Bisbibenzyl Formation in Aseptic Cultures of *Marchantia polymorpha* L.

Klaus-Peter Adam and Hans Becker*

Fachrichtung 12.3 der Universität des Saarlandes, Pharmakognosie und Analytische Phytochemie, D-66041 Saarbrücken, Bundesrepublik Deutschland

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Dedicated to Prof. Huneck on the occasion of his 65th birthday

Marchantia polymorpha, Liverwort, *in vitro* Culture, Bisbibenzyl Formation

Three new aseptic cultures of *Marchantia polymorpha* from different collecting sites have been induced from the gametophytes. The formation of bisbibenzyls in differentiated cultures and cell suspension cultures was investigated. We observed that the production of the main bisbibenzyl, Marchantin A was induced by lack of nitrate and the addition of cupric sulfate to the medium. The comparison of bisbibenzyl patterns in three different cultures suggest the existence of chemical races of *M. polymorpha*. Bisbibenzyl patterns in differentiated cultures and cell suspension cultures appeared to be similar.

Introduction

Marchantia polymorpha L. (Marchantiales) is a widely distributed thalloid liverwort containing bisbibenzyls as characteristic phenolic constituents [1]. The qualitative bisbibenzyl pattern of plant material obtained from different collecting sites is well investigated [2–4]. Marchantin A (**4**), the main compound of many samples exhibits cytotoxic effects against tumor cells, antimicrobic and muscle relaxing activity [5, 6].

This paper describes the identification and quantitative determination of five bisbibenzyls in axenic cultures of *M. polymorpha*. *In vitro* cultures are very useful in the investigation of the formation of this interesting group of secondary metabolites. They offer the great advantage of studying product formation under well defined and reproducible conditions. Effects of nutrients, organic and inorganic elicitors on the bisbibenzyl content were examined.

Materials and Methods

Plant material and culture conditions

Sterile cultures of *Marchantia polymorpha* L. were initiated by surface disinfection of gametophytes [7] obtained from Börfink (B), Dietrichingen (D) and the green house of the Botanical Garden of the University of Heidelberg (HD). Cultures were grown in 200 ml flasks under constant illumination (2000 Lux) on Gamborg B 5 liquid medium without phytohormons, containing 2% sucrose [8]. For the different experiments the composition of the nutrient media was changed: 10% P of the basic medium, 10% N of the basic medium, 5 g/l yeast extract, 4 g/l chitosan, 1 mM $CuSO_4$. To obtain cell suspension cultures 1 ppm 2.4 D was added to the medium. The unchanged medium was used as control in all experiments. Cultures of *Marchantia plicata*, *Marchantia berteroana* and *Marchantia chenopoda* were also available in our institute.

Voucher specimen are deposited in the "Herbarium des Fachbereich Botanik der Universität des Saarlandes", Saarbrücken.

Isolation and identification of bisbibenzyls

Cultured plant material was extracted with Et_2O. After removal of the solvent, the residue was fractionated on silica gel using vacuum liquid chromatography [9] with a hexane/ethylacetate gradient (0–40% EtOAc (*v*/*v*)). The resulting four fractions were chromatographed on Sephadex LH-20. Fraction 1 gave compound **5** after HPLC on Lichrospher RP 18 ($MeCN/H_2O$ (60 + 40 volume parts)). Compound **4** was obtained by HPLC from fraction 2 with the same solvent system as for fraction 1. Compounds **2** and **3** were isolated from fraction 3 by HPLC with $MeCN/H_2O$ (45 + 55 volume parts) as solvent. **1** was obtained directly from the Sephadex LH-20 separation.

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Reprint requests to Prof. Dr. H. Becker.

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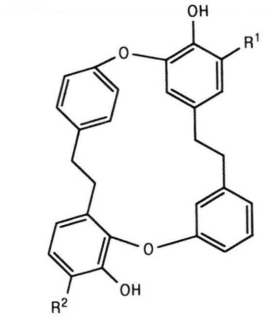
Quantitative determination

Bisbibenzyls. Cultured plant material (40 mg dry weight) was extracted with hot MeOH. After removal of the solvent *in vacuo,* the residue was redissolved in 0.5 ml MeOH/H_2O (80 + 20 volume parts) and passed through a Merck Adsorbex C_{18} cartridge (100 mg) using the same solvent system in order to remove non polar compounds and chlorophyll. The eluate (2 ml) containing the bisbibenzyls was analyzed by HPLC using a Lichrospher RP 18 column (4 × 250 mm) and MeCN/H_2O (172 g + 270 g) containing 0.01% H_3PO_4 (*w*/*w*) as solvent and monitoring with UV at 275 nm. Carvacrol (Serva, Heidelberg) was used as internal standard.

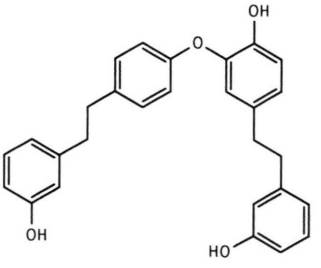
Nitrate. Nitrate content of the media was determined electrometrically using a nitrate selective electrode.

Results and Discussion

Compounds **1**–**5** (Fig. 1) were isolated from the diethylether extract of *M. polymorpha* and identified as Marchantin A, Marchantin B, Marchantin C, Marchantin H and Perrottetin E, according their ^1H NMR, ^{13}C NMR and MS data [9, 10]. An analytical HPLC method for the quantification of those five compounds was established as a combination of a solide phase extraction on reversed silicagel as sample preparation and a RP HPLC separation and quantification method.



	R^1	R^2
1 Marchantin B	OH	OH
2 Marchantin A	OH	H
3 Marchantin H	H	OH
5 Marchantin C	H	H



4 Perrottetin E

Fig. 1. Bisbibenzyls from *Marchantia* species.

Comparison of different Marchantia cultures

The HPLC quantification method was used for the comparison of different *Marchantia* cultures.

The different bisbibenzyl patterns found in three cultures (HD, D, B) grown under the same conditions, reflect the chemical variability of *M. polymorpha* (Fig. 2).

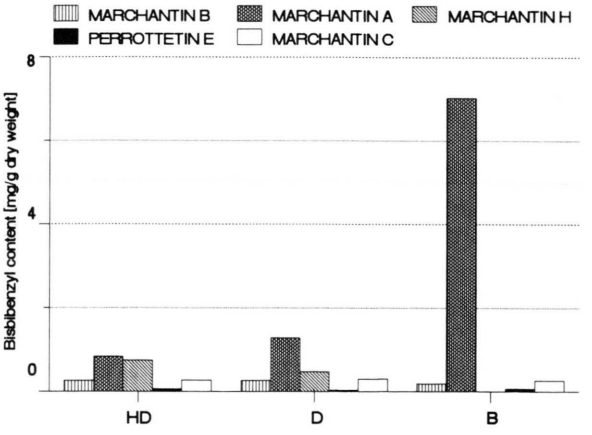


Fig. 2. Bisbibenzyl pattern of different sterile cultures of *M. polymorpha.*

Furthermore the bisbibenzyl content of differentiated cultures and cell suspension cultures of the origin "HD" were compared. The five bisbibenzyls investigated could be traced in similar amounts in differentiated cultures as well as in cell suspension cultures (Fig. 3).

The analysis of three other *Marchantia* species, *M. plicata, M. berteroana* and *M. chenopoda,* demonstrated that the HPLC method is also useful as a screening method for bisbibenzyls in liverworts.

Marchantia plicata and *Marchantia berteroana* contain Marchantin A as the main compound. The main bisbibenzyl of *Marchantia chenopoda* is Marchantin C.

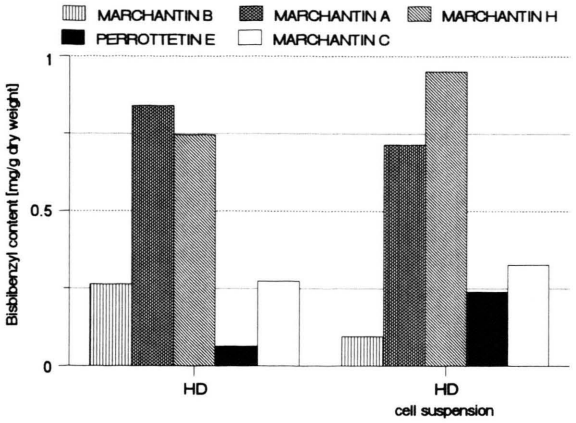


Fig. 3. Comparison of bisbibenzyl content in differentiated cultures and cell suspension cultures of *M. polymorpha*.

Constitutive formation of bisbibenzyls

Fig. 4 shows the bisbibenzyl profile in the differentiated cultures (HD) during growth for 8 weeks. Perrottetin E, Marchantin C and Marchantin B concentrations remain at a low level. After an initial increase caused by the subculturing stress Marchantin H content reaches a constant level of about 0.65 mg/g dry weight. The most interesting time course shows the Marchantin A content.

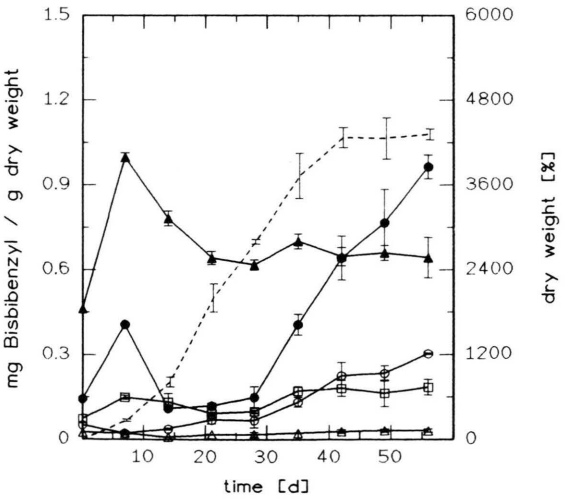


Fig. 4. Growth and bisbibenzyl content in *M. polymorpha* cultures (Inoculum 0.028 g dry weight = 100%). (– – –) Dry weight, (△—△) Perrottetin E, (●—●) Marchantin A, (▲—▲) Marchantin H, (○—○) Marchantin B, (□—□) Marchantin C.

After a relative maximum level on the 8th day of growth also caused by subculturing, Marchantin A accumulation starts during exponential growth phase after 28 days and is maintained in the stationary phase. This observation suggests that nutrient limitation might stimulate the Marchantin A accumulation.

To investigate the influence of nutrient limitation, media with reduced contents of phosphate, nitrate and glucose were examined and compared

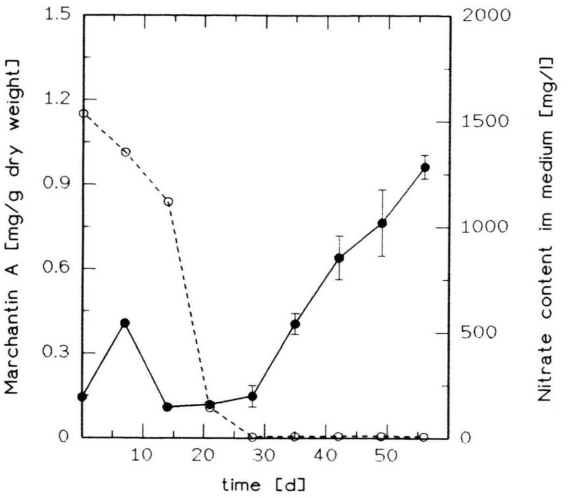


Fig. 5A. Marchantin A content and nitrate content during growth of *M. polymorpha*, unmodified medium. (●—●) Marchantin A; (○—○) nitrate.

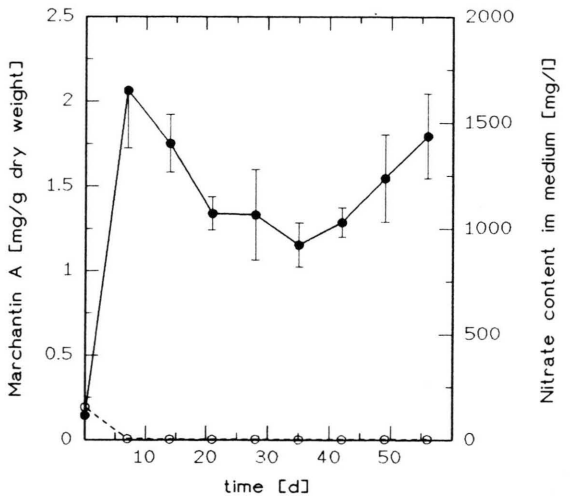


Fig. 5B. Marchantin A and nitrate content during growth of *M. polymorpha* in nitrate reduced medium. (●—●) Marchantin A, (○—○) nitrate.

with the non-modified medium. Lack of phosphate and glucose did not have any significant effect on the bisbibenzyl content. As demonstrated in Fig. 5, nitrate limitation is responsible for the Marchantin A accumulation. In the unmodified medium, Marchantin A started to accumulate as soon as nitrate was depleted (Fig. 5A). Immediately after subcultivation of *M. polymorpha* in a nitrate reduced medium (containing only 10% of the normal nitrate amount) a high concentration of Marchantin A was observed (Fig. 5B). These results show the accumulation of Marchantin A to be inverseley correlated with the presence of nitrate in the medium.

Influence of organic and inorganic elicitors

Yeast extract contains glucans which can elicit the formation of new phenolic secondary metabolites [11] or stimulate the production of constitutive phenolics such as rosmarinic acid in *Orthosiphon aristatus* cell cultures [12]. Chitosan is also known as elicitor [13] as well as cupric sulfate [14].

The effects on bisbibenzyl production of yeast extract, chitosan, and cupric sulfate have been tested. Yeast extract and chitosan did not affect the bisbibenzyl content.

As shown in Fig. 6, addition of cupric sulfate to the medium at day 28 of growth, in a final concentration of 1 mM induced a strong formation of Marchantin A in comparison to the control. The content of Marchantin A increased within two days from 0.45 mg/g to 1.1 mg/g dry weight. This formation was selective for Marchantin A compared to the other bisbibenzyls.

From these results it can be concluded that Marchantin A is a secondary metabolite with biological importance for the plant. Generally liverworts do not possess lignified tissue as mechanical barriers against microbial destruction. Furthermore, liverworts are in strong competition with microbes in their natural habitat. The formation of the cytotoxic and antimicrobial Marchantin A might be explained as a protective reaction against microbial destruction under stress conditions.

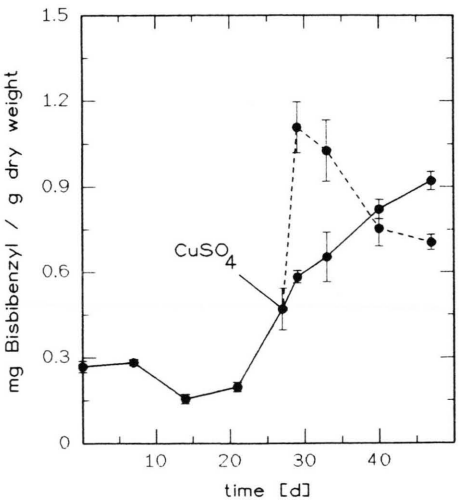


Fig. 6. Marchantin A content after addition of $CuSO_4$ to the medium. (●—●) Control, (●– – –●) with $CuSO_4$.

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