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Cytochromes P-450 catalyze the formation of marchantins A and C in *Marchantia polymorpha*

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Abstract

Two specific cytochrome P-450 enzymes were detected in cell suspension cultures of *Marchantia polymorpha*; the first catalyzes the coupling of two molecules of lunularic acid to form marchantin C and CO_2 and the second hydroxylates marchantin C to marchantin A. Cell free experiments using $^{3}H/^{14}C$ doubly-labeled substrates demonstrated that lunularic acid, and neither lunularine nor prelunularic acid, is the sole substrate for the coupling reaction. Both enzymes are dependent on the presence of oxygen and NADPH. Both reactions were inhibited in the presence of CO in the dark; this inhibition was partially reversed by white light. In addition, both reactions were inhibited by typical inhibitors of cytochrome P-450 enzymes such as cytochrome c and ancymidol.

Keywords: *Marchantia polymorpha*; Marchantiaceae; Biosynthesis; Bibenzyls; Cyclic bis(bibenzyls); Phenolic coupling; Cytochrome P-450

1. Introduction

Marchantin A, the first characterized member of a large number of cyclic bis(bibenzyls) found in the liverwort *Marchantia polymorpha* L. (Asakawa, Tokunaga, Toyota, Takemoto & Suire, 1979; Asakawa, 1995) exhibits a range of interesting biological effects including cytotoxic, antibacterial, antifungal, 5-lipoxygenase- and calmodulin-inhibiting activities (Asakawa, 1990; Kámory, Keseru & Papp, 1994; Schwartner et al. 1995; Panossian, Gabrielan, Schwartner & Wagner, 1996; Taira, Takei, Endo, Hashimoto, Sakiya & Asakawa, 1994; Keseru & Nógrádi, 1995). Little is known about the biosynthesis of this group of compounds. Asakawa and Matsuda (1982) suggested that the bibenzyls lunularic acid and lunularine could be the monomeric precursors for the condensation reaction to the bis(bibenzyl) molecules. Lunularic acid could be derived from the phenylpropane/polymalonate pathway via cinnamic acid and *p*-coumaric acid as already proposed by Pryce (1971). By analogy with the results of Fritzemeier and Kindl (1983) and Fritzemeier, Kindl and Schlösser (1984), obtained for the biosynthesis of the bibenzyls in species of the families Dioscoreaceae and Orchidaceae, one might assume that it is not coumaric acid itself but its dihydro-form that is the building block for lunularic acid. Recently, we obtained evidence for the involvement of the phenylpropane/polymalonate pathway in the biosynthesis of the marchantins in *M. polymorpha* by demonstrating the incorporation of the ^{13}C- or radioactively-labeled putative precursors L-phenylalanine, acetate, malonate, cinnamic acid, *p*-coumaric acid and dihydro-*p*-coumaric acid into marchantins A and C (Fig. 1) (Friederich, Maier, Deus-Neumann, Asakawa & Zenk, 1999). The incorporation of labeled lunularic acid and prelunularic acid into marchantin A supported the original hypothesis (Asakawa & Matsuda, 1982) for the involvement of the monomeric bibenzyls in the pathway leading to the marchantins.

At the enzymatic level, phenylalanine ammonia lyase

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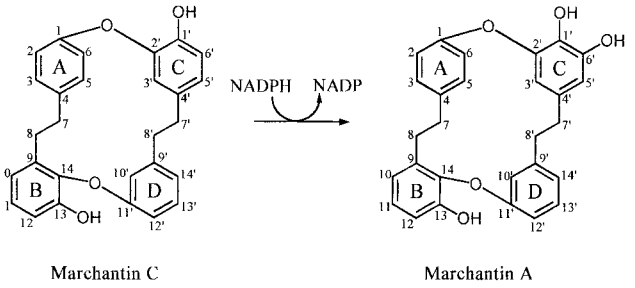

Fig. 1. Structures of the cyclic bis(bibenzyls) marchantin A and marchantin C.

(PAL) and cinnamic acid-4-hydroxylase, catalyzing the formation of *p*-coumaric acid from L-phenylalanine via cinnamic acid, have been detected in *M. polymorpha* L. (Löffelhardt, Ludwig & Kindl, 1973; Gorham, 1977, 1978). The formation of the bibenzyl prelunularic acid has not yet been proven at the enzymatic level. Most likely this reaction is analogous to the formation of the bibenzyls in the Dioscoreaceae and Orchidaceae, which involves dihydro-*p*-coumaroyl-CoA and three units of malonyl-CoA as shown by Fritzemeier and Kindl (1983).

A further step in the putative biosynthetic pathway to the marchantins could be the postulated decarboxylation (Asakawa & Matsuda, 1982) from lunularic acid to lunularine, a compound which may be regarded as a monomeric building block for marchantin biosynthesis. This decarboxylase was detected and shown to be membrane bound in *Conocephalum conicum* (L.) Dum. (Pryce & Linton, 1974) and *M. polymorpha* L. (Gorham, 1977). The crucial step in the biosynthesis of the bis(bibenzyls), however, is the intermolecular phenolic coupling reaction of the bibenzyl monomers to the cyclic dimers. During the past years intra- as well as intermolecular phenolic coupling reactions have been shown to be catalyzed by cytochrome P-450 enzymes (Zenk, Gerardy & Stadler, 1989; Stadler & Zenk, 1993; Kraus & Kutchan, 1995; Nasreen, Rueffer & Zenk, 1996) without introduction of catalytically activated oxygen into either of the substrate molecules.

The aim of this study was to explore the possible involvement of cytochrome P-450 enzymes in the condensation of bibenzyl monomers to form bis(bibenzyl) dimers. By incubation of different radioactively-labeled precursors with microsomal preparations from cell cultures of *M. polymorpha*, we also attempted to clarify which of the putative precursors, lunularic acid, lunularine or both, are the immediate building units of the condensation reaction to form marchantin C. In addition, the metabolic fate of marchantin C in these cell-free incubations was investigated, since this reaction formally would imply a possible cytochrome P-450 catalyzed hydroxylation of marchantin C in 6′ position (Fig. 1) to yield marchantin A.

2. Results and discussion

2.1. Enzyme detection

Microsomal preparations from cell cultures of *M. polymorpha* cultivated for 14 days were used to detect the formation of the marchantins from the potential ^{14}C-labeled precursors prelunularic acid, lunularic acid and lunularine in the presence of NADPH. With prelunularic acid and lunularine as substrates, no product formation was observed. The putative precursor molecules were recovered quantitatively and unchanged. Incubation with ^{14}C-labeled lunularic acid resulted, however, in a small but significant formation (2.5%, 0.2 nmol) of a new metabolite which could be detected after TLC with the same R_f value as the authentic marchantin C (R_f 0.71) and in addition the decarboxylation product lunularine (R_f 0.43) was formed in 90% yield. To optimize the yields a crude organelle preparation was subjected to a sucrose-step-gradient centrifugation. Assays with the fraction from the boundary layer between 20 and 33% (w/w) sucrose yielded high transformation rates to lunularine (55.7%, 5.0 nmol), putative marchantin C (34%, 3.1 nmol) and a third product with the R_f value (0.5) of marchantin A (10.3%, 0.9 nmol). No reaction could be observed in assays with heat inactivated protein or without NADPH. Electron microscopy (A. Bock & G. Wanner, unpublished) of this organelle fraction showed small membrane vesicles with diameters up to 100 nm. The origin of such vesicles, which appear similar to those obtained from *Papaver somniferum* cell cultures and which contain a cytochrome P-450 enzyme catalyzing the coupling reaction from (*R*)-reticuline to salutaridine (Gerardy & Zenk, 1993), is unclear.

2.2. Product identification

For the identification of the reaction products, 14 standard assays with lunularic acid were combined after incubation, the products were isolated by TLC and diluted with authentic references to 2.1×10^4 dpm μmol^{-1} (marchantin A) and 1.5×10^4 dpm μmol^{-1} (marchantin C). After dilution, the products were methylated with diazomethane, purified by TLC and the specific activities again determined. The marchantin A-1′,6′,13-O-methyl ether had a specific activity of 2.6×10^4 dpm μmol^{-1} and the marchantin C-1′,13-O-methyl ether of 1.5×10^4 dpm μmol^{-1}. Furthermore, radio-HPLC analysis of the undiluted product isolated by TLC (with an R_f value identical to that of marchantin A) together with unlabeled marchantin A reference showed only one peak with the retention time of 19 min. The specific activity before and after HPLC remained constant at 2.2×10^6 dpm μmol^{-1}.

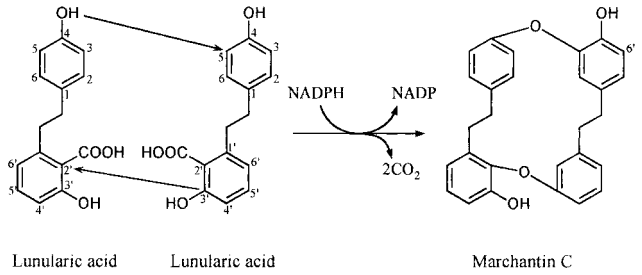

Fig. 2. Coupling reaction between two molecules of lunularic acid.

2.3. The coupling reaction

As shown above, lunularic acid is clearly the substrate for the cell free formation of marchantin C. The main reaction obviously proceeds by decarboxylation of lunularic acid to lunularine. However, lunularine as a substrate did not result in the formation of marchantins C or A. From these experiments it was not clear whether only lunularic acid or a mixture of lunularic acid and lunularine would couple to form the bis(bibenzyl) compounds. This question could be answered by using double-labelling experiments in which a 1 : 1 mixture of [^{3}H]lunularic acid and [^{14}C]lunularine were incubated together in one assay.

If lunularic acid and lunularine would couple enzymatically, the ^{3}H : ^{14}C ratio of the assay should remain constant in the products. On the other hand if only lunularic acid would be coupled, the ^{3}H : ^{14}C ratio should change dramatically. In the case given above the marchantins formed should contain only tritium.

In the first experiment [2,6-^{3}H]lunularic acid and [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]lunularine (^{3}H : ^{14}C = 6.8 : 1) were incubated together in the standard enzyme assay. The products of the reaction were purified by TLC, eluted with methanol and the ^{3}H : ^{14}C ratio determined. A nearly complete loss of ^{14}C could be observed in marchantin A (^{3}H : ^{14}C = 160 : 1); in marchantin C only ^{3}H could be detected. The experiment vice versa with [^{3}H]lunularine and [^{14}C]lunularic acid showed the complete loss of ^{3}H in the products.

From these results it can be concluded that lunularic acid serves as the sole substrate for the coupling enzyme to form marchantin C (Fig. 2). In this coupling reaction, two ether bridges are formed between the two lunularic acid molecules, the first between the hydroxy group at C-4 of one monomer and at C-5 of the other molecule in analogy to the reaction forming the bisbenzylisoquinoline alkaloids (Stadler & Zenk, 1993). The formation of the second ether bridge involves the hydroxy group at C-3′ of ring B of one and the C-2′ of the other molecule. In addition, either during or directly after this reaction both monomers are decarboxylated at C-2′.

2.4. Characterization of the enzymes

A physical separation of both enzymes was not possible in the crude vesicle preparation. Therefore, a simultaneous characterization was attempted. Both enzymes showed a pH optimum at pH 7.5, which is similar to both phenol coupling (Gerardy & Zenk, 1993; Stadler & Zenk, 1993) as well as hydroxylation reactions (e.g. Karp, Mihaliak, Harris & Croteau, 1990; Bouwmeester, Konings, Gershenzon, Karp & Croteau, 1999).

Both the coupling and the hydroxylation reaction were strictly dependent on NADPH (Table 1). Replacement of NADPH by NADH did not result in any product formation. The coupling enzyme showed a higher activity in the presence of a NADPH regenerating system than with NADPH alone or a 1 : 1 mixture of NADPH : NADH. In contrast, the hydroxylation reaction leading from marchantin C to marchantin A was unaffected by the NADPH regenerating system. In the presence of a NADPH/NADH mixture a synergistic effect (59%), well-known from plant hydroxylases, was observed (Benveniste, Salaun & Durst, 1977; Hinderer, Flentje & Barz, 1987; Grand, 1984). The removal of O_2 by a glucose oxidase/catalase system (Kochs & Grisebach, 1987) showed that both enzymes were totally inactive in the absence of O_2.

A strong indication for the involvement of cytochrome P-450 enzymes in both reactions was their inhibition by carbon monoxide (Table 2). The formation of marchantins A and C could be totally inhibited by

Table 1
Influence of cofactors on the formation of marchantins A and C[a]

Cofactor	Marchantin A formed (nmol)	Marchantin C formed (nmol)
NADPH	0.9	2.4
NADH	0	0
Without cofactor	0	0
NADPH regenerating system	0.9	3.1
NADPH/NADH 1 : 1	2.2	2.5

[a] The final concentration of the cofactors in the standard assay was 500 μM. The NADPH regenerating system consisted of 500 μM NADPH, 500 μM NADP, 2.5 mM glucose-6-phosphate and 0.7 U glucose-6-phosphate dehydrogenase.

Table 2
Effect of carbon monoxide and light on marchantin C synthase and marchantin C hydroxylase

Assay condition	Relative enzyme activity (%)	
	Marchantin C synthase[a]	Marchantin C hydroxylase[b]
Air	100	100
N_2/O_2 (9 : 1) (light)	158	117
N_2/O_2 (9 : 1) (dark)	85	80
CO/O_2 (9 : 1) (dark)	0	0
CO/O_2 (9 : 1) (light)	17	31

[a] Marchantin C synthase: 100% = 4.3 pkat/mg protein.
[b] Marchantin C hydroxylase: 100% = 1.3 pkat/mg protein.

CO incubating the assay in the dark. The inhibition of both enzymes could be partially reverted by illumination with white light (Table 2).

Interestingly, the presence of typical cytochrome P-450 inhibitors in the incubation mixture showed different effects on the coupling and hydroxylation reactions (Table 3). The typical N-substituted triazole or imidazole inhibitors such as ketoconazole, propiconazole or prochloraz inhibited only the hydroxylation whereas a considerable stimulation of the phenol coupling reaction could be observed. Only the pyrimidine type ancymidol and the dihydrotriazole tetcyclacis inhibited both hydroxylation and phenol coupling reaction. The naphthoquinone juglone, which interferes with the electron transport in the oxidoreductase complex, and cytochrome c, which competitively extracts electrons from the NADPH–cytochrome c reductase complex, were both very effective inhibitors of the reactions, an observation that supports the involvement of P-450 systems.

Additional evidence for the presence of cytochrome P-450 enzymes could be obtained from recording the CO-difference spectrum of the vesicle preparation (Omura & Sato, 1964). A small but significant amount of 28 pmol mg^{-1} protein P-450 could be detected in the enzymatically active membrane preparations. The activity of the NADPH–cytochrome c reductase could be determined in these preparations at a level of 0.9 pkat mg^{-1} protein.

3. Conclusion

The results presented in this publication suggest the involvement of a cytochrome P-450 enzyme in the formation of the cyclic (bis)bibenzyl marchantin C providing another example of an intermolecular phenol coupling reaction catalyzed by this type of enzyme. For this enzyme we propose the name "marchantin C synthase". In addition, we propose the name "marchantin C hydroxylase" for a second cytochrome P-450 enzyme which catalyzes the hydroxylation from marchantin C to marchantin A.

Based on these results, the biosynthetic sequence leading to the marchantin-type bis(bibenzyls) can be outlined as portrayed in Fig. 3. Starting with phenylalanine, through the action of PAL this aromatic amino acid is converted to cinnamic acid and further

Table 3
Effect of inhibitors of cytochrome P-450 enzymes on the marchantin C synthase and the marchantin C hydroxylase

Inhibitor[a]	Concentration (mM)	Relative enzyme activity (%)	
		Marchantin C synthase[b]	Marchantin C hydroxylase[c]
Ancymidol	0.25	0	0
Ketoconazole	0.5	123	0
Metyrapone	0.5	190	18
Prochloraz	0.5	223	7
Propiconazole	0.5	144	0
Tetcyclacis	0.5	10	0
Triadimefone	0.5	177	29
Cytochrome c	0.25	0	0
Juglone	0.25	0	0

[a] The inhibitors were added to the standard enzyme assay.
[b] Marchantin C synthase: 100% = 5.6 pkat/mg protein.
[c] Marchantin C hydroxylase: 100% = 1.4 pkat/mg protein.

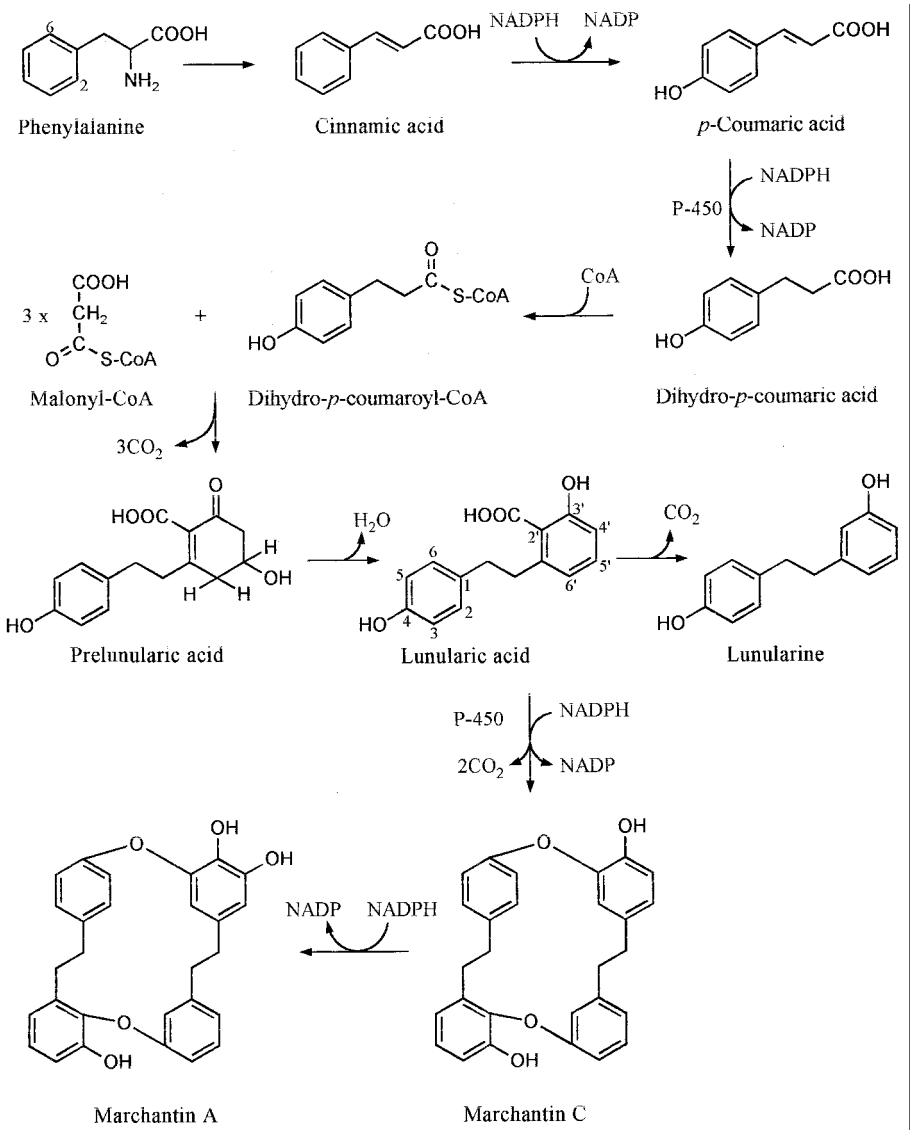

Fig. 3. Proposed pathway for the biosynthesis of marchantins, leading from L-phenylalanine via dihydro-*p*-coumaric acid, prelunularic acid, lunularic acid and marchantin C to marchantin A. The last two steps are catalyzed by the cytochrome P-450 enzymes marchantin C synthase and marchantin C hydroxylase.

hydroxylated to *p*-coumaric acid followed by reduction to dihydro-*p*-coumaric acid. The activated CoA-ester of this phenylpropane derivative most likely couples with three units of malonyl-CoA to form prelunularic acid, a reaction which still needs to be investigated. The subsequent aromatization of prelunularic acid to lunularic acid could be observed in enzyme preparations of *M. polymorpha* suspension cultures (Friederich & Zenk, unpublished results). The previously reported enzymatic decarboxylation of lunularic acid to lunularine (Gorham, 1977) is clearly not involved in the pathway leading to the (bis)bibenzyl molecules of the marchantin type since only lunularic acid serves as substrate for the coupling reaction. The ultimate reaction is the cytochrome P-450 catalyzed hydroxylation of marchantin C to yield marchantin A, which follows the classical substrate hydroxylation for which cytochrome P-450 enzymes are well known.

The solubilization, purification and cloning especially of the coupling enzyme would be desirable to gain further insight into this new reaction mechanism.

4. Experimental

4.1. General

Distribution of radioactivity on TLC plates was monitored with a Berthold (Tracemaster 20) linear analyser. Identification of marchantin A was per-

formed with radio 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, aqueous 2% acetonitrile, 0.1% HOAc; solvent B, 98% acetonitrile, 2% H_2O, 0.1% HOAc; gradient 100–0% A in 20 min, 0% A for 5 min; detection by HPLC radio detector monitor LB 506 C-1 (Berthold) and UV–Vis detector L-4250 (Merck-Hitachi) at 280 nm.

4.2. Plant material

Suspension cultures of *M. polymorpha* obtained from Prof. Ono (Kumamoto) 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 over a period of 14 days at 23°C under constant illumination (650 lux) at 100 rpm. Cells for enzyme preparation were filtered after 2 weeks, and extracted immediately without freezing.

4.3. Chemicals

All solvents and reagents were of the highest purity commercially available. The reference substances marchantin A and C were from the collection of the Tokushima department. The enzymes glucose oxidase and superoxide dismutase were purchased from Sigma-Aldrich, and catalase and glucose-6-phosphate dehydrogenase from Boehringer Mannheim. Ancymidol, cytochrome c, ketoconazole and metyrapone were from Sigma-Aldrich, juglone from Roth, tetcyclacis was a kind gift from the BASF company, prochloraz, propiconazole and triadimefone were kind gifts of the Schering company.

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

[1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Prelunularic acid was obtained by feeding L-[*U*-^{14}C]phenylalanine (5 μCi, 497 μCi μmol^{-1}, Amersham) to 1 ml aliquots of a 14-day-old *M. polymorpha* suspension culture under constant shaking (100 rpm). After 2 days the cells were separated from the medium by centrifugation, washed with 1 ml H_2O and extracted with 1.5 ml MeOH under constant shaking for 30 min at room temperature. The extract was separated by filtration, evaporated 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 average incorporation rate of phenylalanine into prelunularic acid was 20%.

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

For the 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) was incubated in 300 μl 0.13 M H_2SO_4 for 2 h at 56°C. After neutralization with 2 N NaOH, the reaction mixture was freeze dried, the residue 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. [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Lunularine

[1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Lunularine was obtained by enzymatic decarboxylation of [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]lunularic acid. Suspension cultures of *M. polymorpha* were harvested after 7 days of cultivation (typically 20 g fr. wt.) shock frozen with liquid N_2 and ground in 100 mM Tricine/NaOH buffer, pH 7.5, containing 10 mM KCl, 20 mM 2-mercaptoethanol, 10% glycerine (3 ml g^{-1} fr. wt.) in an ice-cold mortar with a pestle for 30 min. The tissue was filtered through four layers of cheesecloth, and the filtrate centrifuged at 2500 × *g* for 10 min. The supernatant was centrifuged again at 39.500 × *g* for 30 min. The pellet was taken up in 1 ml Tricine/NaOH buffer and an aliquot of this solution (100 μg protein) incubated with 10 μmol K–Pi buffer, pH 6.5, 8 nmol [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]lunularic acid (40,000 cpm) in a total volume of 50 μl at 30°C for 120 min. The decarboxylation reaction under these conditions was quantitative. After acidification with HOAc, the assays were extracted three times with 150 μl EtOAc, the organic phases combined and concentrated under vacuum. The residue was purified chromatographically by absorption to an RP18 column (Merck, 150 × 10 mm) and elution with 80% MeOH. Evaporation yielded [1,1',2',3',4',5',6',7,8-^{14}C]lunularine (80% yield) with the sp. act. as the precursor.

4.7. ^{3}H-*Labeled substrates*

^{3}*H*-Labeled lunularic acid and lunularine were obtained in the same way as described above by using L-[2,6-^{3}H]phenylalanine (Amersham, 55 mCi μmol^{-1}, diluted to the same sp. act. as the L-[*U*-^{14}C]phenylnylalanine used, 495 μCi μmol^{-1}). Both the isolated lunularic acid and lunularine had a sp. act. of 3 × 10^{6} dpm μmol^{-1}.

4.8. Enzyme preparation

All enzyme preparation procedures were carried out at 4°C. Freshly filtered cells of a 14-day-old cell culture were grounded for 30 min with the same amount (w/v) of 0.17 M Tricine/NaOH buffer, pH 7.5, containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 M sucrose and 9 g l^{-1} BSA. After fil-

tration through four layers of cheesecloth the crude extract was centrifuged for 10 min at $400 \times g$ and the supernatant again for 30 min at $9900 \times g$. The pellet was suspended in extraction buffer and layered on top of a four-step sucrose density gradient consisting of a 5 ml 50% (w/w) sucrose cushion and each of 15 ml of 44% (w/w), 33% (w/w), and 20% (w/w) sucrose in gradient buffer (20 mM Tricine/NaOH buffer, pH 7.5, 20 mM 2-mercaptoethanol, 1 mM EDTA). After centrifugation for 3 h at $80{,}000 \times g$ in an SW 27 rotor (Ultracentrifuge L8 55M, Beckman) the organelles between the 20 and 33% sucrose layer were carefully collected (10–15 ml), sedimented by centrifugation (20 min, $39{,}500 \times g$), washed with 0.17 M Tricine/NaOH buffer (20 mM 2-mercaptoethanol, 0.5 M sucrose, pH 7.5) sedimented again, taken up in the washing buffer (200 µl, 4.1 mg protein ml^{-1}) and used for the enzyme assays.

4.9. Standard enzyme assay

[1,1′,2′,3′,4′,5′,6′,7,8-^{14}C]Lunularic acid (40,000 cpm, 9 nmol), 100 nmol NADPH, 100 nmol NADP, 500 nmol Glc-6-phosphate, 0.7 U Glc-6-phosphate-dehydrogenase, 50 µmol Tricine/NaOH buffer, pH 7.5 and 0.1 mg protein were incubated in a total volume of 200 µl at 30°C for 120 min. The incubation was terminated by addition of 1 µl HOAc, extracted with 500 µl EtOAc and the phases separated by centrifugation at $10{,}000 \times g$ for 3 min at room temperature. The organic phases were concentrated to dryness, the residues taken up in 50 µl MeOH and 20 µl of this solution subjected to TLC using toluene–HOAc (8 : 2) as solvent system (lunularic acid R_f 0.48, lunularine R_f 0.43, marchantin A R_f 0.50, marchantin C R_f 0.71). The labeled products were quantified by a TLC-radioscanner. For the determination of the pH-optimum, the following buffers were used in the standard assay system in 0.25 M concentration: citrate, pH 4–6, Tris/HCl, 7–9 and Tricine/NaOH 7.5–8.5.

4.10. Inhibition experiments

Inhibition experiments with the different gas mixtures were performed as described by Estabrook and Cooper (1963).

4.11. Product identification

For product identification, 14 standard enzyme assays were combined after incubation, extracted with EtOAc and the products separated by TLC (toluene–HOAc, 8 : 2). The isolated products were diluted with the corresponding reference substances (1.5 µmol marchantin A, 5 µmol marchantin C) and rechromatographed in EtOAc–*n*-hexane (1 : 1, marchantin A R_f 0.62, marchantin C R_f 0.73). After elution from TLC the sp. act. of both products were determined by calculating the concentrations from standard curves taken at 280 nm (linear from 0.1 to 2.4 µmol ml^{-1}) and measuring aliquots in a scintillation counter. The eluted and concentrated marchantins were methylated by incubation for 24 h at room temperature with a freshly distilled solution of diazomethane. The products were chromatographed twice ((1) toluene–EtOAc, 4 : 1, marchantin-A-1′,6′,13-O-methyl ether R_f 0.83, marchantin C-1′,13-O-methyl ether R_f 0.89; (2) toluene–HOAc, 8 : 2, marchantin A-1′,6′,13-O-methyl ether R_f 0.79, marchantin C-1′,13-O-methyl ether R_f 0.84) and the sp. act. after each chromatography step determined again.

4.12. ^{3}H/^{14}C experiments

The first experiment was performed with [2,6-^{3}H]lunularic acid (228,975 dpm; sp act. 3×10^6 dpm µmol^{-1}) and [1,1′,2′,3′,4′,5′,6′,7,8-^{14}C] lunularine (33,670 dpm; sp. act. 4.1×10^6 dpm µmol^{-1}) the second one with [^{3}H] lunularine (106,450 dpm; 4.1×10^6 dpm µmol^{-1}) and [^{14}C]lunularic acid (20,777 dpm ^{14}C; 3×10^6 dpm µmol^{-1}) in the standard assay system. The products were separated by TLC as described in section 4.9, eluted with methanol and the radioactivity determined in a scintillation counter.

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