



Biosynthesis of anthraquinones in cell cultures of *Cinchona* ‘Robusta’ proceeds via the methylerythritol 4-phosphate pathway

Ying-Shan Han^a, Robert van der Heijden^a, Alfons W.M. Lefeber^b,
Cornelis Erkelens^b, Robert Verpoorte^{a,*}

^a*Division of Pharmacognosy, Leiden / Amsterdam Center for Drug Research, Gorlaeus Laboratories,
PO Box 9502, 2300 RA Leiden, The Netherlands*

^b*Division of NMR, Leiden Institute of Chemistry, Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands*

Received 26 March 2001; received in revised form 13 June 2001

Abstract

Robustaquinone B was found as a major anthraquinone in cell cultures of *Cinchona* ‘Robusta’ after treatment with a fungal elicitor. Anthraquinones in *Cinchona* are considered to be of the *Rubia* type, i.e. rings A and B are derived from chorismate and α -ketoglutarate, whereas ring C is formed from isopentenyl diphosphate (IPP). To determine the origin of IPP, either formed via the mevalonic acid pathway or the 2-C-methyl-D-erythritol 4-phosphate pathway, the incorporation of [1-¹³C]glucose into robustaquinone B was studied. The ¹³C labeling of robustaquinone B was analyzed by one- and two-dimensional NMR spectroscopy and the labeling pattern was compared with the hypothetical labeling patterns obtained via the different biosynthetic pathways. The results clearly show that the IPP, constituting the ring C of robustaquinone B, is biosynthesized via the 2-C-methyl-D-erythritol 4-phosphate pathway. Moreover, the data also confirm that rings A and B of robustaquinone B are formed from chorismate and α -ketoglutarate via *o*-succinylbenzoate. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Cinchona* ‘Robusta’; Rubiaceae; Anthraquinone; Isopentenyl diphosphate; Mevalonic acid; 2-C-Methyl-D-erythritol 4-phosphate; NMR spectroscopy

1. Introduction

Cinchona is an evergreen tree of the Rubiaceae family. The bark of the *Cinchona* tree is the source for the pharmaceutically important alkaloids quinine and quinidine. However, cell and tissue cultures of *Cinchona* species produce anthraquinones (AQs) more readily than alkaloids (Wijnsma et al., 1985, 1986). In cell cultures of *Cinchona robusta* (now revised as *C. ‘Robusta’*

according to Snoeijer, 2000), the AQ biosynthesis can be induced by biotic elicitors such as a homogenate of *Phytophthora cinnamomi* (*Pcin*) (Ramos-Valdivia et al., 1997).

One of the remarkable features of AQ biosynthesis in higher plants is that they are derived from a variety of different precursors and pathways (Leistner, 1981; Inouye and Leistner, 1988). There are two main biosynthetic pathways leading to AQs in higher plants: the polyketide pathway (Van den Berg and Labadie, 1989) and the chorismate/*o*-succinylbenzoic acid pathway (Leistner, 1985). In the polyketide pathway, AQs are biosynthesized from acetyl-CoA and malonyl-CoA via an octaketide chain. These types of AQs often exhibit a characteristic substitution pattern, i.e. they are substituted in both rings A and C of AQs. However, AQs in the family Rubiaceae such as those from *Morinda*, *Rubia*, *Galium* species are considered to be formed via the chorismate/*o*-succinylbenzoic acid pathway (Fig. 1). Early work on feeding experiments established that rings A and B of AQs in *Rubia* (Leistner, 1981), *Morinda*

Abbreviations: AQ, anthraquinone; CC, column chromatography; 2,4-D, 2,4-dichlorophenoxyacetic acid; DMAPP, 3,3-dimethylallyl diphosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; HMBC, heteronuclear multiple bond coherence; HPLC, high-performance liquid chromatography; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MVA, mevalonic acid; NMR, nuclear magnetic resonance; NOE, nuclear overhauser enhancement; *Paph*, *Pythium aphanidermatum*; *Pcin*, *Phytophthora cinnamomi*; SAM, S-adenosyl-methionine; TLC, thin-layer chromatography; UV-vis, ultraviolet-visible

* Corresponding author. Tel.: +31-71-5274528; fax: +31-715-274511.

E-mail address: verpoort@lacdr.leidenuniv.nl (R. Verpoorte).

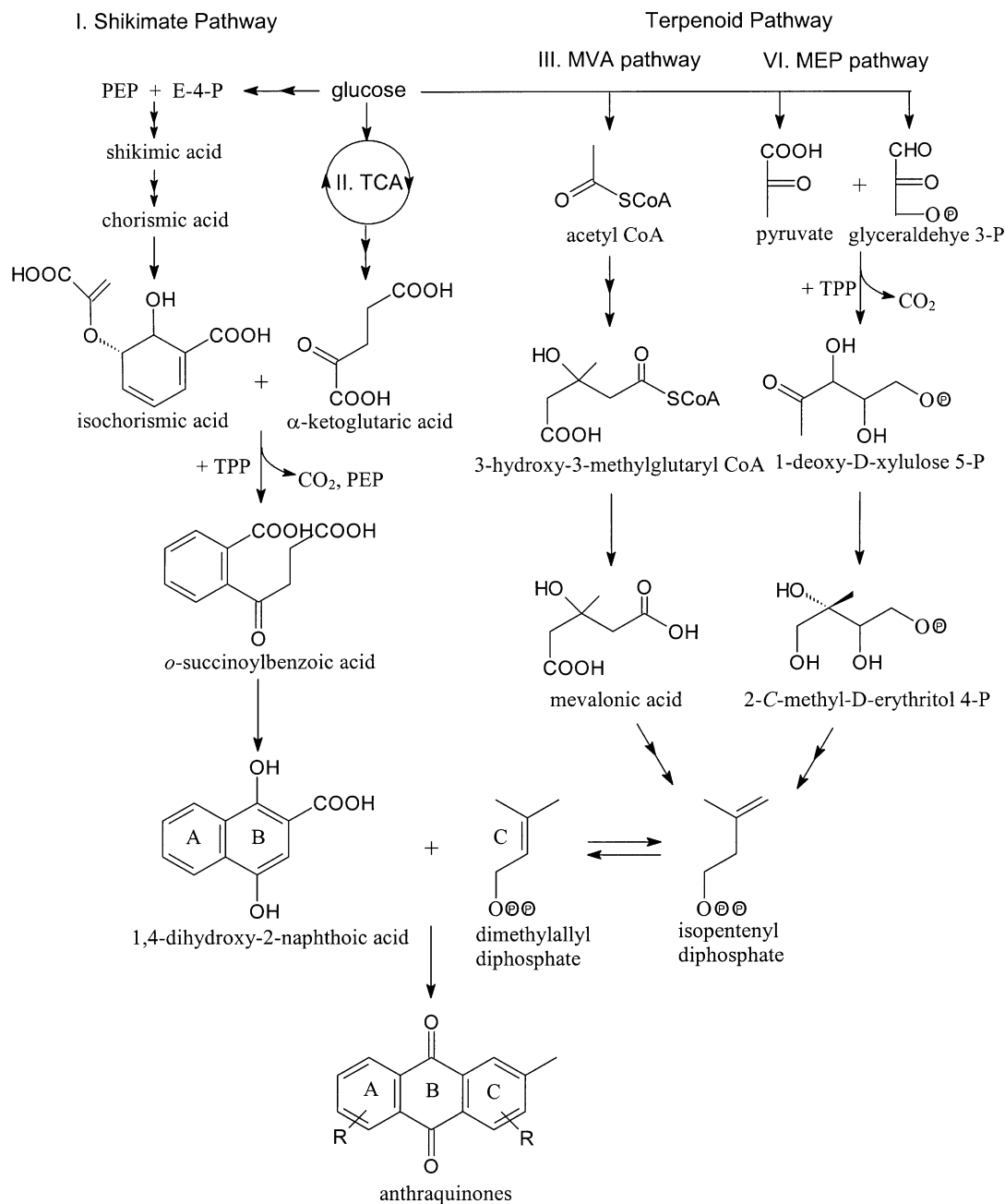


Fig. 1. Biosynthetic pathway leading to anthraquinones in the Rubiaceae. I, II. Rings A and B of AQs are derived from chorismic (or isochorismic) acid and α -ketoglutarate via *o*-succinylbenzoic acid (Leistner, 1981, 1985); III and IV. The origin of ring C is not yet known, either via the MVA (III) or the MEP pathway (IV) (part of the investigation of this paper). E-4-P=erythrose 4-phosphate, P=phosphate residue, PEP=phosphoenolpyruvate, TCA=tricarboxylic acid, TPP=thiamine diphosphate.

(Leistner, 1975) and *Galium* (Bauch and Leistner, 1978; Inoue et al., 1984) are derived from shikimic acid (Leistner and Zenk, 1967), α -ketoglutarate via *o*-succinylbenzoate [Fig. 1(I) and (II)], whereas ring C of *Rubia* type AQs is derived from mevalonic acid [Fig. 1(III)] (Leistner, 1981, 1985), possibly via isopentenyl diphosphate (IPP)/3,3-dimethylallyl diphosphate (DMAPP), a universal building block for all isoprenoids, because feeding of [2-¹⁴C] mevalonic acid to root system (Leistner and Zenk, 1968) and plants

(Burnett and Thomson, 1968) of *Rubia tinctorum* resulted in incorporation into AQs.

For many decades, the formation of IPP had been described as being uniquely derived from the acetate/mevalonic acid (MVA) pathway [Fig. 1(III)], which was accepted as ubiquitous in all living organisms (Qureshi and Porter 1981; Bach et al., 1999; Bochar et al., 1999). Recently, a non-MVA pathway, termed as the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [Fig. 1(IV)], in which IPP is formed from 1-deoxy-D-

xylulose 5-phosphate by condensation of glyceraldehyde 3-phosphate and pyruvate, was found to be existing in bacteria, green algae and higher plants (Lichtenthaler, 1999; Rohmer, 1999; Eisenreich et al., 2001; Schwender et al., 2001). This raises the question about the origin of IPP, constituting ring C of the AQs in the Rubiaceae, whether it is either derived from the MVA or the MEP pathway.

AQs from *Cinchona*, another member of the Rubiaceae, may be an exception, as they are substituted in both rings A and C (Schripsema et al., 1999). To a lesser extent, this substitution pattern has also been found in other genera of the Rubiaceae, such as *Galium* (Koyama et al., 1993) and *Morinda* (Leistner, 1973a). Normally this pattern is typical of AQs derived from acetyl-CoA via the polyketide pathway (Van den Berg and Labadie, 1989). However, the AQs in *Cinchona* are considered to be of the *Rubia* type.

To elucidate the origin of IPP, incorporated to ring C of the AQs in *Cinchona*, either derived from the MVA or the MEP pathway, the biosynthesis of the AQs in *Cinchona* was investigated by using the retro-biosynthetic nuclear magnetic resonance (NMR) analysis (Eisenreich and Bacher, 2000), except without analysis of amino acid labeling patterns. In this study, we have chosen cell cultures of *C. 'Robusta'* for the incorporation experiments using [1-¹³C]glucose as precursor. The use of this *Cinchona* cell line has some advantages. The biosynthesis of AQs in cell cultures of *C. 'Robusta'* can be easily turned on by elicitation with biotic elicitors (Wijnsma et al., 1985; Ramos-Valdivia et al., 1997). The elicited cell cultures accumulated AQs in relatively large amounts. The elicitation process has shown to be stable over the years. Moreover, the cells can grow on glucose as the sole carbon source. However, prior to the incorporation experiments, the production of AQs was optimized to obtain satisfactory amounts of AQs.

2. Results and discussion

2.1. Optimization of the system with respect to AQ accumulation

Cell cultures of *C. 'Robusta'* produce a variety of AQs, when induced by *Pcin* (Ramos-Valdivia et al., 1997; Schripsema et al., 1999) and by addition of the medium filtrate of *Pythium aphanidermatum* (*Paph*). To optimize the AQ production in cell cultures of *C. 'Robusta'*, five elicitors were tested. Methyl jasmonate (100 μ M), yeast extract (1 g/l; w/v), the medium filtrate of *Paph* (2:10 v/v (volume of *Paph* medium filtrate: volume of culture)), salicylic acid (200 μ M), *Pcin* (800 mg mycelium dry wt/l culture) were added to 5-day old cell cultures of *C. 'Robusta'*, respectively. The cell cul-

tures were harvested after an additional three-day growth period. The five elicitors had no large effects on the cell growth, except *Paph* showed some inhibition of biomass accumulation (data not shown). For the induction of AQ biosynthesis, *Paph* was found to be most effective, the content of total AQs was 3.25 μ mol/g (dw). Furthermore, the AQ production was also increased by addition of increasing amounts of *Paph* medium filtrate ranging from 1:10 to 7:10 v/v. The type of carbon source and its concentration further influenced the AQ production. Concentrations of 2% sucrose, 4% sucrose, 2% glucose and 4% glucose were tested. The B5 medium with 2% glucose gave highest content of AQs, 10.2 μ mol/g. This is consistent with the results in cell cultures of *R. cordifolia*, where sucrose was inferior to glucose for AQ production (Suzuki et al., 1984). In contrast, sucrose was better for the formation of AQs in *Morinda citrifolia* (Zenk et al., 1975; Wijnsma et al., 1985). Glutathione, a ubiquitous tripeptide, has been reported to induce a marked increase in AQ production in adventitious root cultures of *R. tinctorum*, particularly lucidin-3-*O*-primeveroside (Sato et al., 1997). Glutathione was added to the medium at a concentration of 2 mM, but it had no effect on the AQ accumulation in *C. 'Robusta'* cell cultures (data not shown).

Therefore, the following condition: B5 medium with 2% glucose instead of sucrose and treatment by *Paph* at 3:10 or 1:10 v/v (concentrated *Paph*) was finally chosen for further experiments. The concentrated *Paph* was used because it gave a satisfactory amount of AQs without addition of too much volume of *Paph*. From the elicited cell cultures of *C. 'Robusta'*, a major AQ was isolated and identified by spectroscopic methods and mass spectrometry as robustaquinone B (1), i.e. the 1-hydroxy-6, 7-dimethoxy-2-methylantraquinone (Fig. 2), which was previously isolated from cell cultures of *C. 'Robusta'* (Schripsema et al., 1999).

2.2. Incorporation of [1-¹³C]glucose into robustaquinone B

The feeding experiments with [1-¹³C] labeled and unlabeled glucose as precursors yielded 1.1 mg labeled 1

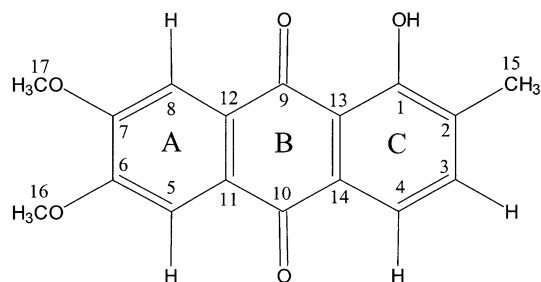


Fig. 2. Structure and carbon numbering of robustaquinone B (1).

from $[1-^{13}\text{C}]$ glucose grown culture and 3 mg unlabeled **1** (as control) from unlabeled glucose grown culture. Both samples were also analyzed by mass spectrometry, which showed the two samples are identical molecular ion $[\text{M}]^+$ at m/z 298. The mass fragmentation of labeled **1** gave the signal $[m/z \text{ (rel. int.)}]$: 298 ($[\text{M}]^+$, 100), 299 ($[\text{M}+1]^+$, 62.13), 300 ($[\text{M}+2]^+$, 19.66), 301 ($[\text{M}+3]^+$, 4.29). The unlabeled **1** exhibited the signal $[m/z \text{ (rel. int.)}]$: 298 ($[\text{M}]^+$, 100), 299 ($[\text{M}+1]^+$, 19.51), 300 ($[\text{M}+2]^+$, 1.78). The UV-Vis λ_{max} (MeOH): 220, 280, 302 (*sh*), 412 and HPLC elution profile (data not shown) were identical for the labeled and unlabeled compounds of **1**.

A prerequisite for the interpretation of biosynthetic tracer studies by quantitative NMR spectroscopy is the unequivocal assignment of all ^1H NMR and ^{13}C NMR signals of **1**. ^1H chemical shifts of **1** in CDCl_3 has been previously reported (Schripsema et al., 1999). ^{13}C chemical shifts of **1** were not yet reported. We performed a more detailed NMR analysis using HMQC, HMBC, APT and NOE spectroscopy experiments (Figs. 3 and 4). In the ^1H NMR spectrum in CDCl_3 , the H-5 and H-8 are very close and it is difficult to distinguish them (Fig. 3A). However, they can be easily distinguished in benzene (C_6D_6) (Fig. 3B), due to solvent shifts (Wilczynski et al., 1968). The ^{13}C NMR signals were assigned on the basis of two-dimensional HMQC and HMBC experiments (Fig. 4). The shifts of the methoxy groups at C-6 and C-7 were determined by a NOE experiment in CDCl_3 . Irradiation of the proton at C-8 resulted in an increase in the signal intensity of the hydroxy group at C-1 and methoxy group at C-6. Irradiation of the proton at C-5 did not result in an increase in the signal

intensity of the hydroxy group at C-1 and methoxy group at C-7 (data not shown). The C-12 signal in C_6D_6 can not be determined due to overlapping with the signal of $\text{C}_6\text{D}_5\text{H}$ in C_6D_6 . However, it can be determined in CDCl_3 . The relative ^{13}C intensities and enrichments for all carbon positions of **1** in C_6D_6 and CDCl_3 are presented in Table 1, respectively. The ^{13}C enrichment ratios in C_6D_6 matched those in CDCl_3 very well. The mass spectrometry analysis of the labeled **1** from $[1-^{13}\text{C}]$ glucose grown culture and unlabeled **1** from unlabeled glucose grown culture revealed a high level of enrichment too. The absolute abundance of each carbon in CDCl_3 was calculated on the basis of the mass intensity and is presented in Table 1.

On the basis of the existing different hypothetical pathways, the predicted labeling patterns of **1** were obtained and are shown in Fig. 5(I)–(III). The observed labeling pattern of **1** is presented in Fig. 5(IV).

In comparing the observed ^{13}C labeling patterns [Fig. 5(IV)] with the predicted labeling patterns via the polyketide pathway [Fig. 5(I)], it is obvious that the observed ^{13}C labeling pattern in **1** is not consistent with the predicted labeling pattern based on a polyketide origin of the entire ring system. Thus biosynthesis of AQs in *Cinchona* via the polyketide pathway can be ruled out conclusively.

The labeling pattern of IPP/DMAPP was interpreted on the basis of the metabolism of glucose in plants (Eisenreich et al., 1996). The label from $[1-^{13}\text{C}]$ glucose is diverted to C-3 of triose phosphate/pyruvate and C-2 of acetyl-CoA. IPP synthesis starting from three C-2 labeled acetyl-CoA molecules via the MVA pathway

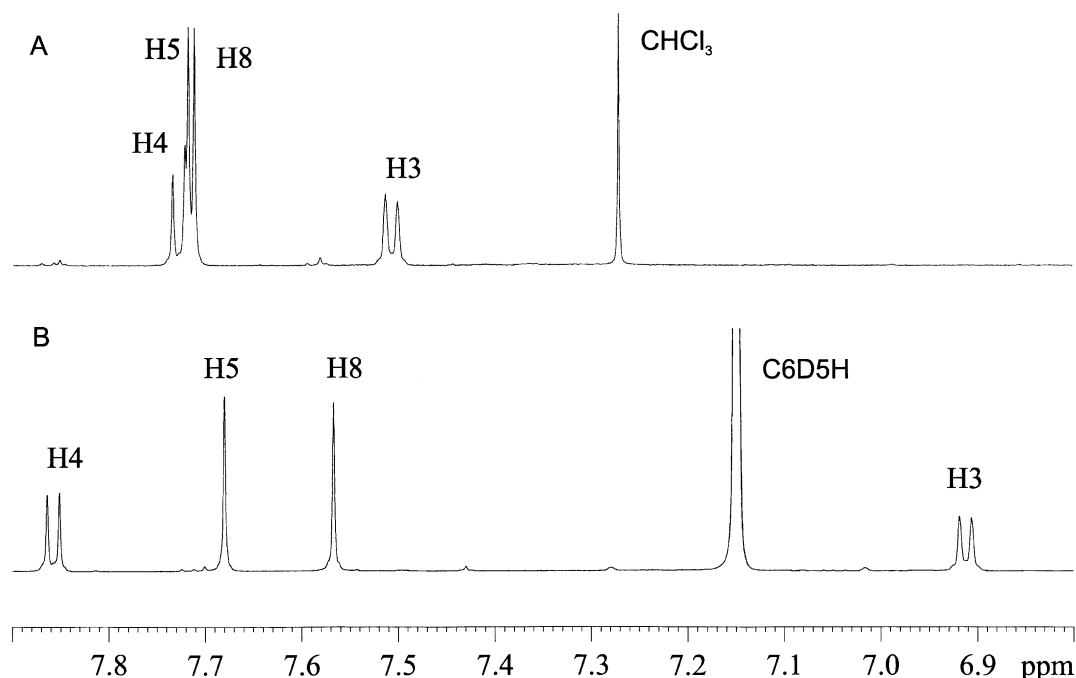


Fig. 3. Parts of the ^1H NMR spectrum of robustaquinone **B** recorded in CDCl_3 (A) and C_6D_6 (B) (600 MHz).

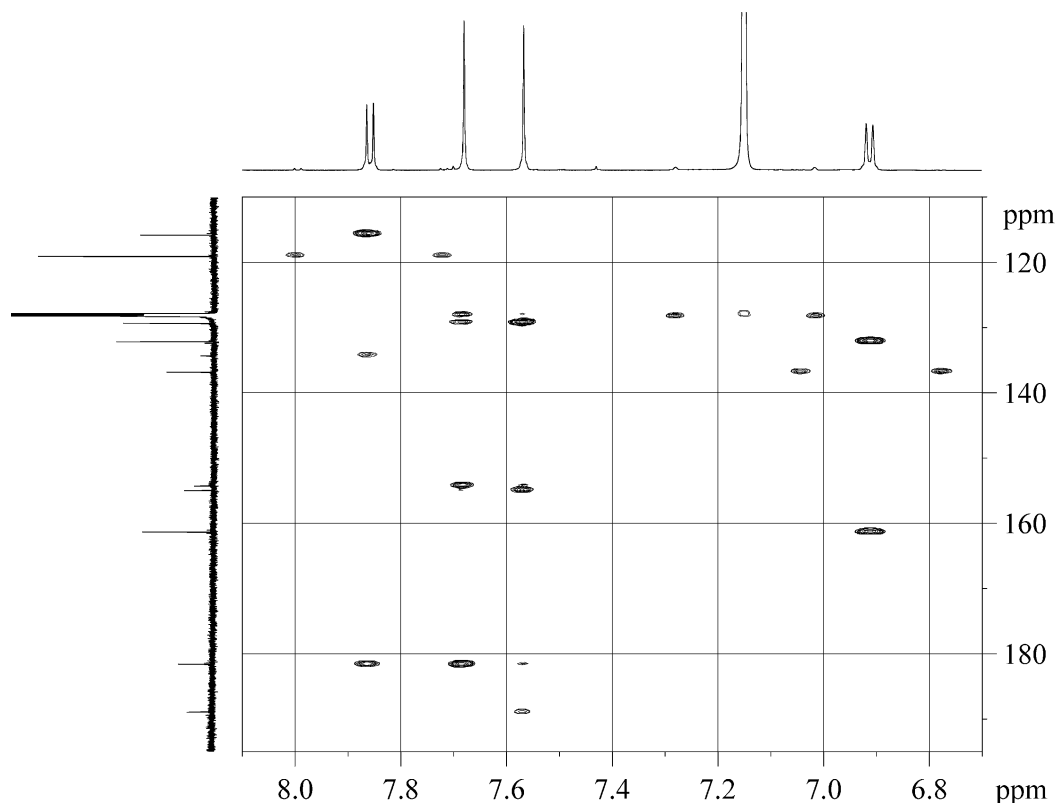


Fig. 4. Part of a two-dimensional HMBC spectrum in C_6D_6 of robustaquinone **B** from $[1-^{13}C]$ glucose grown *Cinchona* 'Robusta' cell suspension culture.

should divert label to C-2, C-4 and C-5 in IPP/DMAPP. This resulted in predicted labeling patterns with high ^{13}C abundance in C-1, C-3 and C-15 of **1** [Fig. 5(II)]. On the other hand, IPP biosynthesis from C-3 labeled pyruvate and glyceraldehyde 3-phosphate via the MEP pathway should divert label to C-1 and C-5 in IPP/DMAPP. This resulted in the predicted labeling patterns with high ^{13}C abundance in C-1 and C-4 of **1** [Fig. 5(III)]. By comparison with the observed labeling patterns of the ring C in **1** [Fig. 5(IV)], it is obvious that it is consistent with the MEP pathway.

Enrichment of ^{13}C in the C-15 (methyl group) was also observed, but the enrichment was lower compared to the data of C-1 and C-4 of **1**. This may be explained that C-4 (unlabeled) and C-5 (labeled) in DMAPP via the MEP pathway can be randomly attached to the ring B of **1** during the cyclization reaction for the formation of ring C. This leads to scrambling of the label (C-5 in DMAPP) between C-1 and C-15 in **1**. This is in good agreement with the observation on scrambling of radioactivity in the AQs in *R. tinctorum* (Burnett and Thomson, 1968). It has been shown that the two pathways are located in different plant cell compartments. The MEP pathway is operative in plastids of higher plants, whereas the MVA pathway occurs in the cytoplasm (Lichtenthaler et al., 1997). In photoautotrophic cell cultures of *M. citrifolia*, irregular or distorted plastids containing starch grains were observed in AQ-producing cells (Yamamoto et al.,

1987). This suggests that plastids play an important role in AQ production. At present the exact process of AQ synthesis within the cell is not yet known. However, it is clear that the biosynthesis of AQs in the Rubiaceae at least requires plastids for the formation of isoprene moiety. In addition, early work has shown that feeding of $[2-^{14}C]$ mevalonic acid in cell cultures of *M. citrifolia*, no incorporation into morindone or alizarin was observed (Leistner, 1973a). Also feeding of $[5-^{14}C]$ mevalonic acid to cell cultures of *R. tinctorum*, the incorporation rate of ^{14}C into alizarin was only 0.003% (Leistner, 1973b). These results are in agreement with the view that ring C of AQs in the Rubiaceae is derived from the MEP pathway, not the classical MVA pathway. However, we can not exclude a very minimal contribution of the MVA pathway for the formation of IPP unit in **1** as well. It was also shown that all higher plants appear to utilize both the MVA and MEP pathways with apparent cross-talk between the two pathways across the compartments (Goese et al., 1999). Since the ^{13}C enrichment in C-15 was much lower than those of C-1 and C-4, and no enrichment in C-3 was found, the former explanation seems more conceivable. Thus it is obvious that IPP/DMAPP units in **1** are biosynthesized via the MEP pathway.

AQs in *Cinchona* have a characteristic 6,7-disubstitution in the A-ring. In comparing the structural relations of the AQs isolated from *Cinchona* species, Schripsema

Table 1
¹³C NMR data and abundance of labeled and unlabeled robustaquinone B in C₆D₆

Carbon position	ppm ^a in CDCl ₃	ppm ^a in C ₆ D ₆	Relative intensity ^b in CDCl ₃ control	Relative intensity ^b in CDCl ₃ labeled	Relative intensity ^b in C ₆ D ₆ control	Relative intensity ^b in C ₆ D ₆ labeled	Enrichment ratio in CDCl ₃	Enrichment ratio in C ₆ D ₆	Absolute abundance in CDCl ₃ ^c
1	160.82	161.32	1.52	4.40	1.53	4.40	5.35 ^{ae}	3.79 [*]	5.47 [*]
2	134.72	134.32	1.48	1.22	1.80	1.40	1.52	1.03	1.55
3	136.77	136.82	3.33	1.89	3.60	3.00	1.06	1.09	1.08
4	119.13	119.06	3.42	9.33	3.67	11.70	5.06 [*]	4.20 [*]	5.17 [*]
5	108.50	108.77	3.48	1.89	3.70	2.80	1.00	1.00	1.02
6	154.16	154.95	1.35	1.10	1.23	1.00	1.50	1.06	1.53
7	153.65	154.24	1.32	1.22	1.27	1.40	1.70	1.45	1.74
8	107.86	108.13	3.48	8.78	3.47	12.60	4.67 [*]	4.78 [*]	4.77 [*]
9	188.36	188.86	1.29	1.10	1.30	1.20	1.57	1.21	1.61
10	181.86	181.58	1.13	3.77	1.00	4.30	6.18 [*]	5.66 [*]	6.32 [*]
11	128.92	129.33	1.36	3.11	2.03	3.95	4.22 [*]	2.56 [*]	4.31 [*]
12	127.96	n.d. ^d	1.32	1.00	n.d. ^d	n.d. ^d	1.41	n.d. ^d	1.44
13	115.15	115.76	1.00	2.89	1.33	3.55	5.35 [*]	3.51 [*]	5.47 [*]
14	131.39	132.16	1.27	4.40	1.57	4.70	6.41 [*]	3.93 [*]	6.55 [*]
15 (CH ₃)	16.17	15.98	3.53	4.78	2.93	6.30	4.37 [*]	2.83 [*]	4.47 [*]
16 (OCH ₃)	56.53	55.43	4.12	11.33	4.13	14.75	5.09 [*]	4.70 [*]	5.20 [*]
17 (OCH ₃)	56.59	55.41	4.06	9.56	4.63	15.90	4.35 [*]	4.51 [*]	4.45 [*]

^a Reference to the TMSi signal as internal standard.

^b Relative intensities of signals in quantitative ¹³C NMR spectrum.

^c Calculated on the basis of mass intensity times with enrichment ratio.

^d Not determined due to signal overlapping with the signal of C₆D₅H in C₆D₆.

^e ¹³C enrichment.

et al. (1999) proposed that these AQs might be derived from caffeic acid or another phenylpropanoid and not from chorismate via *o*-succinylbenzoic acid. According to the labeling patterns of phenylalanine produced in cell cultures of *R. tinctorum* after incorporation of [1-¹³C] glucose (Eichinger et al., 1999), it is known that caffeic acid is derived from phenylalanine via the phenylpropanoid pathway (Fig. 6) (Strack, 1997). If the rings A and B of AQs in *Cinchona* are derived from caffeic acid or another phenylpropanoid, this should result in ¹³C enrichment in either C-8, C-9, C-11 in ring A and B [Fig. 6(I)] or C-5, C-10, C-12 in rings A and B [Fig. 6(II)] of **1**. However, the observed labeling patterns in rings A and B of **1** showed enrichments in C-8, C-10, C-11 in rings A and B of **1** [Fig. 6(III)]. Therefore, this hypothesis is unlikely. So the data clearly show that ring A and B of **1** are formed from chorismate and α -keto-glutarate via *o*-succinylbenzoate. This result is also consistent with the report on the biosynthesis of lucidin primeveroside in cell cultures of *R. tinctorum* (Eichinger et al., 1999).

As shown in Fig. 5(IV), the two methoxy groups (C-16 and C-17) substituted at C-6 and C-7 of ring A in **1** were highly enriched. In the biosynthesis of morindone in cell cultures of *M. citrifolia* (Leistner, 1973a, 1975), it was shown that the hydroxy groups attached to ring A are not derived from the hydroxy groups of shikimic acid. The hydroxy groups are introduced at a later stage of the biosynthetic pathway. So the two methoxy

groups attached to ring A of **1** may be formed in the same way, i.e. the hydroxy groups are first introduced to ring A and subsequently are methylated to give the methoxy groups. The methyl groups are probably derived from the methyl of *S*-adenosyl-methionine (SAM) via an enzymatic transmethylation reaction. It is known that the methyl group of SAM is from *N*⁵-methyl-tetrahydrofolic acid. Whereas the methyl group of *N*⁵-methyl-tetrahydrofolic acid is formed from the glycolytic intermediate 3-phosphoglycerate via serine and cysteine. Since the labeled acetate from [1-¹³C]glucose is channeled into the tricarboxylic acid (TCA) cycle, incorporation of the label via 3-phosphoglycerate is to be expected. Thus this easily explains why the two methoxy groups attached to ring A of **1** were labeled. Since the cells might carry out hydroxylation and methylation in the later stage of AQ biosynthesis, there should be hydroxylase and methyltransferase activity present in the elicited cells. The character of such enzymes remains to be done.

These results clearly show that the isoprenoid unit for AQ biosynthesis in elicited cell cultures of *C. 'Robusta'* is formed via the MEP pathway. However, additional experiments with pathway specific precursors or inhibitors and investigations on the MEP pathway at the enzyme and gene levels might provide further evidence. So far we found that lovastatin (or mevinolin), a highly specific inhibitor of the enzyme 3-hydroxy-3-methylglutaryl CoA reductase in the MVA pathway, did not

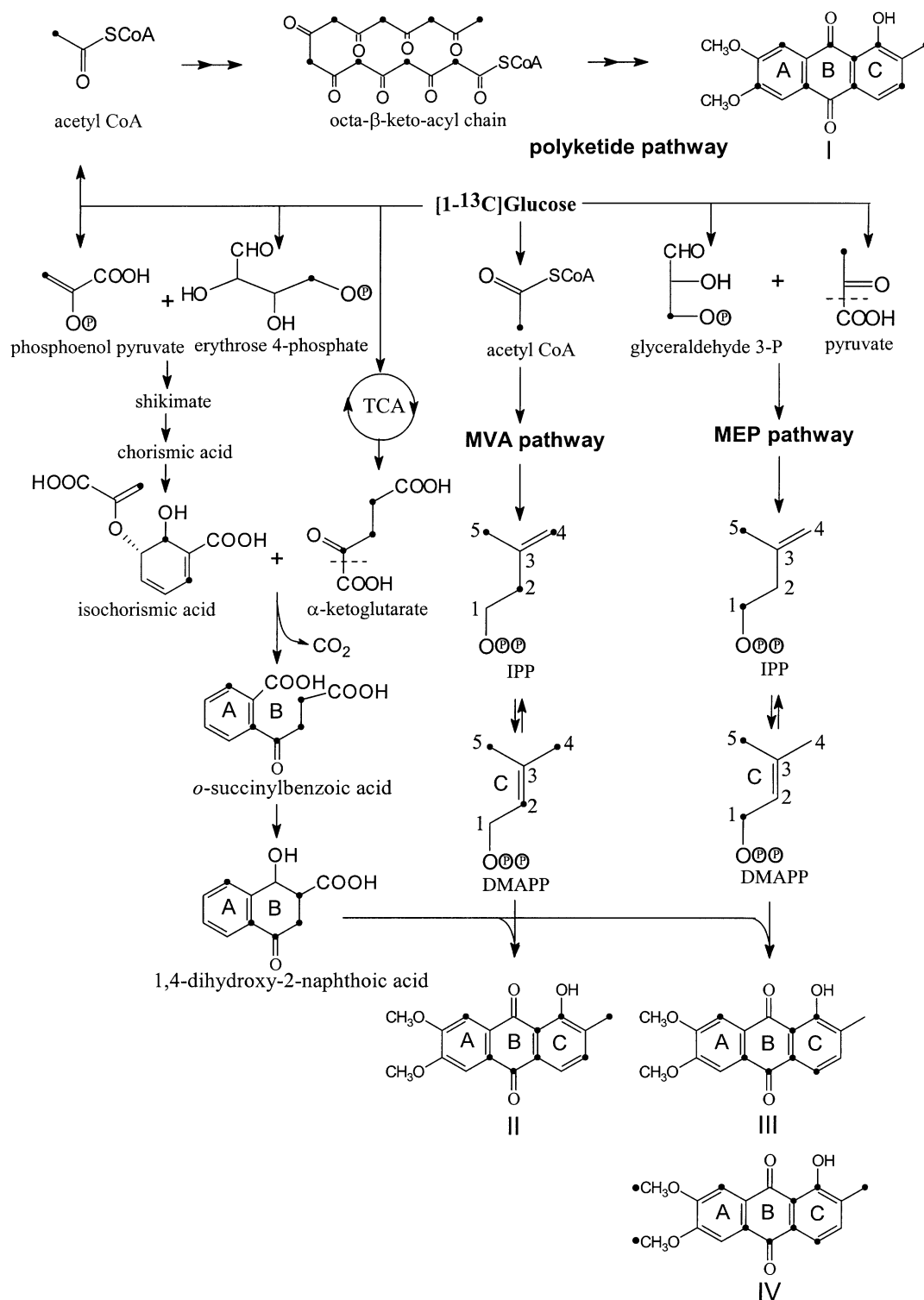


Fig. 5. Labeling patterns of robustaquinone B after feeding of [1-¹³C]glucose diluted with unlabeled glucose (1:1, w/w) to cell cultures of *C. 'Robusta'*. The black dots in I, II and III indicate ¹³C labeled positions predicted from the assumed pathways. I. The predicted labeling pattern of AQs via polyketide pathway on the basis of the labeling data from acetyl CoA. II. The predicted labeling pattern of AQs derived from chorismate via *o*-succinylbenzoate and IPP/DMAPP originated from acetyl-CoA via the MVA pathway. III. The predicted labeling pattern of AQs derived from chorismate via *o*-succinylbenzoate and IPP/DMAPP formed via the MEP pathway. IV. Observed labeling pattern. DMAPP = 3,3-dimethylallyl diphosphate, IPP = isopentenyl diphosphate, TCA = tricarboxylic acid.

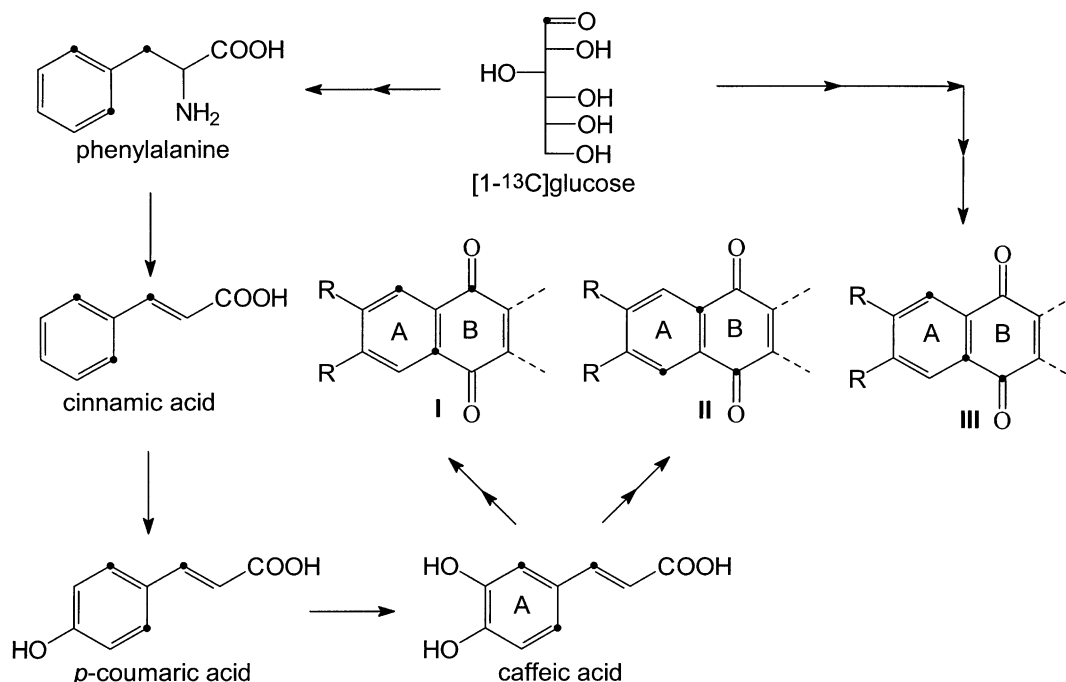


Fig. 6. Observed labeling pattern and the predicted labeling patterns of rings A and B in robustaquinone B via the phenylpropanoid pathway. I and II. The predicted labeling patterns of rings A and B in robustaquinone B via the phenylpropanoid pathway. III. Observed labeling pattern of rings A and B in robustaquinone B from cell cultures of *C. 'Robusta'* grown with [1-¹³C]glucose.

inhibit the biosynthesis of AQs in cell cultures of *C. 'Robusta'* (Han et al., unpublished data). Further studies on the feeding of precursors and the MEP pathway specific inhibitors, the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (catalyzing the initial step of the MEP pathway) and cloning of the gene encoding DXS are also under way.

It was shown that lucidin primeveroside in cell cultures of *R. tinctorum* (Eichinger et al., 1999) was formed via the MEP pathway. In cell cultures of *M. citrifolia* grown in the presence of AQ synthesis-inhibiting auxin 2,4-D (Stalman et al., 2001), it was found that addition of pyruvate at a concentration of 20 mM in the growth medium resulted in a 4-fold increase in AQ production. Feeding ¹³C- and ¹⁴C-labeled pyruvate to the cells demonstrated that pyruvate was incorporated into the AQs and the resulting label was present in the three-ring skeleton of both AQ glycosides and aglycones. When ¹⁴C-labeled pyruvate was fed to the cell cultures in presence of glyphosate, an inhibitor of the shikimate pathway (Amrhein et al., 1980), no label was found in the accumulated shikimate. This indicates that pyruvate is incorporated into AQs, probably via IPP and/or α -ketoglutarate but not via shikimate. Obviously, it appears to be clear that the C-ring of the AQs in cell cultures of the family Rubiaceae is derived from the MEP pathway.

In higher plants, it has been shown that the MEP pathway is also involved in the formation of isoprene (Zeidler et al., 1997), monoterpenoids (Eisenreich et al., 1997), diterpenoids (Eisenreich et al., 1996), as well as

carotenoids, prenyl chain of chlorophylls and plastoquinone-9 (Lichtenthaler et al., 1997). This result affords a further example of the MEP pathway shown to be involved in the formation of isoprenoid unit for AQ biosynthesis in higher plants.

3. Experimental

3.1. Reagents

[1-¹³C]Glucose was purchased from Isotec (Miamisburg, OH). Methyl jasmonate was purchased from Aldrich (Milwaukee, WI, USA). Yeast extract was from Merck (Darmstadt, Germany). Salicylic acid was from Sigma (St. Louis, MO, USA).

3.2. Preparation of the elicitors

Pythium aphanidermatum was purchased from Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands) and was grown in 500 ml Erlenmeyer flasks containing 100 ml MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2 mg/l glycine. After 7 days, the cultures were harvested by filtration under suction and autoclaved. The autoclaved *Paph* cultures were filtered under sterile condition and the obtained medium filtrates were used as elicitor. To reduce the volume of *Paph*, the *Paph* medium filtrates were concentrated by freeze-drying to one third of their original volumes. The concentrated

Paph was autoclaved again and was stored at -20°C . *Phytophthora cinnamomi* (*Pcin*) was prepared as described by Ramos-Valdivia et al. (1997). Yeast extract and salicylic acid were dissolved in water and methyl jasmonate was dissolved in 50% ethanol.

3.3. Plant cell cultures

Cell suspension cultures of *Cinchona* 'Robusta' were established and maintained as described according to Ramos-Valdivia et al. (1997). For the experiments, the cells were taken from the cultures when they were in the exponential phase of the growth cycle (3–5 days old). The 250 ml Erlenmeyer flasks containing 50 ml of medium were inoculated with 5 g fresh weight of cells under sterile conditions.

For the incorporation experiments, one batch of 20 culture flasks was grown on medium containing 10 g/l [$1\text{-}^{13}\text{C}$]glucose and 10 g/l unlabeled glucose. One batch of 40 flasks grown in the medium containing 20 g/l unlabeled glucose under the same conditions was used as control. The cell cultures at day 4 of growth were added with 5 ml of the concentrated medium filtrate of *Paph* under sterile conditions for the induction of AQ production. After an additional 4 days of growth, cell cultures were harvested by filtration under suction.

3.4. Estimation of AQ content

Fresh cells (15–20 g) were extracted at least three times with 25 ml of 60°C 80% ethanol using an Ultra Turrax mixer at the highest speed for 1–2 min until the biomass was colorless. The extracts were centrifuged at 5000 rpm for 15 min and the combined supernatants were made up to 100.0 ml in volumetric flasks. 1 ml of each extract was centrifuged at 13,000 rpm for 5 min and its absorption spectrum between 600 and 200 nm was recorded by a Cary 1 Bio UV-vis spectrophotometer (Varian, Inc., Palo Alto, USA). The content of AQs was calculated from the absorbance values of the extracts at 410 nm, using a molar extinction coefficient of 55,000, according to Schulte et al. (1984).

3.5. Isolation of robustaquinone B

After being harvested by filtration under suction, 498 and 540 g of fresh biomass from the [$1\text{-}^{13}\text{C}$]glucose and unlabeled glucose grown cultures were obtained, respectively. After freeze-drying, dry biomass of 25.5 and 40.6 g were suspended in appropriate amounts of dichloromethane and homogenized with an Ultra Turrax mixer at maximum speed for 2 min, respectively. After centrifugation at $6000\times g$ for 15 min at room temperature, the supernatants were pooled and the residues were extracted additional two to three times until colorless. The combined supernatants were con-

centrated to a volume of 5 ml using a rotary evaporator at room temperature under reduced pressure. The concentrated extract (about 5 ml) was subjected to silica gel column chromatography (CC). The CC system consisted of a Lobar C glass column (40–63 μM , $3.7\times 44\text{ cm}$) (Merck, Darmstadt, Germany), a Pharmacia LKB 2150 pump and an automatic fraction collector (Bromma, Sweden), and a Rheodyne Model 7125 injector (Hamilton Company, Nevada) with a loop. The column was first pre-equilibrated with 500 ml of toluene, and subsequently eluted at a flow rate of 5.0 ml/min with a step-gradient of toluene–methanol mixture at 1% increment of methanol in toluene (from 0 to 5%, v/v) (each 500 ml). Fractions (10 ml) were collected in glass tubes. The yellow fractions containing **1** were eluted at around 1% methanol in toluene, and checked by silica gel 60 F₂₅₄ coated aluminum TLC sheets (Merck, Darmstadt, Germany). The similar fractions containing **1** were combined and concentrated to give crude **1** extract with a volume of 2 ml. The crude **1** extract was further purified by preparative silica gel 60 F₂₅₄ TLC plates (20 \times 20 cm, 0.5 mm layer) with a concentrating zone (20 \times 4 cm) (Merck, Darmstadt, Germany) to yield the pure **1**, using dichloromethane as eluent: 1.1 and 3 mg pure **1** (robustaquinone B) from [$1\text{-}^{13}\text{C}$]glucose and unlabeled glucose grown cultures were obtained, respectively.

3.6. NMR spectroscopy

^1H and ^{13}C NMR spectra of **1** were recorded in C_6D_6 and CDCl_3 using a Bruker DMX 600 spectrometer (equipped with an Indy Silicon graphics computer). ^1H -Decoupled ^{13}C NMR spectra of the sample from the incorporation experiment and of the control sample from unlabeled glucose grown cultures were recorded under identical conditions. ^{13}C NMR spectra were measured as follows: 45° pulse (6 μs); repetition time, 3 s; spectral width, 35 kHz; data set, 64 kilo-words; room temperature; zero-filling to 128 kilo-words prior to Fourier transformation, 1 Hz line broadening, ^1H decoupling by WALTZ 16 during acquisition and relaxation. ^{13}C Abundance in **1** was analyzed by quantitative NMR spectroscopy. Two-dimensional experiments and data processing routines were performed according to standard Bruker software (XWINNMR 2.6). All ^{13}C NMR signals of robustaquinone B were assigned unequivocally on the basis of two-dimensional $^1\text{H}^{13}\text{C}$ correlation experiments (HMQC, HMBC) in conjunction with ^1H homocorrelation experiments (COSY, NOESY).

The relative intensity of individual carbon atoms was calculated by comparison of the ^{13}C signal integrals of the individual carbons and the carbon with the lowest ^{13}C enrichment. The enrichment ratio was calculated by dividing the relative intensities of each carbon atom from the labeled sample by the relative intensities of each carbon atom from the control sample.

3.7. Mass spectrometry

The electronic impact mass spectra of **1** obtained from the incorporation experiments with [1-¹³C]glucose and unlabeled glucose were obtained from a Finnigan MAT 900 spectrometer. Mass characteristics were as follows: 70 eV electron impact ionization energy, 150 °C source temperature and 1 s scanning from 50 to 500 Da.

Acknowledgements

We thank Mr. Barry Karabatak for his measurement of mass spectra at Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories and Mr. Frank ten Hoonte for his discussion on the assignment of NMR signals.

References

- Amrhein, N., Deus, B., Gehrke, P., Steinrücken, H.C., 1980. The site of the inhibition of the shikimate pathway by glyphosate. *Plant Physiol.* 66, 830–834.
- Bach, T.J., Boronat, A., Campos, N., Ferrer, A., Vollack, K.U., 1999. Mevalonate biosynthesis in plants. *Crit. Rev. Biochem. Mol. Biol.* 34, 107–122.
- Bauch, H.J., Leistner, E., 1978. Aromatic metabolites in cell suspension cultures of *Galium mollugo*. *Planta Med.* 33, 105–123.
- Bochar, D.A., Friesen, J.A., Stauffacher, C.V., Rodwell, V.W., 1999. Biosynthesis of mevalonic acid from acetyl-CoA. In: Barton, D., Nakanishi, K. (Eds.), *Comprehensive Natural Products Chemistry*, Vol. 2. Pergamon, Oxford, pp. 15–44.
- Burnett, A. R., Thomson, R. H., 1968. Naturally occurring quinones. Part XV. Biogenesis of the anthraquinone in *Rubia tinctorum* L. (Madder). *J. Chem. Soc. C*, 2437–2441.
- Eichinger, D., Bacher, A., Zenk, M.H., Eisenreich, W., 1999. Quantitative assessment of metabolic flux by ¹³C NMR analysis. Biosynthesis of anthraquinones in *Rubia tinctorum*. *J. Am. Chem. Soc.* 121, 7469–7475.
- Eisenreich, W., Bacher, A., 2000. Elucidation of biosynthetic pathways by retrodictive/predictive comparison of isotopomer patterns determined by NMR spectroscopy. In: Jane, K., Setlow (Eds.), *Genetic Engineering, Principles and Methods*, Vol. 22. Kluwer Academic, New York, pp. 121–153.
- Eisenreich, W., Menhard, B., Hylands, P.J., Zenk, M.H., Bacher, A., 1996. Studies on the biosynthesis of taxol: the taxane carbon skeleton is not of mevalonoid origin. *Proc. Natl. Acad. Sci., USA* 93, 6431–6436.
- Eisenreich, W., Rohdich, F., Bacher, A., 2001. Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Sci.* 6, 78–84.
- Eisenreich, W., Sagner, S., Zenk, M.H., Bacher, A., 1997. Monoterpenoid essential oils are not of mevalonoid origin. *Tetrahedron Lett.* 38, 3889–3892.
- Goese, M., Kammhuber, K., Bacher, A., Zenk, M.H., Eisenreich, W., 1999. Biosynthesis of bitter acids in hops. A ¹³C-NMR and ²H-NMR study on the building blocks of humulone. *Eur. J. Biochem.* 263, 447–454.
- Inoue, H., Leistner, E., 1988. Biosynthesis of quinones. In: Patai, S., Rappoport, Z. (Eds.), *The Chemistry of Quinonoid Compounds*, Vol. 2. John Wiley & Sons, New York, pp. 1293–1349.
- Inoue, K., Shiobara, Y., Nayeshiro, H., Inouye, H., Wilson, G., Zenk, M.H., 1984. Biosynthesis of anthraquinones and related compounds in *Galium mollugo* cell suspension cultures. *Phytochemistry* 23, 307–311.
- Koyama, J., Ogura, T., Tagahara, K., 1993. Anthraquinones of *Galium spurium*. *Phytochemistry* 33, 1540–1542.
- Leistner, E., 1973a. Biosynthesis of morindone and alizarin in intact plants and cell suspension cultures of *Morinda citrifolia*. *Phytochemistry* 12, 1669–1674.
- Leistner, E., 1973b. Mode of incorporation of precursors into alizarin (1, 2-dihydroxy-9,10-anthraquinone). *Phytochemistry* 12, 337–345.
- Leistner, E., 1975. Isolation, identification and biosynthesis of anthraquinones in cell suspension cultures of *Morinda citrifolia*. *Planta Med. (Suppl.)* 214–224.
- Leistner, E., 1981. Biosynthesis of plant quinones. In: Conn, E.E. (Ed.), *The Biochemistry of Plants*, Vol 7. Academic, London, pp. 403–423.
- Leistner, E., 1985. Biosynthesis of chorismate-derived quinones in plant cell cultures. In: Neumann, K.H., Barz, W., Reinhard, E. (Eds.), *Primary and Secondary Metabolism of Plant Cell Cultures*. Springer, Berlin, pp. 215–224.
- Leistner, E., Zenk, M. H., 1967. Incorporation of shikimic acid into 1,2-dihydroxy-anthraquinone (alizarin) by *Rubia tinctorum* L. *Tetrahedron Lett.* 475–476.
- Leistner, E., Zenk, M. H., 1968. Mevalonic acid a precursor of the substituted benzenoid ring of Rubiaceae-anthraquinones. *Tetrahedron Lett.* 1395–1396.
- Lichtenthaler, H.K., 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoids biosynthesis in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 50, 47–65.
- Lichtenthaler, H.K., Schwender, J., Disch, A., Rohmer, M., 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. *FEBS Lett.* 400, 271–274.
- Murashige, T., Skoog, F., 1992. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15, 473–497.
- Qureshi, N., Porter, J.W., 1981. Conversion of acetyl-coenzyme A to isoprenoid pyrophosphate. In: Porter, J.W., Spurgeon, S.L. (Eds.), *Biosynthesis of Isoprenoid Compounds*, Vol. 1. John Wiley & Sons, New York, pp. 47–93.
- Ramos-Valdivia, A., van der Heijden, R., Verpoorte, R., 1997. Elicitor-mediated induction of anthraquinone biosynthesis and regulation of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase activities in cell suspension cultures of *Cinchona robusta*. *Planta* 203, 155–161.
- Rohmer, M., 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat. Prod. Rep.* 16, 565–574.
- Sato, K., Kubota, H., Goda, Y., Yamada, T., Maitani, T., 1997. Glutathione enhanced anthraquinone production in adventitious root cultures of *Rubia tinctorum*. *Plant Biotechnol. (Tokyo)* 14, 63–66.
- Schripsema, J., Ramos-Valdivia, A., Verpoorte, R., 1999. Robust-quinones, novel anthraquinones from an elicited *Cinchona robusta* suspension culture. *Phytochemistry* 51, 55–60.
- Schulte, U., El Shagi, H., Zenk, M.H., 1984. Optimization of 19 Rubiaceae species in cell suspension cultures of *Cinchona ledgeriana*. *Plant Cell Rep.* 3, 51–54.
- Schwender, J., Gemünden, C., Lichtenthaler, H.K., 2001. Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-C-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids. *Planta* 212, 416–423.
- Snøeijer, W., 2000. *Flora of Pharmacognosy*, pp. 27.
- Stalman, M., Swolfs, A. E. M., Klunder, A. J. H., Croes, A. F., van der Plas, L. H. W., Hagendoorn, M. J. M., Wullems, G. J., 2001. Pyruvate stimulates anthraquinone biosynthesis in cell cultures of

- Morinda citrifolia*. PhD thesis, University of Nijmegen, The Netherlands, pp. 53–72.
- Strack, D., 1997. Phenolic metabolism. In: Dey, P.M., Harborne, J.B. (Eds.), *Plant Biochemistry*. Academic Press, San Diego, pp. 387–416.
- Suzuki, H., Matsumoto, T., Mikami, Y., 1984. Effects of nutritional factors on the formation of anthraquinone by *Rubia cordifolia* plant cells in suspension culture. *Agric. Biol. Chem.* 48, 603–610.
- Van den Berg, A.J.J., Labadie, R.P., 1989. Quinones. In: Harborne, J.B. (Ed.), *Methods in Plant Biochemistry*, Vol 1. Academic Press, London, pp. 451–491.
- Wijnsma, R., Go, J.T.K.A., Harkes, P.A.A., Verpoorte, R., Baerheim-Svensen, A., 1986. Anthraquinones in callus cultures of *Cinchona pubescens*. *Phytochemistry* 25, 1123–1126.
- Wijnsma, R., Go, J.T.K.A., van Weerden, I.N., Harkes, P.A.A., Verpoorte, R., Baerheim-Svensen, A., 1985. Anthraquinones as phytoalexins in cell and tissue cultures of *Cinchona* sp. *Plant Cell Rep.* 4, 241–244.
- Wilczynski, J.J., Daves, G.D., Folkers, K., 1968. Structure determination of ubiquinone analogs by solvent shifts in nuclear magnetic resonance spectra. *J. Am. Chem. Soc.* 90, 5593–5598.
- Yamamoto, H., Tabata, M., Leistner, E., 1987. Cytological changes associated with induction of anthraquinone synthesis in photoautotrophic cell suspension cultures of *Morinda lucida*. *Plant Cell Rep.* 6, 187–190.
- Zeidler, J.G., Lichtenthaler, H.K., May, H.U., Lichtenthaler, F.W., 1997. Is isoprene emitted by plants synthesized via the novel isopentenyl pyrophosphate pathway. *Z. Naturforsch.* 52c, 15–23.
- Zenk, M. H., El-Shagi, H., Schulte, U., 1975. Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. *Planta Med.* (Suppl.), 79–101.