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Biosynthesis of cyclic bis(bibenzyls) in *Marchantia polymorpha*

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Abstract

Using sterile thallus tissue of *Marchantia polymorpha*, the biosynthesis of the cyclic bis(bibenzyl) marchantin A was investigated. The synthesis of the radioactively and ^{13}C-labelled precursors for the application experiments is described. Feeding experiments showed that rings A and C of the marchantin molecule are derived from the benzene ring of L-phenylalanine via *trans*-cinnamic acid and *p*-coumaric acid. Further application of ^{13}C-labelled precursor with subsequent ^{13}C NMR spectroscopy proved that dihydro-*p*-coumaric acid is an intermediate in marchantin biosynthesis. A phenylpropane/polymalonate pathway using dihydro-*p*-coumaric acid and acetate/malonate is proposed for the biosynthesis of the bibenzyl monomers which were confirmed to be the building blocks of the marchantin molecule. The bibenzyls in turn are coupled in a unique way to form the bis(bibenzyl) structure. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: *Marchantia polymorpha*; Marchantiaceae; Biosynthesis; Bibenzyls; Cyclic bis(bibenzyls); Marchantin A; Precursor feeding experiments

1. Introduction

The liverwort *Marchantia polymorpha* L. has been used as a diuretic (Asakawa, 1995) in traditional medicine for hundreds of years. Its pharmaceutical potency, however, was only recognized during the last three decades. Phytochemical investigations showed that besides terpenoids and flavonoids, bibenzyls and cyclic bis(bibenzyls) are also present in this plant (Asakawa, 1995). The most prominent examples of the group of bibenzyls are lunularic acid (Pryce, 1971a), its decarboxylation product lunularine (Pryce, 1972) and the 'prearomatic' precursor prelunularic acid (Ohta, Abe, Komura & Kobayashi, 1983).

Most interesting with regard to the biological activity of *M. polymorpha* is the group of cyclic bis(bibenzyls) which were first isolated by Asakawa, Tokunaga, Toyota, Takemoto and Suire (1979); Asakawa, Tori, Takikawa, Krishnamurty and Kanti Kar (1987) and later proved to be characteristic of liverworts (Asakawa, 1995). These substances contain two bibenzyl moieties which are condensed by ether bridges and/or C–C bonds (Asakawa, 1995). Marchantin A, the first bis(bibenzyl) characterized (Fig. 1), shows interesting pharmaceutical properties. Its cytotoxic activity may be utilized for anticancer therapy (Asakawa, Toyota, Tori, Fujiki, Suganuma et al., 1985; Asakawa, 1990; Tori, Masuya, Takikawa, Toyota, & Asakawa, 1986). In addition, its antibacterial effect (Asakawa et al., 1985; Tori et al., 1986), especially against the drug resistant bacterium *Pseudomonas aeruginosa* (Kámory, Keseru & Papp, 1994), and its antifungal activity (Asakawa, 1990), seem to be promising for pharmaceutical application.

In 1982, Asakawa and Matsuda proposed that cyclic bis(bibenzyls) may be synthesized from bibenzyls (Asakawa & Matsuda, 1982), which chemically correspond to dihydrostilbenes. The biosynthesis of stilbenes, in turn, was investigated by feeding radioactively labelled precursors. These experiments demonstrated the incorporation of acetate, *p*-coumaric acid (Billek & Schimpl, 1962) and phenylpropane derivatives (Billek & Ziegler, 1962) into the compounds under investigation. Furthermore, labelled acetate and tyrosine were incorporated into the 2-carboxy-stilbenes hydrangenol and hydrangenic acid (Billek & Kindl,

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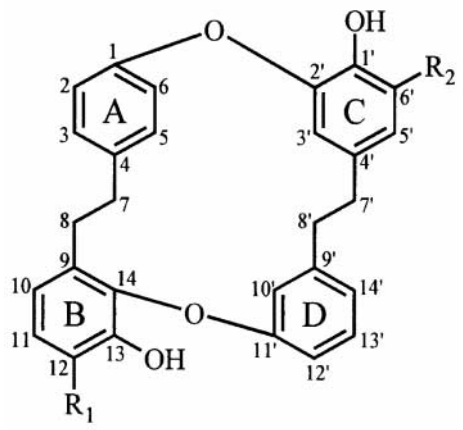

Fig. 1. Structures of the cyclic bis(bibenzyl) isomers marchantin A and marchantin H.

1962), which in turn were assumed to be precursors to the bibenzyl lunularic acid (Pryce, 1971b).

A first hypothesis for the biosynthesis of the bibenzyl lunularic acid was presented by Pryce (1971b). Based on feeding experiments he proposed a phenylpropane/polymalonate pathway starting from L-phenylalanine, which is metabolized to cinnamic acid and then to *p*-coumaric acid. This intermediate should condense with malonyl CoA to form hydrangenol, the proposed immediate precursor to lunularic acid.

A new perspective on the biosynthesis of lunularic acid emerged from the finding that *M. polymorpha* contains prelunularic acid in considerable amounts (Ohta, Abe, Komura & Kobayashi, 1983; Abe & Ohta, 1984), while hydrangenol was not detectable in liverworts (Pryce, 1971b). Therefore, prelunularic acid is probably a precursor to lunularic acid (Ohta, Abe, Komura & Kobayashi, 1983). Since prelunularic acid is a bibenzylic compound, its synthesis may proceed via dihydro-*p*-coumaric acid as has been demonstrated for bibenzyl-derived natural compounds of Orchidaceae and Dioscoreaceae (Fritzemeier & Kindl, 1983; Fritzemeier, Kindl & Schlösser, 1984). In this way, dihydro-*p*-coumaric acid may take part in the bibenzyl biosynthesis of *M. polymorpha*.

The committed precursors for the biosynthesis of the phenylpropane moiety of the bibenzyl molecule are up to now not identified. Detection of the enzymes phenylalanine ammonia lyase (Gorham, 1977; Löffelhardt, Ludwig & Kindl, 1973), tyrosine ammonia lyase (Rudolph, 1990) and cinnamic acid-4-hydroxylase (Gorham, 1978) in *M. polymorpha*, however, made a phenylalanine- or tyrosine-derived synthesis conceivable.

In order to verify which of these possible compounds take part in the biosynthesis of cyclic bis(bibenzyls), radioactively labelled potential precursors were synthesized and applied to *M. polymorpha* plant material. Feeding of ^{13}C-labelled precursors and NMR analysis of the isolated product subsequently proved the participation of the postulated precursors in the biosynthesis of marchantin A.

2. Results

2.1. Synthesis of labelled precursors

Prior to the application experiments it was necessary to isolate or to synthesize the previously suggested potential precursors in labelled form. In particular, the feeding of labelled prelunularic acid and lunularic acid (Fig. 2) should give a clue as to which of these substances is a precursor to the group of marchantins.

Since a chemical synthesis of labelled prelunularic acid was not possible, labelling was achieved by application of L-[*U*-^{14}C]phenylalanine to cell cultures of *M. polymorpha*. In preliminary experiments it was observed that metabolism of this labelled amino acid resulted in prelunularic acid carrying considerable label in positions 1′,2′,3′,4′,5′ and 6′ of ring B as well as position 1 of ring A and positions 7 and 8 of the aliphatic side chain.

L-[*U*-^{14}C]Phenylalanine was applied to a 14-day-old *M. polymorpha* cell suspension culture. After two days of incubation the cells had taken up 98% of the radioactivity applied. [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Prelunularic acid was purified by TLC. A 20% incorporation with a specific activity of 0.4 μCi μmol^{-1} was observed. To avoid a spontaneous conversion to lunularic acid, the labelled prelunularic acid was stored in methanol at −20° until use.

For the production of radioactively labelled lunularic acid, the previously isolated [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]prelunularic acid was incubated with H_2SO_4 at 56°. After 2 hr of incubation the labelled precursor was completely converted to [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]lunularic acid. Alternatively, labelled prelunularic acid could be directly converted within the cell culture extract. [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Lunularic acid was separated from the incubation mixtures by TLC and the specific activity determined to be 0.4 μCi μmol^{-1}.

Radioactively labelled *p*-coumaric acid was enzymically synthesized from labelled tyrosine using the commercially available enzyme phenylalanine ammonia lyase (PAL), which is known to also act on tyrosine. The labelled product was converted to [*U*-^{14}C]dihydro-*p*-coumaric acid by catalytic hydrogenation.

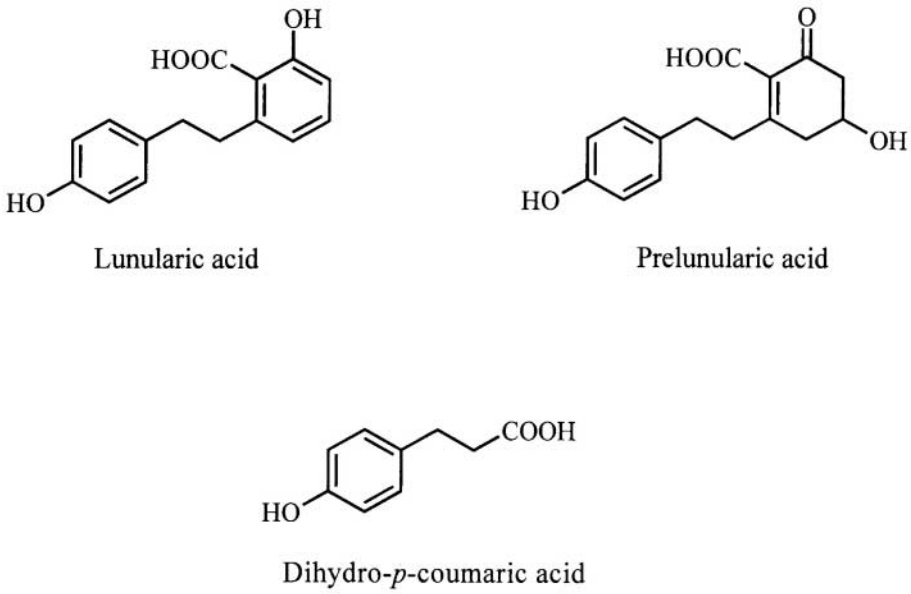

Fig. 2. Structures of putative precursors of marchantin A biosynthesis.

^{13}C-labelled dihydro-*p*-coumaric acid was analogously synthesized from an L-[3,5-^{13}C]tyrosine substrate as given above and was then used for the catalytic reduction to the respective dihydro compound. The identity of the product, [3,5-^{13}C]dihydro-*p*-coumaric acid, was verified by ^{13}C NMR analysis.

2.2. Application of potential precursors

In addition to the above described substances, L-phenylalanine, *trans*-cinnamic acid, acetate, propionate and malonate, which are all assumed to take part in the biosynthesis of bibenzyls, were also fed to *M. polymorpha* tissue. The feeding of L-tyrosine should clarify the origin of the phenylpropane moiety and the possible involvement of tyrosine ammonia lyase (Rudolph, 1990) in marchantin formation in this plant. Lunularic acid and prelunularic acid were also investigated as possible immediate precursors to the bis(bibenzyl) molecule.

Since in preliminary investigations aseptically grown *M. polymorpha* thallus tissue had been shown to contain the most abundant spectrum of natural products, this plant material was chosen for the following application experiments. Pieces of *Marchantia* tissue (*ca* 0.5 cm^2) were transferred under sterile conditions to freshly prepared solid culture medium. The radioactively labelled potential precursors in neutralized aqueous solution (10–100 μl depending on the solubility of the substances) were then administered to the plant material. After incubating for 24 h in continuous light, the *Marchantia* thalli were extracted and analysed as described in the Experimental. The results of this investigation are shown in Table 1.

Depending on the substance applied, the amount of radioactivity that was taken up by the plant tissue ran-

Table 1
Feeding of radioactively labelled precursors to *Marchantia polymorpha* thalli. The aqueous precursor solutions were applied to 0.5 cm^2 of aseptic thallus tissue and incubated for 24 h. The incorporation into marchantin A was, after TLC separation of the extracts, determined by radio-scanning.

Precursor	Radioactivity applied (μCi)	Specific activity (μCi μmol^{-1})	Incorporation into marchantin A (%)
L-[*U*-^{14}C]Phenylalanine	0.3	497	10.0
L-[*U*-^{14}C]Tyrosine	0.3	443	0
[*U*-^{14}C]*p*-Coumaric acid	0.3	443	1.5
[*U*-^{14}C]Dihydro-*p*-coumaric acid	0.3	443	4.0
trans-[3-^{14}C] Cinnamic acid	0.3	55	2.7
[2-^{14}C]Acetate	0.3	56	2.5
[2-^{14}C]Malonate	0.3	6.3	0.6
[2-^{14}C]Propionate	0.4	12	0.95
[1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Prelunularic acid	0.5	0.4	1.6
[1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Lunularic acid	0.4	0.4	0.2

ged between 10 and 60%. While tyrosine was taken up but not incorporated into marchantin A, significant labelling was found when L-phenylalanine and dihydro-*p*-coumaric acid were fed to the thalli yielding incorporation rates of 10 and 4%, respectively. Cinnamic acid and *p*-coumaric acid, substances which are known intermediates derived from L-phenylalanine in secondary metabolism were incorporated to 2.7 and 1.5%, respectively. Acetate yielded the highest incorporation rate (2.5%) among the group of aliphatic organic acids tested. Interestingly, there was a distinct difference in the incorporation rates of lunularic acid and prelunularic acid. While lunularic acid was incorporated to only a very low extent, prelunularic acid yielded an incorporation rate of 1.6%.

Furthermore it was apparent that the radioactively labelled precursors were not only incorporated into marchantin A. In addition, lunularine was labelled when lunularic acid, malonate and cinnamic acid were applied. Moreover, the precursors incorporated into marchantin A were also incorporated into marchantin C, albeit to a lower extent.

2.3. Feeding of ^{13}C-labelled compounds

The non random participation of the above assumed precursors in the biosynthesis of cyclic bis(bibenzyls) had to be experimentally proven and to get insight into the mode of precursor incorporation into the molecular structure, ^{13}C-labelled compounds were fed to *Marchantia* thalli and the resulting marchantin A isolated and analysed by NMR spectroscopy. For this investigation precursors were chosen which, after incorporation, should label all four aromatic rings of the marchantin molecule. Since radioactively labelled L-phenylalanine and acetate had been incorporated, these substances were used for ^{13}C-application. Furthermore, administration of [3,5-^{13}C]dihydro-*p*-coumaric acid should prove whether this compound indeed is precursor of cyclic bis(bibenzyls).

[2-^{13}C]Acetate was applied to *Marchantia* tissue in aqueous solution (10 µl) at a concentration of 0.39 µmol per 0.5 cm^2 of thallus. After 24 h of incubation, marchantin A was separated by TLC and further purified by HPLC. The incorporation rate was 3.4% and the bis(bibenzyl) was subsequently subjected to NMR spectroscopy.

The ^{13}C NMR spectrum of marchantin A after incorporation of [2-^{13}C]acetate as shown in Fig. 3 confirmed the incorporation of the applied compound. A total of six molecules of acetate had been taken up into one molecule of marchantin A, showing increased signals at 112.0 ppm (C-12′), 114.3 ppm (C-12), 115.5 ppm (C-10′), 121.9 ppm (C-10), 123.2 ppm (C-14′), and 139.6 ppm (C-14), respectively. In addition, NMR spectroscopic investigation of marchantin H, also isolated after application of [2-^{13}C]acetate, showed that the signals for C-12′ (112.0 ppm), C-10′ (115.5 ppm), C-10 (121.6 ppm), C-14′ (123.5 ppm), and C-12 (143.4 ppm) were clearly enhanced. In the spectrum of unlabelled marchantin H, the signal for C-14 is the weakest of all signals. For this reason, the enhanced signal for C-14 in the spectrum of labelled marchantin H is also not detectable. Since marchantin

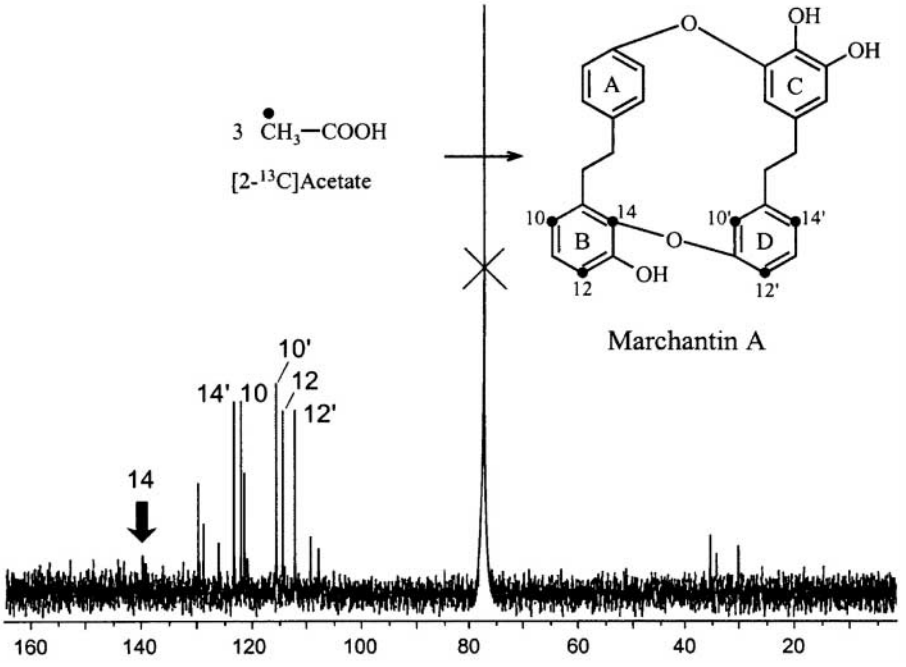

Fig. 3. Partial proton-decoupled ^{13}C NMR spectrum of marchantin A after incorporation of [2-^{13}C]acetate.

A and H are isomers it can be postulated that they have a similar mode of biosynthesis. The application of ^{13}C-labelled acetate to *Marchantia* thalli confirms without doubt that rings B and D of the marchantin molecule are composed of six C-2 units derived from acetate and/or malonate.

The application of L-phenylalanine ^{13}C-labelled in the carboxy group should clarify whether two of the aromatic rings of marchantin A are synthesized via condensation of malonyl CoA with a phenylpropane derivative. L-[^{13}COOH]Phenylalanine was supplied in aqueous solution (10–20 μl) to *M. polymorpha* thallus tissue at a concentration of 0.3–0.6 μmol 0.5 cm^{-2}. After 24 h of incubation the purified marchantin A showed a 6.6% incorporation of the labelled compound.

The ^{13}C NMR spectrum of marchantin A after incorporation of L-[^{13}COOH]phenylalanine, shown in Fig. 4., demonstrated that the signals of C-9 and C-9′ at 136.1 ppm and 143.1 ppm, respectively, were clearly enhanced due to the incorporation of the ^{13}COOH-labelled precursor. This finding indicates that the carboxy groups of two phenylalanine molecules were integrated into rings B and D of marchantin A, while rings A and C of the marchantin structure were formed from the aromatic rings of this amino acid. The incorporation of two molecules of L-[^{13}COOH]phenylalanine into marchantin A was further confirmed by the mass spectrum showing besides the molecular ion (M + H) peak an enhanced (M + H + 2) peak: CI MS: *m/z* (rel. int.): 441 ([M + H]$^+$, 100), 443 (11).

The ^{13}C-labelled L-phenylalanine was not only incorporated into marchantin A. In addition, marchantin H was labelled as shown by an enhancement of the signals corresponding to C-9 and C-9′ at 127.2 ppm and 143.1 ppm, respectively. Therefore, the incorporation of L-[^{13}COOH]phenylalanine into marchantin A and H unequivocally confirms that L-phenylalanine participates in the biosynthesis of cyclic bis(bibenzyls).

To investigate whether dihydro-*p*-coumaric acid is a true precursor, [3,5-^{13}C]dihydro-*p*-coumaric acid was administered to *Marchantia* tissue in a concentration of 0.5 μmol per 0.5 cm^2 in 10 μl solution. After 2 days of incubation the purified marchantin A had taken up 1% of the labelled precursor. Surprisingly, this was a lower incorporation rate than was found for the much less concentrated radioactively labelled precursor. Nevertheless, the target compound was sufficiently labelled for ^{13}C NMR spectroscopy.

Fig. 5 shows the ^{13}C NMR spectrum of marchantin A after incorporation of [3,5-^{13}C]dihydro-*p*-coumaric acid. The enhanced signals for C-2 and C-6 (121.2 ppm) and C-6′ (144.2 ppm) are clearly visible. Due to the low intensity of the signal for C-2′ (146.4 ppm) in the spectrum of unlabelled marchantin A, the presumably enhanced signal for C-2′ in the spectrum of labelled marchantin A could not be seen. The labelling of four carbon atoms is proven by the mass spectrum of marchantin A, which displayed an

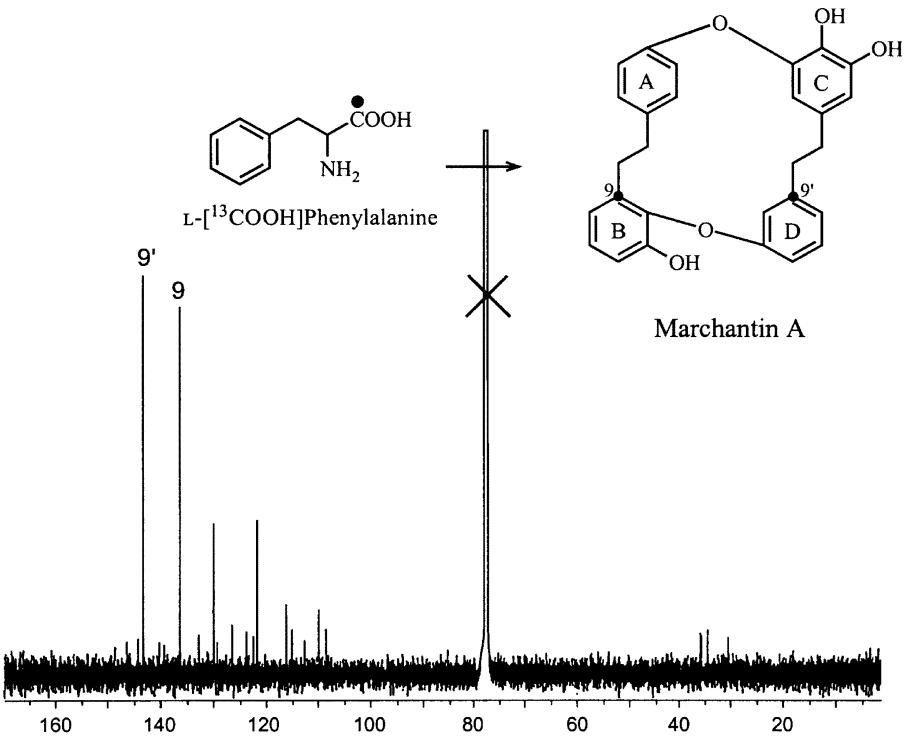

Fig. 4. Partial proton-decoupled ^{13}C NMR spectrum of marchantin A after incorporation of L-[^{13}COOH]phenylalanine.

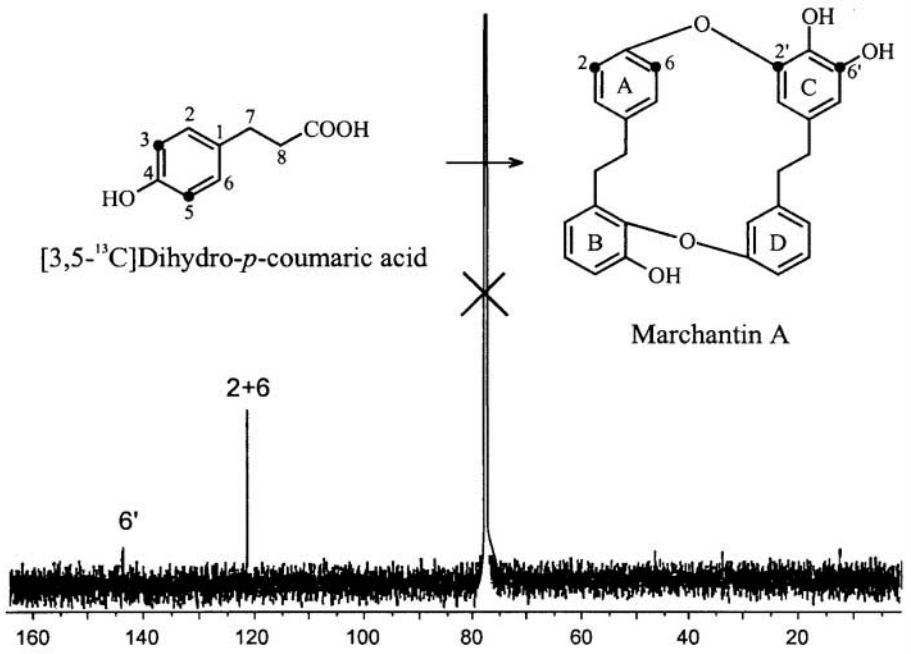

Fig. 5. Partial proton-decoupled ^{13}C NMR spectrum of marchantin A after incorporation of [3,5-^{13}C]dihydro-*p*-coumaric acid.

enhanced (M + H + 4) peak: CI MS: *m*/*z* (rel. int.): 441 ([M + H] + , 100), 445 (13). The label of positions 3 and 5 in the precursor molecule were found in marchantin A at positions C-2/6 and C-2′/6′, respectively.

Taken together, the results obtained with ^{14}C- and ^{13}C-labelled dihydro-*p*-coumaric acid confirmed that dihydro-*p*-coumaric acid is a precursor in bis(bibenzyls) biosynthesis. Its arrangement within the marchantin A molecule corresponds to the incorporation of L-phenylalanine. The aromatic rings of dihydro-*p*-coumaric acid are the building blocks for rings A and C of marchantin.

Results obtained from precursor feeding experiments using ^{13}C-labelled compounds supported the conclusions which were drawn from the application of radioactively labelled substrates. Bibenzyls are formed from L-phenylalanine and C_2 units with the carboxy group of L-phenylalanine integrated into the second ring of the molecule. Moreover, the previous observation that dihydro-*p*-coumaric acid is an intermediate in bibenzyl biosynthesis in Orchidaceae (Fritzemeier & Kindl, 1983) is now confirmed for the bis(bibenzyls) biosynthesis in *M. polymorpha*. L-Phenylalanine is assumed to be metabolized to dihydro-*p*-coumaric acid which is integrated into the marchantin A molecule in a manner that is reminiscent of the flavonoid pathway. Therefore, the hypothesis of Pryce (1971a); Pryce (1971b) for the biosynthesis of lunularic acid can thus be extended to the bis(bibenzyl) compounds found in liverworts (Asakawa, 1995) and dihydro-*p*-coumaric acid is confirmed as an intermediate.

3. Discussion

A new class of natural compounds was found in 1979 when Asakawa et al. (1979) discovered the cyclic bis(bibenzyl) marchantin A, a major secondary metabolite in *Marchantia polymorpha*. In addition to marchantins, a considerable number of related compounds, namely riccardins, perrottetins and paleatins were subsequently isolated from liverworts (Asakawa et al., 1987; Asakawa & Matsuda, 1982; Asakawa, 1982). Due to the multiple structures possible from ring subunit combinations and substitution, more than 50 different cyclic bis(bibenzyls) have been characterized up to now (Asakawa, 1995).

Besides bis(bibenzyls) the class of bibenzyls are typical for liverworts with lunularine (Pryce, 1972), lunularic acid (Valio, Burdon & Schwabe, 1969), and prelunularic acid (Ohta et al., 1983) as the most prominent members.

As early as 1982, Asakawa and Matsuda had proposed that bibenzyls are the building blocks for the biosynthesis of cyclic bis(bibenzyls) (Asakawa & Matsuda, 1982). Due to their structural similarity, knowledge about the formation of bibenzyls might be adopted to the biosynthesis of cyclic bis(bibenzyls). A first hypothesis for the biosynthesis of the bibenzyl lunularic acid was presented by Pryce (1971b) who proposed a phenylpropane/polymalonate pathway starting from L-phenylalanine and yielding hydrangenol, the proposed immediate precursor to lunularic acid. Hydrangenol, however, has never been isolated from *M. polymorpha*.

The feeding experiments presented here partially verified the hypothesis of Pryce concerning the biosynthesis of lunularic acid in *Lunularia cruciata* (Pryce, 1971b), but have also provided a more complete characterization of bibenzyl biosynthesis. The application of potential precursors demonstrated the incorporation of the following into marchantin A (see Table 1): L-[*U*-^{14}C]phenylalanine (10%), *trans*[3-^{14}C]cinnamic acid (2.7%), [*U*-^{14}C]*p*-coumaric acid (1.5%) and L-[*U*-^{14}C]tyrosine (0%). Tyrosine was the only substance applied which was not incorporated at all into marchantin A, excluding the possibility (Rudolph, 1990) that tyrosine ammonia lyase is catalytically active in *M. polymorpha*.

The application of ^{13}C-labelled L-phenylalanine with subsequent NMR spectroscopy clearly verified that two molecules of the amino acid were incorporated into marchantin A, contributing rings A and C of the bis(bibenzyl) structure.

The participation of hydrangenol as an intermediate in marchantin biosynthesis was previously ruled out by the isolation of prelunularic acid from *M. polymorpha* (Ohta et al., 1983) as a major secondary metabolite. Since prelunularic acid easily can be converted into lunularic acid, the former may participate in the biosynthesis of bibenzyls. Prelunularic acid is a known bibenzylic precursor, therefore, the hypothesis of Pryce (1971b) required modification. Investigations of Fritzemeier and Kindl (1983) had previously demonstrated that bibenzyls in Orchidaceae are synthesized from dihydro-*p*-coumaric acid and malonyl CoA, which suggested dihydro-*p*-coumaric acid as a possible intermediate in cyclic bis(bibenzyl) biosynthesis.

The participation of dihydro-*p*-coumaric acid in the biosynthesis of marchantin A was verified by the experiments presented here. Application of this potential precursor in radioactively labelled form resulted in 4% incorporation into marchantin A; a result that was verified using [3,5-^{13}C]dihydro-*p*-coumaric acid as a precursor. In addition, [*U*-^{14}C]*p*-coumaric acid was incorporated into the substance under investigation at a rate of 1.5% (see Table 1). Therefore, both precursors participate in bis(bibenzyls) biosynthesis in sequence.

Feeding of the radioactively labelled malonate, acetate and propionate led to incorporation rates of 0.6, 2.5, and 0.95%, respectively (Table 1). The participation of acetate in the biosynthesis of marchantin A was verified by administration of the ^{13}C-labelled compound. Subsequent NMR spectroscopy demonstrated that a total of six molecules of acetate formed rings B and D of the marchantin molecule. These results further confirmed that a phenylpropane/polymalonate pathway using dihydro-*p*-coumaric acid and acetate units is responsible for the biosynthesis of bibenzyls.

The assumption (Asakawa & Matsuda, 1982) that bibenzyls are the monomers of cyclic bis(bibenzyls) is now experimentally confirmed. Radioactively labelled lunularic acid and prelunularic acid were incorporated into marchantin A up to 0.2 and 1.6%, respectively (see Table 1). The reason for the difference in incorporation rates may be the fact that prelunularic acid and not lunularic acid is the immediate precursor to the marchantin molecule.

The fact that application of L-[^{13}COOH]-phenylalanine, [3,5-^{13}C]dihydro-*p*-coumaric acid and [2-^{13}C]acetate labelled two, four and six positions, respectively, in marchantin A, verified that bibenzyls, indeed, are the building blocks for the cyclic bis(bibenzyl) system.

The biosynthesis of marchantin A as elucidated from precursor feeding experiments is depicted in Fig. 6. L-Phenylalanine is deaminated to yield cinnamic acid which is converted to *p*-coumaric acid by hydroxylation. Enzymic hydrogenation yields dihydro-*p*-coumaric acid, which in activated form, condenses with three molecules of malonyl CoA to form prelunularic acid. Aromatization yields lunularic acid and possibly lunularine. Two molecules of lunularine or lunularic acid condense to yield marchantin A.

The mechanism of this final coupling step is unknown. Since the condensation reaction of the bibenzyl monomers obviously does not incorporate oxygen into the bis(bibenzyl) structure, an oxidative phenol-coupling reaction may be conceivable. Analogously to the formation of bisbenzylisoquinoline alkaloids (Stadler & Zenk, 1993; Kraus & Kutchan, 1995) and related mechanisms (Gerardy & Zenk, 1993; Nasreen, Rueffer & Zenk, 1996; Zenk, Gerardy & Stadler, 1989), highly specific cytochrome P-450 enzymes may serve as catalysts for this phenol-coupling reaction.

A complete elucidation of the pathway leading to marchantins in the genus *Marchantia* will only be possible at the enzyme level. Therefore, future research will have to be directed to the enzymes underlaying the reaction sequence leading to cyclic bis(bibenzyls), this unique class of pharmacologically active compounds from liverworts.

4. Experimental

4.1. General

All NMR spectra were obtained on a Bruker AM 360 spectrometer. CDCl3 was used as an int. standard. ^{13}C NMR spectra were obtained at 90 MHz. Mass spectra were recorded with a Finnigan-MAT SSQ 700 in the CI mode. Distribution of radioactivity on TLC plates was monitored with a Berthold (Tracemaster 20)

linear analyser. Separation of marchantin A was performed with HPLC using a Vydac Sc-201 RP column (50×4 mm, Macherey and Nagel) and a Nucleosil 100 C18 column, 5 μm (250×4 mm, Knauer); solvent A, aq. 2% acetonitrile, 0.1% HOAc; solvent B, 98% acetonitrile, 2% H_2O, 0.1% HOAc; gradient 1, 100–0% A in 20 min, 0% A for 5 min; gradient 2, 100–50% A in 15 min, 50–40% A in 10 min, 40–38% A in 10 min, 38–0% A in 5 min, 0% A for 5 min, flow rate: 1 ml min^{-1}, detection at 280 nm.

4.2. Plant material

Suspension cultures of *Marchantia polymorpha* were kindly provided by Prof. E. Beck (Bayreuth) and cultivated in 200 ml MS medium (Murashige & Skoog, 1962) with 2% glucose instead of sucrose in 1 l flasks at 23° under constant illumination (650 lux) at 100 rpm. Sterile thallus cultures of *M. polymorpha* were established in the cell culture laboratory of the Munich department and were grown on solid medium

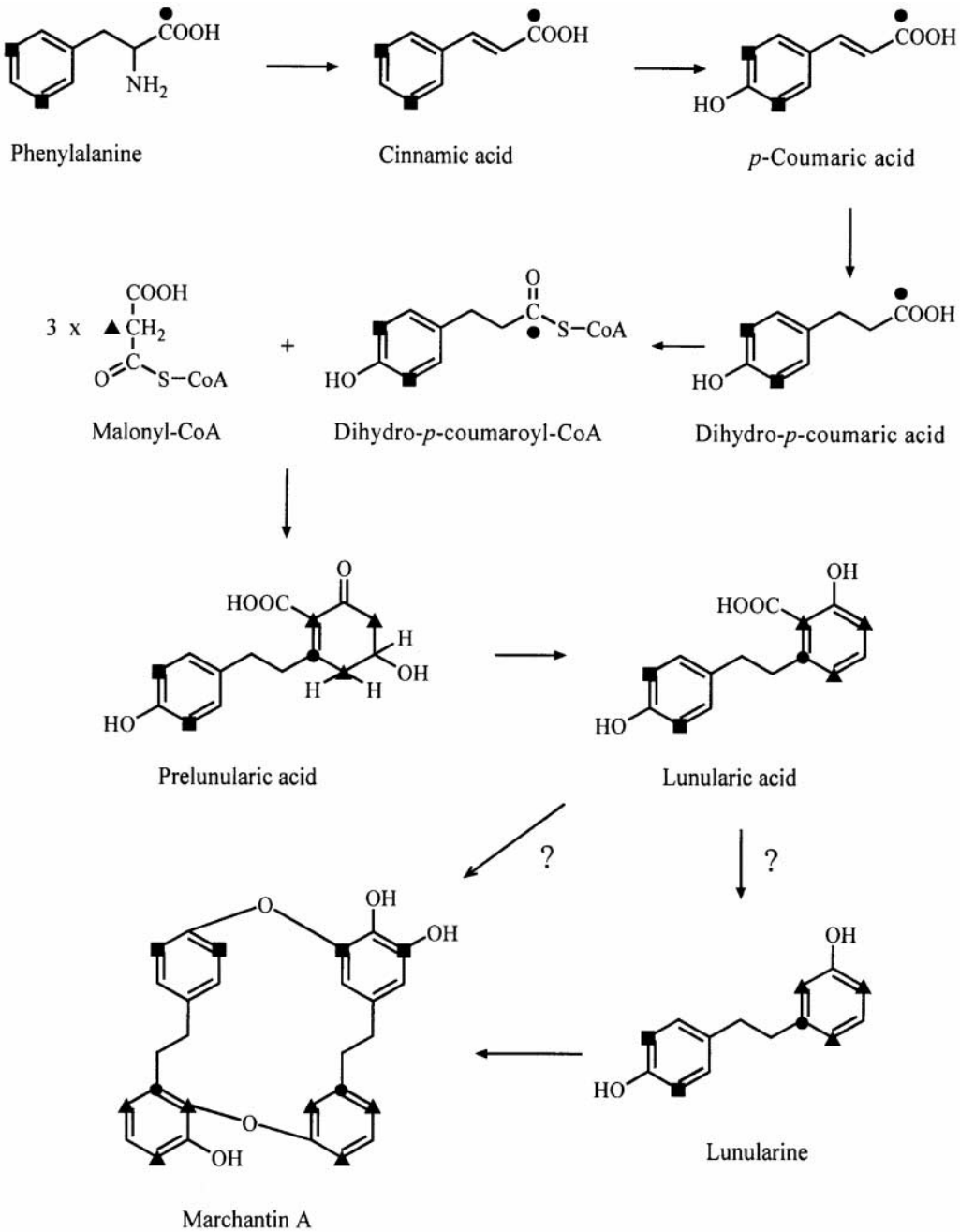

Fig. 6. Reaction sequence in *Marchantia polymorpha* thallus leading from L-phenylalanine via dihydro-*p*-coumaric acid to marchantin A. The markers (●, ▲ and ■) show the labelling after application of ^{13}C-labelled precursors.

of (Nitsch & Nitsch, 1969) with a cultivation period of 4–6 weeks.

4.3. Chemicals

All solvents and reagents were of the highest purity commercially available. The reference substances marchantin A, C and H were from the collection of the Tokushima department. Radioactively labelled compounds were purchased from Amersham Buchler and NEN DuPont. L-[^{13}COOH]Phenylalanine, [2-^{13}C]acetate and L-[3,5-^{13}C]tyrosine were purchased from Isotec. The phenylalanine ammonia lyase enzyme was from Sigma.

4.4. Synthesis of [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]prelunularic acid

L-[*U*-^{14}C]Phenylalanine (5 μCi, 497 μCi μmol^{-1}) was applied to 1 ml of a 14-day-old *M. polymorpha* suspension culture in a well of a multi-well plate and incubated under constant shaking (100 rpm) for 2 days. Thereafter, the cells were transferred to an Eppendorf tube (2.5 ml) and sepd by centrifugation. After washing with 1 ml H_2O, the cells were extracted with 1.5 ml MeOH under constant shaking at room temp. for 30 min. The extract was sepd by filtration, evapd to dryness with N_2, the residue taken up in 100 μl MeOH and purified by TLC (Polygram Sil G/UV254, Macherey and Nagel, $CHCl_3$–MeOH–H_2O, 6:4:1, R_f 0.6). The isolated [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]-prelunularic acid had a sp. act. of 0.4 μCi μmol^{-1} and was stored at −20° until use.

4.5. Synthesis of [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]lunularic acid

[1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Prelunularic acid (40.000 cpm) in aq. soln was incubated with 20 μl 2 M H_2SO_4 in a total volume of 300 μl for 2 h at 56°. Thereafter, the reaction mixt. was neutralised with 2N NaOH and freeze dried. The residue was taken up in 50 μl MeOH and purified by TLC (toluene-EtOAc-HOAc, 8:2:0.1, R_f 0.36). The isolated [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]lunularic acid had a sp. act. of 0.4 μCi μmol^{-1}.

4.6. Synthesis of [U-^{14}C]p-coumaric acid

[*U*-^{14}C]Tyrosine (5 μCi, 100 μl) in an Eppendorf tube was mixed with 200 μl borate buffer, pH 8.5. Phenylalanine ammonia lyase (0.45 U, 300 μl) was added and the mixt. incubated at 30° overnight in the dark. The reaction was terminated by the addition of 20 μl HOAc, and the mixt. extracted ×4 with a total of 2 ml EtOAc. The extracts were combined, dried over Na_2SO_4 and the solvent evapd. The residue was taken up in 1 ml MeOH and the product purified by TLC (toluene–HOAc, 8:2, R_f 0.54). The isolated [*U*-^{14}C]*p*-coumaric acid (83% yield) had a sp. act. of 443 μCi μmol^{-1}.

4.7. Synthesis of [U-^{14}C]dihydro-p-coumaric acid

The above product (4 μCi) was dissolved in 2 ml MeOH, a small volume of palladium activated charcoal was added and the mixt. treated with H_2 for 2 h. Thereafter, the soln was filtered and washed with *ca* 30 ml hot MeOH. The solvent was evapd and the residue taken up in 200 μl MeOH. Purification of the product by TLC (Polygram CEL 300 UV254, Macherey and Nagel, *n*-BuOH–H_3N–EtOH–toluene, 5:3:2:1, R_f 0.51) yielded [*U*-^{14}C]dihydro-*p*-coumaric acid (90% yield) with a sp. act. of 443 μCi μmol^{-1}.

4.8. Synthesis of [3,5-^{13}C]dihydro-p-coumaric acid

L-[3,5-^{13}C]Tyrosine (13 mg, 70 μmol) diluted in 200 μl 1 M NaOH was mixed with 700 μl 0.5 M borate buffer, pH 8.5, and the pH adjusted to 8.5 with 100 μl 1 M HCl. The soln was mixed with 0.5 M borate buffer, pH 8.5, to a final volume of 4 ml and divided into 50 portions. To every portion, 0.015 U phenylalanine ammonia lyase (30 μl, 3 mg ml^{-1} protein) was added and incubated at 30° for 20 h in the dark. Thereafter, the reaction mixts were combined, acidified with 1 M HCl, and extracted ×4 with a total of 20 ml EtOAc. The extracts were combined, dried over Na_2SO_4 and the solvent evapd yielding 56 μmol [3,5-^{13}C]*p*-coumaric acid (80% yield).

The ^{13}C-labelled *p*-coumaric acid (56 μmol) was then used for the catalytic reduction to the respective dihydro compound. With the method described above for the synthesis of the radioactively labelled compound, 50 μmol [3,5-^{13}C]dihydro-*p*-coumaric acid were obtained. The identity of the product was verified by ^{13}C NMR analysis showing the following characteristics: ^{13}C NMR ($CDCl_3$): δ(ppm) = 32.3 (C-8), 39.9 (C-7), 116.1 (C-3/5), 130.2 (C-2/6), 133.9 (C-1), 156.5 (C-4).

4.9. Application experiments

Precursor substances for feeding experiments (0.3–0.5 μCi) were dissolved in 10–100 μl H_2O (3.3% EtOH in the case of [3,5-^{13}C]dihydro-*p*-coumaric acid) and were then applied to 0.5 cm^2 pieces of aseptic *M. polymorpha* thallus tissue. Prior to precursor application, the thallus pieces had been transferred to freshly prepared solid medium of (Nitsch & Nitsch, 1969) to induce growth. The petri dishes were sealed with para-

film and incubated for 24 h in continuous light (650 lux).

For a subsequent analysis, the thallus was removed from the solid medium, freed of residual agar, cut into small pieces and extracted in 1 ml MeOH at 56° for 30 min. The extract was separated by filtration, evapd to dryness and the residue taken up in 100 µl MeOH. The extracts (30 µl) were subjected to TLC on Polygram Sil G/UV254 with toluene-HOAc (8:2) as solvent system. The products sepd were localized on the plates by their respective radioactivity.

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