Schultze · H. Kassner

The moss *Physcomitrella patens* releases a tetracyclic diterpene

Abstract The presence of the tetracyclic diterpene 16α-hydroxykaurane (16α-hydroxy-*ent*-kaurane, $C_{20}H_{34}O$, CAS 5524–17–4) was detected in sterile cell cultures of the moss *Physcomitrella patens* (Hedw.) B.S.G. using gas chromatography and mass spectrometry. 16α-hydroxykaurane was found to be a major lipid compound in *P. patens*, with an estimated intracellular concentration of up to 0.84 mmol/l and an extracellular concentration of up to 9.3 µmol/l. The overall content of 16α-hydroxykaurane (in milligrams) produced per culture reached 0.37-fold that of chlorophyll *a+b*. In agar cultures with low air exchange, 16α-hydroxykaurane forms needle-like crystals on tissue and on the inner surface of the culture vessels, indicating that it is being released into the atmosphere. Solid phase microextraction confirmed the air-bound release of 16α-hydroxykaurane. To our knowledge this is the first report on the release of a plant-derived tetracyclic diterpene into the air.

Keywords *Physcomitrella patens* · *Saelania glaucescens* · 16α-Hydroxykaurane · Kauranol · Diterpene

K. von Schwartzenberg (✉) · H. Kassner
Biozentrum Klein Flottbek und Botanischer Garten,
Universität Hamburg, Ohnhorststr. 18, 22609 Hamburg, Germany
e-mail: kvschwartzenberg@iangbot.uni-hamburg.de
Tel.: +49-40-42816599
Fax: +49-40-42816571

W. Schultze
Institut für Pharmazie,
Bundesstr. 45, 20146 Hamburg, Germany

Introduction

Diterpenes are present in all plants in the form of phytol, a ubiquitous constituent of chlorophyll *a* and *b*. Tetracyclic diterpenes have been reported as secondary substances for a variety of plant groups. These compounds are widespread in lichens (Lehn and Hunek 1965; Hunek 1968) and have been also detected in species of various families of higher plants—for example, Annonaceae (Takahashi et al. 2001), Asteraceae (Le Quesne et al. 1985), Labiatae (Fujita and Node 1984) and Leguminosae (Hugel et al. 1963). In the bryophytes tetracyclic diterpenes represent a large class of terpenoid compounds (Mues 2000). They mainly occur in liverworts, and novel derivatives are being discovered in searches for new bioactive compounds (Tori et al. 1993; Perry et al. 1999). In contrast, tetracyclic diterpenes are absent in hornworts and rare in mosses (Mues 2000; Asakawa 1995) although Nilsson and Martensson (1971) did describe the presence of 16α-hydroxykaurane in the moss *Saelania glaucescens*, where this compound contributes to the bluish colour of the gametophores; its presence is microscopically obvious as a white cover on leaves and stems, especially in the lower part of the plants.

Although tetracyclic diterpenes have been described for many plant species the functions of these compounds in planta are difficult to assess. We report here on the production of a tetracyclic diterpene by the moss *Physcomitrella patens*, which as a model organism has many advantages for genetic and physiological studies (Cove 2000). The feasibility of highly efficient reverse genetic approaches as well as the accessibility of large expressed sequence tag (EST) collections (Rensing et al. 2002; Schaefer 2002) could help future investigators gain valuable information on the biosynthesis and function of plant secondary substances. We describe and discuss the identification, in vitro production and release of a tetracyclic diterpene that has been found to be a major lipid compound in *P. patens*.

Materials and methods

Plant material

Wild-type *Physcomitrella patens* (Hedw.) B.S.G. was cultivated on agar-solidified ABC medium (Knight et al. 1988). Either polystyrene petri dishes sealed with Parafilm, glass jars, or glass tubes were used as in vitro culture vessels.

Funaria hygrometrica was cultivated on agar solid medium according to Hahn and Bopp (1968).

For the aerated liquid cultures we used a medium described by Wang et al. (1981): 0.359 mM Ca(NO$_3$)$_2$, 0.035 mM FeSO$_4$, 1.01 mM MgSO$_4$, 1.84 mM KH$_2$PO$_4$, 10 mM KNO$_3$; 1 ml of Hoaglents trace element solution per liter (Ashton and Cove 1977). Di-ammonium tartrate was added to a final concentration of 5 mM and the pH was adjusted to 6.5 with KOH. Initially 360 ml of culture medium were inoculated with about 300 mg of protonema filaments that had been freshly cut up with an Ultra-Turrax blender (IKA, Staufen, Germany) to filaments of 10–20 cells each. Culture flasks (1,000-ml Pyrex flasks) closed with cotton stoppers were aerated with water-saturated, sterile air (ca. 600 ml/min).

All cultures were grown at 25°C under white light (Philips TLM) at 100 µmol m^{-2} s^{-1} (400–700 nm) and a 16/8-h (light/dark) cycle. All cultures were checked regularly for microbial contamination by using agar medium containing 2% glucose (w/v).

The moss species listed in Table 1, except for *P. patens*, *F. hygrometrica* and *Saelania glaucescens*, were collected in 2002 from forests in the region of Hamburg, Germany. One sample of the moss *S. glaucescens* (Hedw.) Broth. was obtained from the Herbarium Hamburgense [Biozentrum Klein Flottbek und Botanischer Garten, Hamburg] and dated from 18 August 1903 (collected by J. Bornmüller in Austria, Tirol, Stubaier Alpen, Obernbergersee am Tribulaun, 1,600 m, 9034/1; HBG 4647). Additonal samples of *S. glaucescens* were collected and identified in August 2002 by H. Köckinger (Weisskirchen, Austria) in Austria, Kärnten, Hohe Tauern, Sadnig Gruppe 2,200–2,300 m; HBG 4649.

Preparation of diterpene compound

Extracellular diterpene from Physcomitrella agar cultures

Needle structures were washed off the *Physcomitrella* cultures growing on the agar-solidified medium in petri dishes (9.5 cm diameter) with 10 ml distilled water. The needles from ten plates were sedimented by centrifugation and dissolved in 1 ml hexane for gas chromatography-mass spectrometry (GC-MS) analysis.

Needle-like crystals with a high degree of purity were also harvested by dissolving sublimated material from the inner surface of the lid of several petri dishes or glass jars with 10 ml of absolute ethanol. The sample was dried by rotary evaporation and the residue dissolved in hexane for GC analysis and in CDCl$_3$ for NMR measurements.

Intracellular diterpene from moss tissue

Fresh tissue material (50–300 mg FW) growing on agar media or in liquid culture (or 50 mg DW of collected material) was separated from the medium and transferred into a 2-ml screw cap vial containing 30 mg of glass beads (0.25–0.5 mm in diameter; Serva, Heidelberg, Germany). After the addition of 1 ml of absolute ethanol (containing 37.5 µg cholesterol; Sigma, Taufkirchen, Germany) as an internal standard, the material was ground up in a fast prep shaker (BIO101-Qbiogene, Heidelberg, Germany) for two cycles of 45 s each at speed 4.5. Following a 10-min centrifugation at 15,000 g for 15 min, the supernatant was transferred to a new tube and the residue re-extracted with 0.6 ml of solvent. The re-extract was again centrifuged and the supernatants pooled. The supernatant was stored at –20°C until the analysis of diterpenes and chlorophylls.

Diterpene from S. glaucescens

We isolated 16α-hydroxykaurane from the moss *S. glaucescens* (Nilsson and Martensson 1971) by briefly rinsing about 10 g of dry moss tissue with 30 ml of hexane. The hexane was immediately separated from the moss tissue by filtration, centrifuged (5,000 g, 15 min) and evaporated to dryness. About 10 mg of 16α-hydroxykaurane (with approx. 90% purity as determined by GC-MS) was obtained and used as reference substance.

Diterpene from Physcomitrella culture medium

The culture medium of aerated liquid cultures was separated from the protonema by filtration through a mesh with a 50-µm pore size and 52 µg of cholesterol (Sigma, St. Louis, Mo.) was added as an internal standard. The medium was extracted twice against 30 ml hexane. The hexane phases were then combined and evaporated to dryness. These samples were stored dry at 4°C.

Detection of diterpene as a volatile organic compound in Physcomitrella cultures

A solid phase microextraction was carried out using a fiber coated with a 100-μm film of polydimethylsiloxane (SPME; Supelco, Bellefonte Pa.) by placing the fiber in the air 1 cm above the gametophores of *P. patens* cultured on agar in a sealed glass tube. The microsampler was left for 3 days in the culture vessel to accumulate organic volatile compounds. The microsampler was then introduced into a gas chromatograph injector equipped with a low-volume inlet liner (0.75 mm ID). Desorption was performed for 30 s at 240°C (injector temperature). Gas chromatography and mass spectrometry were carried out as described.

Gas chromatography/mass spectrometry

For qualitative studies a HP (Hewlett Packard, Palo Alto, Calif.) gas chromatograph (5890 Series II) coupled with a MSD 5970 and HP Chem station G1034C (software version C.03.00) were used in EI mode. The column was a 25-m fused-silica capillary column (DB-1; 0.25 mm ID × 0.25 µm film thickness; J&W Scientific, Folsom, Calif.) and was used at the following parameters: an initial temperature of 140°C for 1 min; temperature increments at a rate of 15°C/min up to 240°C; a final temperature of 240°C for 15 min; carrier gas consisted of helium 4.6 at 110 kPa column head pressure. Samples were diluted in hexane or absolute ethanol, and 2-µl aliquots were injected splitless. Mass spectra were compared to the Wiley library (V 38; Wiley, Hoboken, N.J.).

For quantification, a gas chromatograph 5890 Series II equipped with a FID and an autosampler was used. The temperature regime consisted of an initial temperature of 140°C for 1 min; temperature increments at a rate of 20°C/min up to 260°C; a final temperature of 260°C for 15 min. The carrier and make-up gas consisted of nitrogen 5.0 with a flow of 1 and 30 ml/min respectively, hydrogen 5.0 at 30 ml/min and synthetic air 5.0 at 300 ml/min. The carrier gas was used at a column headpressure of 110 kP. The injector temperature was 260°C; the detector temperature was 280°C. GC control and data processing were performed using the GC Chem station (Rev. A.08.03; Agilent technologies, Palo Alto, Calif.). Quantification was based on a standard curve obtained for a 16α-hydroxykaurane reference standard prepared from *S. glaucescens* as described above.

Nuclear magnetic resonance analysis

[^{1}H]-NMR and [^{13}C]-NMR spectra were recorded on a Bruker AMX 400 NMR spectrometer (Bruker, Rheinstetten, Germany) using CDCl$_3$ as a solvent and TMS as the internal standard.

782

Chlorophyll measurements

Chlorophyll *a* and chlorophyll *b* content were determined in the ethanol extracts of the *P. patens* tissue prepared for diterpene determination as described above. The optical density was measured at 648.6 nm and 664.2 nm, respectively, and chlorophyll *a+b* content was determined according to the equation for the solvent ethanol as described by Lichtenthaler (1987): Chl *a+b* (μg/ml) = $5.24\ OD_{664.2} + 22.4\ OD_{648.6}$

Results and discussion

Observation of crystal-like structures in *Physcomitrella* cultures

We observed the formation of needle-shaped and crystal-like structures of an unknown origin on the agar cultures of *P. patens*. The structures were first observed on moss tissue (colour photo supplied as electronic supplementary material) and on the surface of the agar medium. The needles were formed on cultures consisting of pure protonema as well as on cultures differentiated to gametophores. The number and size of the crystal-like structures increased with the amount of *P. patens* tissue grown per culture vessel. However, it was essential to seal the petri dish with Parafilm; cultures without Parafilm sealing showed only very little or no formation of the needle-like structures. No crystals were observed when *P. patens* was cultured in liquid culture (air-lift cultures).

The needles observed on the moss tissue could easily be washed off and did not seem to have a visible connection with the inside of the cells. The crystal-like structures were also found on the inner surface of the vessels used for agar culture (Fig. 1), where the compound sublimated and formed clusters 50–200 μm apart. In general, several needle-like structures originated as a bunch from a point of crystallization (Fig. 1). The diameter of the needles ranged from 0.1 μm to 6 μm as determined by scanning electron microscopy (SEM). Their length depended on the age of the cultures and could be up to 200 μm (or more) for cultures several months old. Occasionally the needle structures showed branching.

Needle-like structures isolated from moss tissue or from culture vessels were found to be soluble in ethanol (absolute and 70% v/v), methanol and acetonitrile. No visible solubilization occurred in water (20°C and 70°C), hydrochloric acid, acetic acid (100%) or 50% (v/v) ethanol.

GC-MS studies

Crystal-like structures were collected from *P. patens* tissue grown on agar and from the inner surface of the lids of culture vessels in which *P. patens* had been cultivated for 8–16 weeks. The crystal-like structures were solubilized in hexane and submitted to GC-MS analysis.
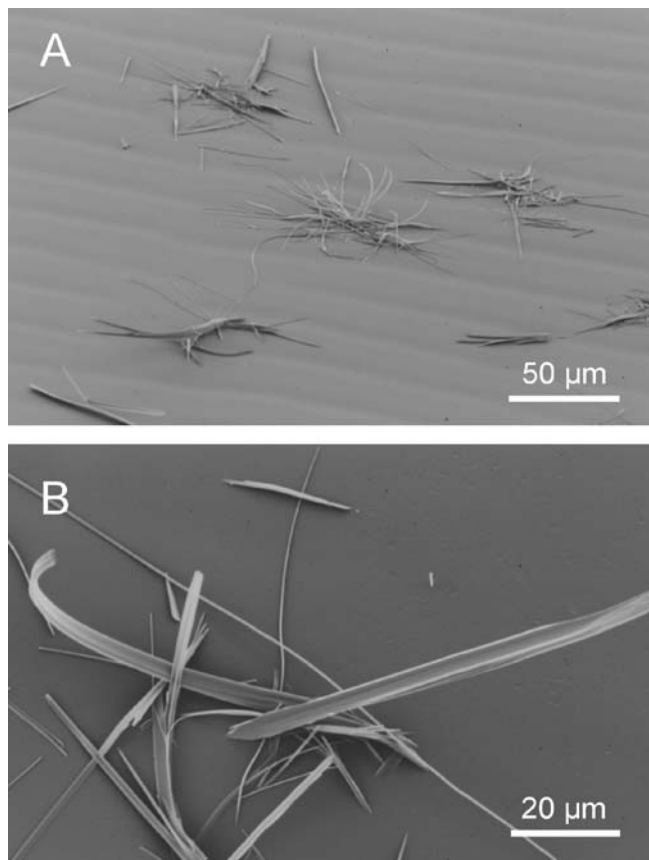

Fig. 1 Scanning electron micrographs (SEM) of sublimated 16-hydroxykaurane on the inner surface of the lid of a polystyrene petri dish of a *Physcomitrella patens* agar culture (sealed with Parafilm) after 16 weeks of growth. Pieces of the lid were sputtered with gold (Balzers SCD-050, Bla-Tec, Witten/Ruhr, Germany) and visualized using a Philips XL-20 SEM (Fei-Company, Kassel, Germany)

The total ion current chromatogram (TIC) from the crystal-like structures obtained from the lid of the culture vessel showed one major peak with approximately 90% of the total ion current (not shown). When the mass spectra were compared with the Wiley-database, this compound matched very well (99% hit quality) with that of 16α-hydroxykaurane (1), a tetracyclic diterpene ($C_{20}H_{34}O$; CAS no. 5524-17-4; Wiley entry no. 133122) with a molecular mass of 290.5 amu. We use the term 16-hydroxykaurane in the following sections of this report.

In order to further validate the chemical identity of the crystals from *P. patens*, we isolated 16-hydroxykaurane as a reference sample from the moss *S. glaucescens*, an organism previously shown to contain this compound (Nilsson and Martensson 1971) (Scheme 1).

A comparison of these two products revealed that their retention times on a GC capillary column were identical and that their mass spectra (EI) were in very good accordance (99%), thus further confirming that our compound isolated from *P. patens* was 16α-hydroxykaurane (the enantiomeric form was not determined). Characteristic ions of its mass spectrum were: m/z (rel. int.) = 290 [M]+ (11), 123 (100), 94 (77), 105 (77), 272 (71), 134
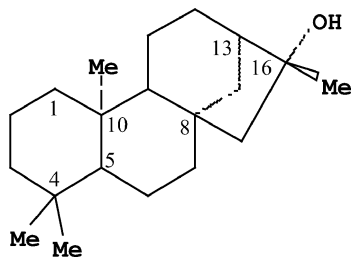
Scheme 1

(68), 257 (61), 232 (60). The mass spectra obtained matched with those for (-)-16α-hydroxykaurane as published by Hunek (1968) and Kalinowsky et al. (1970). Since the mass spectra of the 16-α and ß-hydroxy-isomers are different, we were thus able to confirm the 16α-hydroxy configuration.

16α-Hydroxykaurane was first described as kauranol by McGimpsey and Murray (1960) who detected it in extracts of *Podocarpus spicatus* (Podocarpaceae) from New Zealand. In other publications this compound has been named γ-podocarprene and γ-kaurene (Nishida and Uota 1931; Briggs et al. 1963). There is confusion in the literature concerning the nomenclature for kaurane-related compounds, as some authors including Chemical Abstracts use the name kaurane for the *ent*-kaurane enantiomere (see IUPAC Recommendations, 1999). Here we use the term 16-hydroxykaurane for the 16α-hydroxy-*ent*-kaurane compound.

[^{1}H]- and [^{13}C]-NMR spectroscopy

A [^{1}H]-NMR spectrum (400 MHz) of the 16-hydroxykaurane isolated from the inner surface of culture vessels (petri dishes and glass jars) of *P. patens* provided the following data: δ= 0.8 (3H, s), 0.84 (3H, s), 1.02 (3H, s),1.36 (3H, s), 1.55 (22H, br m). These signals correspond well to those of the reference compound isolated from the *S. glaucescens* material. The chemical shifts in the four methyl groups are in good accordance with data published on 16α-hydroxykaurane from *S. glaucescens* (Nilsson and Martensson 1971).

Additionally, we compared the [^{13}C]-NMR spectra of 16-hydroxykaurane isolated from *P. patens* and *S. glaucescens*. However, since only very small amounts of the substance from *P. patens* were available, several carbon signals were below the NMR detection limit. Nevertheless, we found the signals of 15 carbon atoms, which corresponded well to those of the *S. glaucescens* compound.

With respect to identical GC retention times and the conclusive MS- and NMR data, we are convinced, that the needle-like structures from *P. patens* are deposits of 16α-hydroxykaurane.

16-hydroxykaurane is an abundant diterpene compound in *Physcomitrella* tissue

We prepared ethanol extracts of *P. patens* tissue cultivated for 2–8 days in liquid medium (where no crystals could be seen by microscopical observation) and examined them using GC-MS. In the total ion current the major peak had a mass spectrum and retention time comparable to 16-hydroxykaurane isolated from needle-structures (not shown). Based on these criteria, we concluded that 16-hydroxykaurane is a major lipophilic compound in *P. patens* tissue.

We added cholesterol as an internal standard to the *P. patens* ethanol extract and estimated the amount of 16-hydroxykaurane by referring to a calibration curve obtained for the reference sample prepared from *S. glaucescens*. Using the approximation that 1 g (FW) of tissue equals approximately 1 ml of cell volume, we estimated that the tissue-bound concentration of 16-hydroxykaurane ranged between 0.5 m*M* and 1 m*M* (not shown).

We performed a time course experiment with eight independent liquid cultures and collected samples over a period of 8 days. During this time we monitored the fresh weight and the levels of chlorophyll *a+b* and 16-hydroxykaurane within the cultures (Figs. 2, 3). The time course of fresh weight and levels of chlorophyll *a+b* showed similar curves: the amount of tissue increased approximately 3.5-fold from approximately 0.4 g (FW) to 1.4 g (FW) per culture; the chlorophyll *a+b* content increased from 0.8 mg to 4 mg per culture (Fig. 2A). The tissue-bound amount of 16-hydroxykaurane calculated for a whole culture of 360 ml increased with the growing biomass and reached values of 0.23 mg per culture after 8 days (Fig. 2B).

Hydroxykaurane is released into the culture medium

We separated the culture medium from the moss tissue by filtration and extracted the organic compounds from the medium by two rounds of phase partitioning against *n*-hexane. Methodological tests with pure medium to which 16-hydroxykaurane had been added showed recovery rates of more than 90%. We were able to clearly demonstrate that 16-hydroxykaurane is also released into the culture medium and that it accumulates to about 1 mg per 360 ml of culture medium after 8 days of growth (Fig. 2C). In older cultures up to 3 mg 16-hydroxykaurane per 360 ml culture medium was measured (not shown). At day 8 the extracellular level of 16-hydroxykaurane was approximately 4.2-fold higher than the tissue-bound 16-hydroxykaurane, indicating that approximately 80% of the diterpene produced was located extracellularly (Fig. 2B, C).

The combined level of intra- and extracellular hydroxykaurane (in milligrams) formed per culture was calculated and related to the amount of chlorophyll (in milligrams). We found that the amount of 16-hydrox-
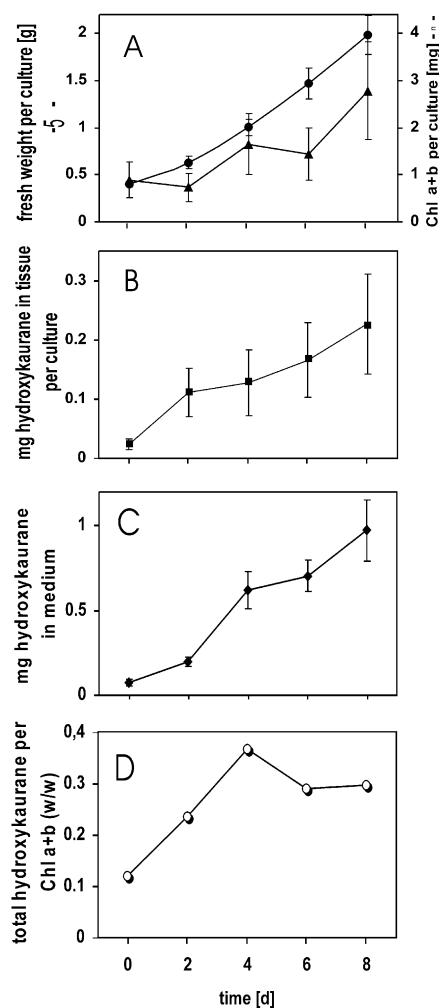
Fig. 2 Time course of fresh weight and chlorophyll *a+b* content (**A**), 16-hydroxykaurane content in tissue (**B**) and in culture medium (**C**) and the ratio of total 16-hydroxykaurane content (medium and tissue) and chlorophyll *a+b* (**D**). Liquid cultures had a volume of 360 ml. **A–C** Representative data (mean values and standard deviation) of eight independent cultures

ykaurane is about 0.37-fold the amount of chlorophyll *a+b*, as shown in Fig. 2D (day 4). These data indicate that a considerable amount of carbon is invested into the production of 16-hydroxykaurane. Consequently, this compound can be considered to be a major terpenoid lipid released into the medium of *P. patens* cultures.

The release of bioactive substances into the culture medium by *P. patens* has already been described for proteins (Neuenschwander et al. 1994) and growth regulating substances (Wang et al. 1981; Reutter et al. 1998; Schulz et al. 2000, 2001; Schwartzenberg et al. 2003). The significance of the extracellular space seems to be related to the biology of this lower plant, which lives in close contact to the substrate and does not have specialized tissues for within-plant storage.
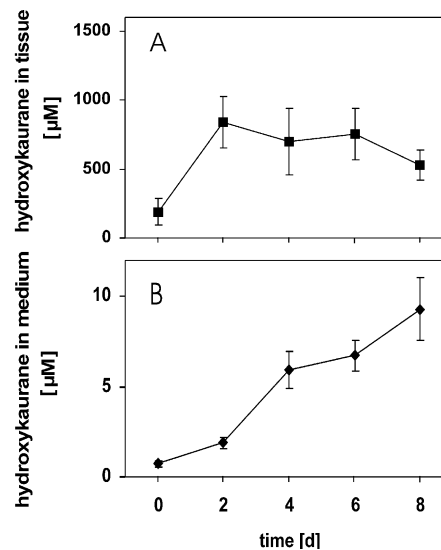

Fig. 3 A Concentration of 16-hydroxykaurane in tissue during growth of *P. patens* in liquid culture. Data were calculated with the approximation that 1 g FW of tissue equals 1 ml of tissue. **B** Concentration of 16-hydroxykaurane determined in culture medium. Values are the means and standard deviation of eight independent cultures

Intra- and extracellular distribution of hydroxykaurane

When we compared the curves for the estimated intracellular and extracellular concentrations of 16-hydroxykaurane over time we found two different slopes (Fig. 3). The intracellular concentration ranged between a minimal value of 190 μM at day 0, when the cells were washed and resuspended in fresh culture medium, and a maximum of 840 μM at day 2. The concentration of intracellular 16-hydroxykaurane apparently does not increase further with the growth time (Fig. 3A). With respect to the concentration of extracellular 16-hydroxykaurane, however, a steady increase was observed, and the levels rose from approximately 0.7 μM at day 1 to 9.3 μM at day 8. At the latter time the concentration of extracellular 16-hydroxykaurane was 57-fold lower than its estimated concentration in the tissue. Although less concentrated, the amount of 16-hydroxykaurane in the culture medium exceeds the tissue-bound amount by approximately 4.2-fold when the volume of the culture medium (60-fold greater than the tissue volume) is taken into consideration (Fig. 2B, C; day 8).

We conclude that 16-hydroxykaurane is released from the tissue to the culture medium where it apparently accumulates with increasing culture age (Fig. 3A, B).

Hydroxykaurane is a rare compound for species in the group of musci

Apart from *P. patens*, we tested 18 other species from the group of musci and analysed ethanol extracts for the presence of 16-hydroxykaurane. However, 16-hydroxykau-

Table 1 Comparative search for 16-hydroxykaurane in moss species. Fifty-milligram (DW) samples of moss were extracted with absolute ethanol and the extracts subjected to GC/MS analysis

Genus/species	16-Hydroxykaurane detectable[a]
Atrichum undulatum	–
Dicranum majus	–
D. norvegicum	–
D. scoparium	–
Hypnum judlandicum	–
H. judlandicum var. *minor*	–
Leucobryum glaucum	–
Mnium affine	–
M. hornum	–
M. undulatum	–
Plagiothecium curvifolium	–
Pleurozium schreberi	–
Polytrichum formosum	–
Rhytidiadelphus squarrosus	–
Sphagnum flagellare	–
Thuidium tamariscinum	–
Saelania glaucescens	+
Funaria hygrometrica[b]	–
Physcomitrella patens[b]	+

[a] Detection limit of approximately 420 ng/mg DW
[b] In vitro cultured material

rane was detectable only in *P. patens* and *S. glaucescens*. Surprisingly, *Funaria hygrometrica*, which belongs to the same family as *P. patens* (Funariaceae), contains no detectable amounts of hydroxykaurane (Table 1).

Hydroxykaurane is released by *Physcomitrella* as a volatile organic compound

We have demonstrated that 16-hydroxykaurane can sublimate on the inner surface of culture vessels during in vitro culture under low air exchange conditions. This can lead to the formation of visible crystals up to 200 μm in length (see Fig. 1). It is obvious that for the formation of sublimated 16-hydroxykaurane a release into the atmosphere is essential. The air-borne release of 16-hydroxykaurane was confirmed using solid phase microextraction (SPME) by placing the sampler approximately 1 cm above the gametophores of a *P. patens* agar culture. After 3 days of sampling the microsampler was directly introduced into the injector of a gas chromatograph. Under these experimental conditions a loss of water from the molecule (M-18) occurred for both the reference (*S. glaucescens*) and the sample, resulting in the mass spectrum of the dehydration product. The retention time of the dehydration product was identical for the substance isolated from *S. glaucescens* and *P. patens*. This result confirmed the release of a diterpene into the air. There is sufficient evidence that the original substance released into the air is 16-hydroxykaurane as we have shown that this compound is deposited on the inner surface of the culture vessels and the loss of water during thermodesorption is a common phenomenon. At this time we are unable to estimate the amount of 16-hydroxykaurane released into the atmosphere. To our knowledge this is the first report on the release of a plant-derived diterpene into the atmosphere.

As *P. patens* agar cultures without Parafilm sealing showed little or no wax crystals, it can be assumed that the 16-hydroxykaurane crystals, which were observed on the tissue (see ESM) and on the inner surface of the culture vessels (Fig. 1), are formed as a consequence of low air exchange rates. Presumably the compound can only visibly sublimate when its concentration is high enough and, in accordance to this, no wax crystals have ever been reported for *P. patens* growing under natural conditions (Nyholm 1954).

Possible biological functions

The biological function of 16-hydroxykaurane in *P. patens* can only be speculated. Given that this compound can not be considered a common constituent in the group of musci and that we are even unable to detect it in the closely related species *F. hygrometrica* (Table 1), we can assume that the formation of 16-hydroxykaurane is probably not essential for basic cellular functions. We also know from the emission of isoprene and monoterpenes that closely related plant species can behave in different ways (Kesselmeyer and Staudt 1999).

The possible role of 16-hydroxykaurane can be seen in the context of defence mechanisms. *Ent*-kauranes like 16-hydroxykaurane from *Solidago* species have been described as allelochemicals with insect antifeeding properties against *Trirhabda canadensis* (Le Quesne et al. 1986; Cooper-Driver and Le Quesne 1987). The release of 16-hydroxykaurane into the medium and atmosphere would be compatible with a possible role as an allelochemical in *P. patens*. However, other explanations involving 16-hydroxykaurane as a possible volatile signal molecule can not be excluded (for review, see Farmer 2001).

Our measurements of the content of 16-hydroxykaurane in culture (medium plus tissue) showed that the amount of diterpene is considerable and can reach approximately 0.4-fold the content of chlorophyll *a+b* (Fig. 2D). With respect to overall production, this value still seems to be an underestimation as it does not include the amount released into the air. This remarkable loss of reduced carbon in the form of tetracyclic diterpene could also be explained as a means to prevent the moss from photooxidation or light damage by dissipating excessive energy from the electron transport chain into organic compounds that are then released from the cells. For the emission of the monoterpene isoprene, which is widespread in higher plants and extremely common in mosses, a similar protective effect has been discussed Hanson et al. (1999).

The putative photoprotective role of diterpene release in *P. patens* is supported by the fact that this species is a seasonal moss and adapted to growing in habitats with a high light intensity—for example, on humid clayey soil, in open acres and on the moist mud by pools and streams (Amann and Meylan 1912)—and reaches maturity in seasons with a high solar radiation (Nyholm 1954).

Approaches involving the use of molecular tools available for *P. patens*, such as EST collections (Rensing et al. 2002; Nishiyama et al. 2003) and gene targeting (Egener et al. 2002; Schaefer 2002), could help produce *P. patens* mutants impaired in 16-hydroxykaurane biosynthesis in order to clarify the physiological role of the release of high amounts of tetracyclic diterpene in this model plant.

References

Amann J, Meylan C (1912) Flore des mousses de la suisse. Impremeries Reunies, Lausanne, vol 22

Asakawa Y (1995) Chemical constituents of the bryophytes. In: Herz W, Kirby GW, Moore RE, Steglich W, Tamm C (eds) Progress in the chemistry of organic natural products, vol 65. Springer, New York, pp 391–403

Ashton NW, Cove DJ (1977) The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants of the moss, *Physcomitrella patens*. Mol Gen Genet 154:87–95

Briggs LH, Cain BF, Cambie RC, Davis BR, Rutledge PS, Wilmshurst JK (1963) Diterpenes-part 7. Kaurene. J Chem Soc 1345–1355

Cove D (2000) The moss, *Physcomitrella patens*. J Plant Regul 19:275–283

Cooper-Driver GA, Le Quesne PW (1987) Dipertenoids as insect antifeedants and growth inhibitors: role in *Solidago* species. In: Waller RG (ed) Allelochemicals: role in agriculture and forestry, American Chemical Society, Washington, pp 534–550

Egener T, Granado J, Guitton MC, Hohe A, Holtorf H, Lucht JM, Rensing S, Schlink K, Schulte J, Schween G, Zimmermann S, Duwenig E, Rak B, Reski R (2002) High frequency of phenotypic deviations in *Physcomitrella patens* plants transformed with a gene disruption library. BMC Plant Biol 2:6

Farmer EE (2001) Surface-to-air signals. Nature 411:854–856

Fujita E, Node M (1984) Diterpenoids of *Rabdosia* species. Progr Chem Org Nat Prod 46: 77–157

Hahn H, Bopp M (1968) A cytokinin test with high specificity. Planta 83:115–118

Hanson DT, Swanson S, Graham LE, Sharkey TD (1999) Evolutionary significance of isoprene emission from mosses. Am J Bot 86:634–639

Hugel G, Lods L, Mellor JM, Theobald DW, Ourisson G (1963) Structure de diterpenènes isolés de *Trachylobium verrucosum*. Bull Soc Chim Fr 1974–1976

Hunek S (1968) Lichen substances. In: Reinhold L, Liwschitz Y (eds) Progress in Phytochemistry, vol 1. Interscience, London, pp 260–261

IUPAC Recommendations (1999) http://www.chem.qmw.ac.uk/iupac/sectionF/RF10.html

Kalinowsky AI, Serebryakov EP, Zolotarev BM, Simolin AV, Kucherov VF, Chizhov OS (1970) Mass spectrometry of kaurene derivatives—1. the mass spectra of some (−)-Kauran-16-ols. Org Mass Spectrom 3:1393–1400

Kesselmeyer J, Staudt M (1999) Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. J Atmos Chem 33:23–88

Knight CD, Cove DJ, Boyd PJ, Ashton NW (1988) The isolation of biochemical and developmental mutants in *Physcomitrella patens*. In: Glime JM (ed) Methods in bryology. Proc Bryol Method Workshop. Mainz Hattori Bot Lab, Nichinan, pp 47–58

Le Quesne PW, Honkan V, Onan KD, Morrow PA, Tonkyn D (1985) Oxidized kaurane derivatives from leaves of *Solidago missouriensis* and *S. rigida*. Phytochemistry 24:1785–787

Le Quesne PW, Cooper-Driver GA, Villani M, Do MN, Morrow PA, Tonkyn DA (1986) Biologically active diterpenoids from *Solidago* species—plant insect interactions. In: Rahman A, Le Quesne PW (eds) New Trends Nat Prod Chem 26:271–282

Lehn J-M., Huneck S (1965) Die erstmalige Isolierung des Diterpens (−)-16-α-Hydroxykauran aus einer Flechte. Z Naturforschung 20b:1013

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Method Enzymol 148:350–382

McGimpsey JR, Murray J (1960) Essential oils of New Zealand Podocarpaceae. II. *Podocarpus spicatus*. J Appl Chem 10:340–344

Mues R (2000) Chemical constituents and biochemistry. In: Shaw AJ, Goffinet B (eds) Bryophyte biology. Cambridge University Press, Cambridge, pp 150–181

Neuenschwander U, Fleming AJ, Kuhlemeier C (1994) Cytokinin induces the developmentally restricted synthesis of an extracellular protein in *Physcomitrella patens*. Plant J 5:21–31

Nilsson E, Martensson O (1971) Chemical studies on bryophytes—11. (−)-16-Hydroxykaurane from *Saelania glaucescens* (Hedw.) Broth. Acta Chem Scand 25:1486–1487

Nishida K, Uota H (1931) Untersuchung über das ätherische Öl aus *Podocarpus macrophylla*, Don. Bull Agric Chem Soc Jpn:7: 1, 157, 957

Nishiyama TT, Fujita T, Shin IM, Seki H, Nishide I, Uchiyama A, Kamiya P, Carninci Y, Hayashizaki K, Shinozaki Y, Kohara M, Hasebe (2003) Comparative genomics of *Physcomitrella patens* gametophytic transcriptome and *Arabidopsis thaliana*: Implication for land plant evolution. Proc Natl Acad Sci USA 100:8007–8012

Nyholm E (1954) II. Musci. In: Swedish Natural Science Research Council, The Botanical Society of Lund (ed) Illustrated moss flora of Fennoscandia. Lund, Sweden

Perry NB, Burgress EJ, Baek S-H, Weavers RT, Geis W, Mauger R (1999) 11-Oxygenated cytotoxic 8,9-secokauranes from a New Zealand liverwort *Lepidolaena taylorii*. Phytochemistry 50: 423–433

Rensing SA, Rombauts S, Van de Peer Y, Reski R (2002) Moss transcriptome and beyond. Trends Plant Sci 7: 535–538

Reutter K, Atzorn R, Hadeler B, Schmülling T, Reski (1998) Expression of the bacterial *ipt* gene in *Physcomitrella* rescues mutations in budding and in plastid division. Planta 206:196–203

Schaefer DG (2002) A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. Annu Rev Plant Biol 53:477–501

Schulz P, Reski R, Maldiney R, Laloue M, Schwartzenberg K v (2000) Kinetics of cytokinin production and bud formation in *Physcomitrella*: analysis of wild type, a developmental mutant and two of its *ipt* transgenics. J Plant Physiol 156:768–774

Schulz P, Hofmann A, Russo V, Hartmann E, Laloue M, Schwartzenberg K von (2001) Cytokinin overproducing *ove* mutants of *Physcomitrella patens* show increased riboside to base conversion. Plant Physiol 126:1224–1231

Schwartzenberg K von, Pethe C, Laloue M (2003) Cytokinin metabolism in *Physcomitrella patens*—differences and similarities to higher plants. Plant Growth Regul 39:99–106

Takahashi JA, Vieira HS, Boaventura MAD, Hanson JR, Hitchcock PB, de Oliveira AB (2001) Mono and diterpenes from seeds of *Xylopia sericea*. Quim Nova 24:616–618

Tori M, Arbyiyanti H, Taira Z, Asakawa Y (1993) Terpenoids of the liverwort *Frullanoides densifolia* and *Tricholejeunea sandvicensis*. Phytochemistry 32:335–348

Wang TL, Horgan R, Cove D (1981) Cytokinins from the moss *Physcomitrella patens*. Plant Physiol 68:735–738