

Occurrence of (–)-Geosmin and Other Terpenoids in an Axenic Culture of the Liverwort *Symphyogyna brongniartii*

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An axenic culture of *Symphyogyna brongniartii* has been initiated from spores on modified Benecke medium. Growth was best on Gamborg B5 medium with 0.3% sucrose, with a doubling time of 36 days. The culture produced the same terpenoids as plants growing in their natural habitat, perrottetianal A being the major constituent. Also found were bicyclo-germacrene, β -barbatene, δ -selinene, β -cubebene, spathulenol, and (–)-geosmin. The latter compound was hitherto only known from microorganisms. Synthesis rate of terpenoids during the passage period and quantitative differences in terpenoid production on different mineral media were investigated.

Introduction

Liverworts are a rich source of both, terpenoids and aromatic compounds [1], some of which show interesting biological activities [2]. Along with terpenoids identical or enantiomeric to those isolated from higher plants, many terpenoid skeletons unique to liverworts were found during the last two decades [3, 4].

However, the increasing interest in secondary metabolites of liverworts is often limited by the difficulty to collect enough plant material for analysis. In those cases, *in vitro* culture of liverworts offers a possibility to obtain the required amounts of plant material [5, 6].

Symphyogyna brongniartii Mont. (Pallaviciniaceae, Metzgeriales) is a thallose liverwort widespread in the submontane to subalpine zones throughout tropical America, growing dispersed on soil, rocks and rotten wood [7–9]. Hitherto no information was available on chemistry of

S. brongniartii or of other species of this genus. In the course of our phytochemical screening of panamanian liverworts, we initiated an axenic culture of *S. brongniartii*, starting from spores.

Materials and Methods

Cultivation

Symphyogyna brongniartii Mont. with developed sporophytes was collected in November 1987 in Fortuna region (8°45' N, 82°15' W), Chiriquí province, Western Panamá. Voucher specimens are deposited in the herbaria of the Departamento de Botánica, Escuela de Biología, University of Panamá and Institut für Pharmakognosie und Analytische Phytochemie, Universität des Saarlandes, Germany.

Mature but still closed capsules of *Symphyogyna brongniartii* were surface-sterilized by subsequent treating with aqueous solutions of 0.1% benzalconium chloride for 10 min, and 2% sodium hypochlorite for 5 min. Capsules were washed three times with sterile water, opened under aseptic conditions, and the spores were sowed onto a modified Benecke agar medium (milligrams per liter of medium): KH_2PO_4 (136), KNO_3 (100), $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$ (10.6), $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ (10),

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$\text{CuSO}_4 \times 5\text{H}_2\text{O}$ (0.055), $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ (0.055), H_3BO_3 (0.614), $\text{MnCl}_2 \times 4\text{H}_2\text{O}$ (0.389), $\text{CoCl}_2 \times 6\text{H}_2\text{O}$ (0.055), KI (0.028), $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$ (0.025), $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ (13.9), $\text{Na}_2\text{-EDTA}$ (18.7), glucose (3000) and Difco-Agar (10000). Further culture experiments were based on Gamborg B5 medium [10] with the trace elements of Murashige and Skoog [11]. In all cases pH was adjusted to 6.3 before autoclaving. Cultures were kept in 200 ml Erlenmeyer flasks at $22^\circ\text{C} \pm 1.5^\circ$, and 12 h illumination per day with white fluorescent tubes (2000 lux). Experiments for growth characteristics were performed with $3\text{ g} \pm 0.2\text{ g}$ inoculum per flask, which contained 70 ml medium. Every 5 or 10 days, respectively, four flasks were harvested, fresh and dry weight (room temperature, 48 h) were determined separately for each flask, and the pH of the remaining agar was measured after homogenization with demineralized water.

Isolation and structure elucidation

The air-dried plant material (224 g) was extracted three times with CH_2Cl_2 using an Ultraturrax homogenizer. The resulting crude extract (6 g) was separated by vacuum liquid chromatography [12] on silicagel with a hexane-ethylacetate gradient (0–50%) to divide into 4 fractions. From fraction 1 (100% *n*-hexane), the sesquiterpene hydrocarbons bicyclogermacrene (**1**) [13], δ -selinene (**2**) [14], β -barbatene (**3**) [3] and β -cubebene (**4**) [15] were identified by GC-MS and comparison of the mass spectra with literature data and, in the case of **1–3**, additionally by comparison with authentic samples. Fraction 2 was rechromatographed on Sephadex LH-20 (CH_2Cl_2 –MeOH 1:1), followed by HPLC (Lichrospher Si 60, *n*-hexane–EtOAc 94:6) to give spathulenol (**5**) [16] (3 mg) and (–)-geosmin (**6**) [17, 18], (27 mg) $[\alpha]_D^{20} = -10.32^\circ$; EI-MS, 70 eV: 182 (M^+ , 7), 149 (6), 126 (13), 125 (15), 112 (100), 97 (10), 55 (17). ^1H NMR (δ_{H} , 400 MHz, CDCl_3): 0.75 (d, H-12), 1.00 (s, H-11), 1.63 (m, H-6). ^{13}C NMR (δ_{C} , 100 MHz, CDCl_3): 14.9 (q), 20.3 (q), 20.7 (t), 20.8 (t), 21.4 (t), 29.9 (t), 30.5 (t), 34.3 (d), 35.1 (t), 35.7 (t), 37.3 (s), 74.5 (s). Column chromatography of fraction 3 on silicagel with *n*-hexane-ethylacetate (0–15%) and Sephadex LH-20 (CH_2Cl_2 –MeOH 1:1) afforded 300 mg of a triglyceride, which was not further examined. From fraction 4, (+)-perrottetianal A (**7**) [19] (1600 mg) was obtained by column chromatogra-

phy on Sephadex LH-20 with CH_2Cl_2 –MeOH 1:1.

Quantitative analysis of the major terpenes

Quantitative analysis of **1**, **3**, **5** and **7** was carried out by capillary gas chromatography, with cedrol (Roth, Karlsruhe) as internal standard.

Sample preparation

To 200 mg of the dried plant 50 μl of the solution of the standard in CH_2Cl_2 (1 mg/ml) and 40 ml CH_2Cl_2 were added and the mixture twice homogenized for 30 sec at an interval of 5 min. The solution was filtered and the solvent evaporated. The remaining crude extract was chromatographed on a silica gel column (6 $\times$ 30 mm) with CH_2Cl_2 –EtOAc 99:1 until the chlorophyll eluted. The resulting terpene fraction was dissolved in 300 μl of CH_2Cl_2 and submitted to GC. From each extract, three samples were injected (1 μl) and the values averaged.

GC conditions

GC 6000 Vega Series 2 (Carlo Erba Strumentazione, Milano), split injector 1:20, detector FID, Integrator SP 4290 (Spectra Physics INC, San José, C.A., U.S.A.), capillary column fused silica DB1, 30 m $\times$ 0.25 mm (J & W Scientific, Folsom, C.A., U.S.A.), carrier gas He, 155 kPa; temperature program: 130–150 $^\circ\text{C}$, $2^\circ/\text{min}$, 150–250 $^\circ\text{C}$, $7^\circ/\text{min}$, 250 $^\circ\text{C}$ 5 min isotherm, 250–285 $^\circ\text{C}$, $20^\circ/\text{min}$, 285 $^\circ\text{C}$ 12 min isotherm. Retention times (response factors) of the compounds: (–)-geosmin 6.68 min (1.241), β -barbatene 7.84 min (0.781), bicyclogermacrene 9.20 (0.678), cedrol 12.12 min (1.000, int. standard), (+)-perrottetianal A 27.33 min (1.468).

Results and Discussion

Ten days after sowing the spores onto modified Benecke agar medium, germination was observed. The developing plantlets showed a fairly different aspect from those growing in nature as the thallus wings of the pale green liverwort were extremely reduced so that the whole plant consisted almost only of the midrib. The plants showed a thread-like aspect, and formed spherical aggregates on the surface of the agar medium. Growth was rather

slow, with a doubling time of 61 days (dry weight 6.3% of fresh weight).

In order to increase growth, the medium composition was varied. In experiments with other liverworts, *e.g.* *Ricciocarpos natans* [20] and *Fossombronina pusilla* [21], the Gamborg B5 medium [10] had good results. The main difference in the two media is the carbon source, which is 0.5% glucose for Benecke and 2% sucrose for Gamborg B5, and the nitrogen source, which is about 1 mmol nitrate for Benecke and about 25 mmol nitrate plus 2 mmol ammonium for Gamborg B5. On the latter medium, however, *Symphyogyna brongniartii* died within four weeks. In a following experiment nitrate concentration of Gamborg B5 medium was reduced to 1 mmol the same as in Benecke medium. Under these conditions plants survived showing a yellowish color and a dense rhizoid cover, but growth was extremely poor.

Low nitrate (1 mmol) and reduction of sucrose concentration to 0.3% led to an appearance and a growth rate of the culture similar to that on modified Benecke medium (doubling time 60 days).

Growth was best on Gamborg B5 medium with an unchanged mineral composition but with a sucrose concentration reduced to 0.3%. Under these conditions, a doubling time of 36 days could be reached, which is, compared with other liverworts [20, 21], still a relatively slow growth. The plants showed a dark green color and the thalli were clearly differentiated into midrib and thallus wings, although the latter were smaller than in the

naturally growing plant (dry weight 5.2% of fresh weight). On this medium, it was possible to produce within 18 months 4350 g fresh material of *Symphyogyna brongniartii* for phytochemical investigation.

The corresponding growth curve (Fig. 1) does not show the sigmoid shape of quickly growing cultures of other plants or microorganisms, but a nearly linear rise. This is certainly due to the fact that growth is relatively poor and a shortage of nutrients at the end of the passage does not occur. The limiting factor of passage length is most likely the loss of water from the medium. During the whole passage, the pH was nearly stable at 5.8 ± 0.2 .

Addition of cyanocobalamine (vitamin B₁₂, 12 µg/l), which was found to act as a growth stimulator in the liverworts *Scapania nemorea*, *Jungermannia leiantha*, *Gymnocolea inflata* [22], and *Fossombronina pusilla* [21] did not have any effect on growth, differentiation or terpene production (Fig. 2) of the culture of *Symphyogyna brongniartii*.

The dichloromethane extract of the air-dried plant material was prefractionated by vacuum liquid chromatography on silica gel with a *n*-hexane-ethylacetate gradient, followed by column chromatography on Sephadex LH-20 and HPLC on silica gel.

The first fraction contained the sesquiterpene hydrocarbons bicyclogermacrene (1) [13], δ -selinene (2) [14], β -barbatene (3) [3], and

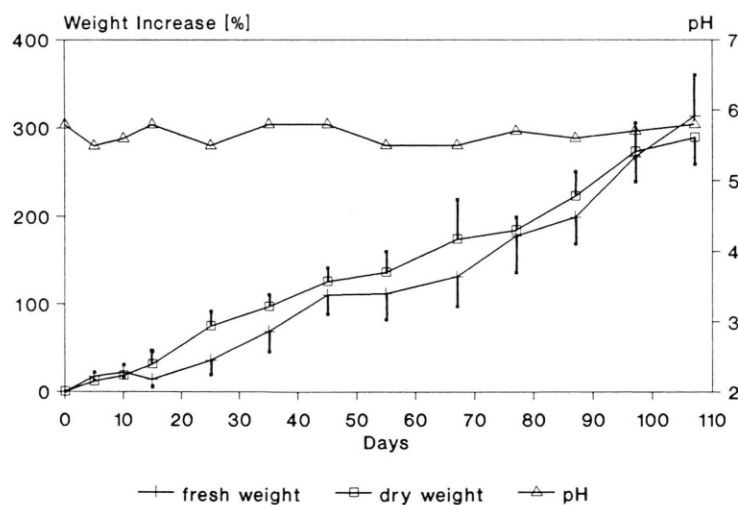


Fig. 1. Growth of an axenic culture of *Symphyogyna brongniartii* and changes in the pH of the medium. Gamborg B5 medium, 0.3% sucrose. Standard deviation bars are only drawn in one direction.

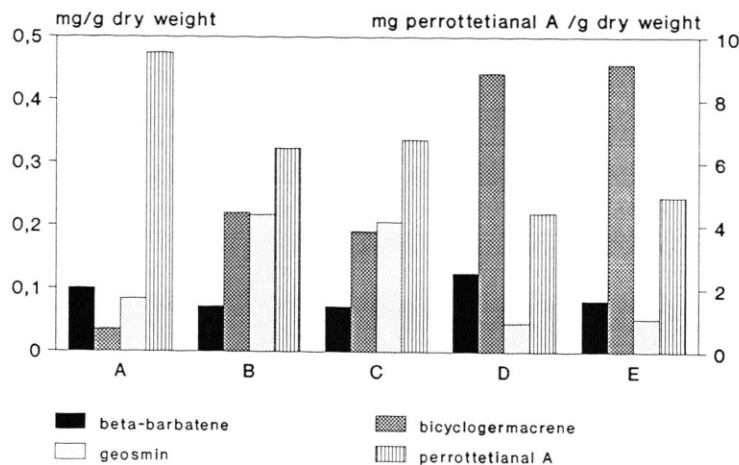
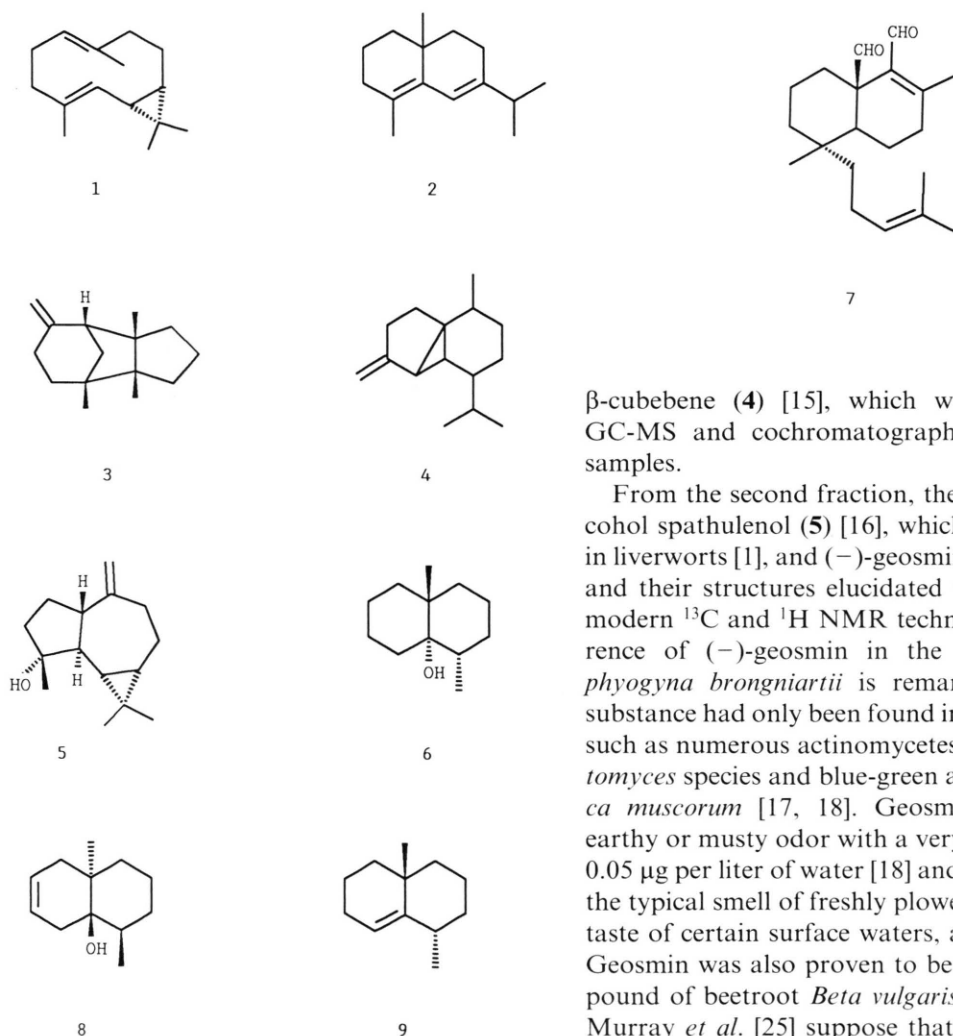


Fig. 2. Effects of medium variation on the terpenoid production of an axenic culture of *Symphyogyna brongniartii*. A = mod. Benecke medium; B = Gamborg B5 medium, 0.3% sucrose; C = Gamborg B5 medium, 0.3% sucrose, 12 µg/l vitamin B12; D = Gamborg B5 medium, 0.3% sucrose, 1 mmol NO₃⁻; E = plant growing in nature.



β-cubebene (4) [15], which were identified by GC-MS and cochromatography with authentic samples.

From the second fraction, the sesquiterpene alcohol spathulenol (5) [16], which is widely spread in liverworts [1], and (–)-geosmin (6) were isolated and their structures elucidated with IR, MS and modern ¹³C and ¹H NMR techniques. The occurrence of (–)-geosmin in the culture of *Symphyogyna brongniartii* is remarkable, since this substance had only been found in microorganisms, such as numerous actinomycetes, especially *Streptomyces* species and blue-green algae, e.g. *Symploca muscorum* [17, 18]. Geosmin has a strong, earthy or musty odor with a very low threshold of 0.05 µg per liter of water [18] and is responsible for the typical smell of freshly plowed soil, the muddy taste of certain surface waters, and trout [23, 24]. Geosmin was also proven to be an odorous compound of beetroot *Beta vulgaris*, but in this case, Murray *et al.* [25] suppose that the compound is

taken up by the plant from the soil. Recently the related dehydrogeosmin (**8**) was found to be a constituent of the flower scent of various Cactaceae [26], which, like *Beta vulgaris*, belong to the Caryophyllales. From the liverworts *Bazzania fauriana* and *B. angustifolia* (Lepidoziaceae, Jungermanniales), the dehydration product of geosmin (**9**), was also isolated [27].

The diterpene dialdehyde perrottetianal A (**7**) formed the major constituent of the lipophilic extract of *Symphyogyna brongniartii*. This substance, hitherto only known from liverworts, has been isolated for the first time from *Porella perrottetiana* (Porellaceae, Jungermanniales) [28]. Later it was also found in other *Porella* species as well as in *Makinoa* species [1] and *Fossombronia pusilla* [21], the latter two belonging to the Metzgeriales.

Quantitative analysis of **1**, **3**, **5**, and **7** was done to determine changes in synthesis rate of terpene metabolites during the culture passage and to compare the terpene spectra of cultures grown on the different media and of wild material.

As could be expected from the growth curve, there were no significant changes in terpene synthesis rate, neither on the B5 medium with 0.3% sucrose nor on the other media during on passage. This supports the assumption that, due to the slow growth, no major changes in medium composition occur, and the plant does not have to adapt to new conditions, which would be accompanied by changes in secondary metabolism.

Comparing the quantitative terpene composition of *Symphyogyna brongniartii* grown on the different media and of the wild plant, pronounced differences are detected (Fig. 2): Reduction of the nitrate content in the B5 medium from 25 mmol to 1 mmol resulted in an increased synthesis of sesquiterpene hydrocarbons (**1**) and (**3**) (Fig. 2B, D). Terpene composition on the B5 medium with 1 mmol nitrate and 0.3% sucrose was most similar to the naturally growing plant (Fig. 2D, E). On all media, perrottetianal A was synthesized in a very high amount, compared to other liverworts [21]. Synthesis rate was highest on Benecke medium (9.8 mg/g of dry plant) reaching nearly double of the content found in the wild growing plant, but the sesquiterpene content was rather low on this medium. The Gamborg B5 medium with 0.3% sucrose finally offered the best possibility to grow *Symphyogyna brongniartii* for the isolation of terpenoids, since growth was optimal and all terpenoids found in the naturally growing plant were synthesized in appreciable amounts.

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