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Robustaquinones, novel anthraquinones from an elicited *Cinchona robusta* suspension culture

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Abstract

A suspension culture of *Cinchona robusta* which under normal culture conditions does not produce anthraquinones, produces a range of these anthraquinones after elicitation. Eight new anthraquinones were identified, robustaquinones A–H, in addition to two known anthraquinones, 1,3,8-trihydroxy-2-methoxyanthraquinone and copareolatin 6-methyl ether. Their oxygenation pattern, characterized by a 6,7-disubstitution in the A-ring, is rare and raises questions about their biogenetic origin. They might be derived from phenylpropanoids and not from *o*-succinylbenzoic acid.

Keywords: Cinchona robusta; Rubiaceae; anthraquinones; elicitation;biosynthesis.

1. Introduction

Anthraquinones are a class of natural products encompassing several hundreds of compounds, differing in the nature and positions of the substituents (Thomson, 1971, 1987, 1996). They are found in a large number of plant families. They are particularly widespread in the subclass Asteridae, comprising among others the plant families Rubiaceae, Gesneriaceae and Scrophulariaceae. In these plants they are considered to be biosynthetically derived from shikimic acid, α-ketoglutarate and mevalonate. Most of them are only substituted in the C-ring. Anthraquinones can also be formed from acetate and malonate along the polyketide pathway, e.g. those which occur in the plant families Rhamnaceae, Polygonaceae, Leguminosae, in fungi and in lichen. Typical representatives of this pathway are emodin and chrysophanol.

Anthraquinones are not only common constituents of plants of the Rubiaceae, but also of their tissue and cell cultures. From *Cinchona ledgeriana* callus and suspension cultures 25 anthraquinones have been reported (Mulder-Krieger, Verpoorte, de Water, Van Gessel, Van Oeveren, & Baerheim Svendsen, 1982; Wijnsma, Verpoorte, Mulder-Krieger, & Baerheim Svendsen, 1984; Robins, Payne, & Rhodes, 1986). and from callus cultures of *Cinchona pubescens* 16 anthraquinones were reported (Wijnsma, Go, Harkes, Verpoorte, & Baerheim Svendsen, 1986).

In the present study suspension cultures of *Cinchona robusta* were investigated.

2. Results and discussion

The suspension cultures of *Cinchona robusta*, used in the present experiments are normally light yellow and do not contain detectable amounts of anthraquinones. Several hours after addition of a fungal elicitor the cultures show a strong orange colouration, due to anthraquinone formation. In the present study, elicitor-treated cultures were harvested after 72 h and the anthraquinones extracted, separated and identified. The elucidation of the substitution pattern of the anthraquinones was greatly facilitated by the paper of Schripsema & Dagnino (1996), in which the characteristic chemical shifts for a wide variety of substitution patterns are given. In this paper, it was also demonstrated that the chemical shifts of *peri*-hydroxyl protons are useful indicators for the substitution pattern of the other benzenoid ring. Additive

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substituent effects were derived from the collected literature data (Schripsema & Dagnino, 1996).

The main component of the anthraquinone mixture was robustaquinone A (**1**). The mass spectrum indicated a M_r of 330, corresponding to $C_{17}H_{14}O_7$. The VIS spectrum with a maximum at 465 nm was typical for a 1,4-dihydroxyanthraquinone. In the ^{1}H NMR spectrum signals of *peri*-hydroxyl protons were observed at δ 13.75 and 12.93. Together with the methyl singlet at δ 2.36 and the singlet at δ 7.11 these signals established the 1,4-dihydroxy-2-methyl substitution of one ring (Schripsema & Dagnino, 1996). The remaining signals in the ^{1}H NMR spectrum: two signals of methoxyls, at δ 4.12 and 3.99, and a singlet at δ 7.75 established the 2-hydroxy-1,3-dimethoxy substitution of the other ring (Schripsema & Dagnino, 1996). The chemical shifts of the *peri*-hydroxyl protons indicated the presence of a 8-methoxy substituent (Schripsema & Dagnino, 1996) and thus robustaquinone A was identified as 1,4,7-trihydroxy-6,8-dimethoxy-2-methylanthraquinone (**1**). The identification was further confirmed by the HMBC spectrum, which also permitted the complete assignment of the ^{13}C NMR spectrum.

Robustaquinone B (**2**): The mass spectrum of this component indicated an M_r of 298, corresponding to $C_{17}H_{14}O_5$. The VIS spectrum (λ_{max} 412 nm) showed the compound to have only one *peri*-hydroxyl group, which in the ^{1}H NMR spectrum gave a singlet at δ 13.00. Adjacent to this hydroxyl, a methyl substituent was present at position 2 as indicated by the methyl signal at δ 2.38 and the two doublets at δ 7.50 and 7.73 of H-3 and H-4 (Schripsema & Dagnino, 1996). The other ring contained two methoxyl groups in the 6 and 7 positions, as indicated by the 6-proton singlet at δ 4.07 and the two proton singlet at δ 7.71 (Schripsema & Dagnino, 1996). It was thus concluded that robustaquinone B has the structure 1-hydroxy-6,7-dimethoxy-2-methylanthraquinone (**2**).

Robustaquinone C (**3**): The mass spectrum indicated an M_r of 300, corresponding to $C_{16}H_{12}O_6$. The VIS spectrum (λ_{max} 408 nm) suggested the presence of a single *peri*-hydroxyl. In the ^{1}H NMR spectrum two methoxyl groups at δ 4.12 and 4.00 and a singlet at δ 7.72 indicated a similar ring substitution as in robustaquinone A (**1**). The other ring only had one substituent, a *peri*-hydroxyl, as shown by the proton signals at δ 7.29 (*d* of 8 Hz), 7.61 (*t*) and δ 7.77 (*d*) and the hydroxyl signal at δ 13.00. The position of this hydroxyl signal indicated that there should be a 8-methoxy substituent (Schripsema & Dagnino, 1996). Robustaquinone C was thus identified as 2,8-dihydroxy-1,3-dimethoxyanthraquinone (**3**).

Robustaquinone D (**4**) has an M_r of 284, corresponding to $C_{16}H_{12}O_5$. The VIS spectrum (λ_{max} 412 nm) indicated the presence of one *peri*-hydroxyl. The ^{1}H NMR spectrum was quite similar to that of robustaquinone B (**2**), except that instead of a 6 proton singlet at δ 4.07 there was now a three proton singlet at δ 4.10. The mass indicated that one of the methoxyl groups has been replaced by a hydroxyl. The unchanged chemical shift of the *peri*-hydroxyl proton showed that the hydroxyl and methoxyl should be present at positions 6 and 7 (Schripsema & Dagnino, 1996). On the basis of the spectral data one cannot distinguish between the structures 1,6-dihydroxy-7-methoxy-2-methylanthraquinone or 1,7-dihydroxy-6-methoxy-2-methylanthraquinone. However, based on the proposed biosynthetic relationship between these anthraquinones, which will be discussed below, it was concluded that robustaquinone D most probably is 1,7-dihydroxy-6-methoxy-2-methylanthraquinone (**4**). The spectral data of robustaquinone D are very similar to those of a compound which was isolated from a *Cinchona pubescens* callus culture and for which the structure 6,7-dihydroxy-1-methoxy-2-methylanthraquinone was reported (Wijnsma, Go, Harkes, Verpoorte, & Baerheim Svendsen, 1986). This structure would be impossible considering the reported spectral data. The reported VIS spectrum with a maximum at 406 nm indicated the presence of a *peri*-hydroxyl and the reported ^{1}H NMR chemical shifts are incompatible with the structure proposed (Wijnsma, Go, Harkes, Verpoorte, & Baerheim Svendsen, 1986). The compound isolated from the *C. pubescens* callus culture most probably was identical to robustaquinone D (**4**).

Robustaquinone E (**5**) displayed a maximum at 465 nm in the VIS spectrum showing this anthraquinone to be 1,4-dihydroxy substituted. The mass spectrum of this component indicated an M_r of 316, corresponding to $C_{16}H_{12}O_7$. The substitution of ring A was identical to that of robustaquinones A (**1**) and C (**3**). Two signals of *peri*-hydroxyls, at δ 12.87 and 13.30, and a two proton signal at δ 7.25 indicated the 1,4-dihydroxy substitution of the other ring (Schripsema & Dagnino, 1996). Robustaquinone E was thus identified as 2,5,8-trihydroxy-1,3-dimethoxyanthraquinone (**5**).

Robustaquinone F (**6**): The mass spectrum of this component indicated a M_r of 302, corresponding to $C_{15}H_{10}O_7$. The VIS spectrum with a maximum at 487 nm, indicated a 1,4,5-trihydroxy substitution, consistent with the three signals of *peri*-hydroxyl protons at δ 13.00, 12.67 and 12.32 in the ^{1}H NMR spectrum. By comparison with the spectra of robustaquinone E (**5**) and 1,3,8-trihydroxy-2-methoxyanthraquinone (Table 1) the structure 1,3,5,8-tetrahydroxy-2-methoxyanthraquinone was derived for robustaquinone F (**6**), which was confirmed by the calculation of the chemical shifts for the peri-hydroxyl protons as described in Schripsema & Dagnino (1996): δ 13.06 (position 5), 12.63 (position 1) and 12.29 (position 8).

Robustaquinone G (**7**): The VIS spectrum (λ_{max} 465 nm) showed that this anthraquinone was 1,4-dihydroxy substituted. The mass spectrum indicated a M_r of 358, corresponding to $C_{18}H_{14}O_8$. The NMR spectrum resembled the one of robustaquinone A (**1**) showing that this compound was also a 2-methylquinizarin derivative.

Table 1
^{1}H NMR chemical shifts of the anthraquinones from *C. robusta* ($CDCl_3$)

H*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	13.75 s	13.00 s	13.00 s	13.00 s	13.30 s	12.32 s	13.78 s	12.73 s	13.00 s	12.10 s
2	2.36 s	2.38 s	7.29 dd (8.4; 0.9)	2.37 s	7.25 d (8)	7.27 s	2.36 s	7.23 d (8)	2.38 s	7.28 dd (8.4; 0.9)
3	7.11 s	7.50 d (7.8)	7.61 dd (8.4; 7.4)	7.49 d (7.5)	7.28 d (8)	7.27 s	7.11 s	7.60 dd (8; 8)	7.52 d (8)	7.65 dd (8.4; 7.4)
4	12.93 s	7.73 d (7.8)	7.77 dd (7.4; 0.9)	7.72 d (7.5)	12.87 s	13.00 s	12.98 s	7.70 d (8)	7.73 d (8)	7.81 dd (7.4; 0.9)
5	7.75 s	7.71 s	7.72 s	7.73 s	7.77 s	7.52 s		4.06 s	13.28 s	7.48 s
6	3.99 s	4.07 s	4.00 s	4.10 s	4.00 s		6.30 s	3.97 s	4.17 s	
7		4.07 s				4.17 s	3.95 s	3.97 s		4.14 s
8	4.12 s	7.71 s	4.12 s	7.77 s	4.12 s	12.67 s	4.19 s	4.05 s	7.46 s	12.50 s

*The substituent numbering used in this table follows the presumed biosynthetic origin of the compounds, as indicated in Fig. 1. In some cases this numbering does not correspond to the chemical numbering.

A two proton signal at δ 6.30 indicated the presence of a methylenedioxy group. The large difference between the shifts of the methoxy groups indicated that one point of attachment of the methylenedioxy group was in a *peri* position. The chemical shifts of the *peri*-hydroxyl protons indicated that a methoxy group was located at position 8 (Schripsema & Dagnino, 1996), thus establishing the structure as: 1,4-dihydroxy-7,8-dimethoxy-2-methyl-5,6-methylenedioxyanthraquinone (**7**). It should be mentioned that in Schripsema & Dagnino (1996) no substituent effect was given for the methylenedioxy group, due to a lack of data. The calculations on robustaquinone G (**7**) did show that the substituent effect of the 5,6-methylenedioxy group should be nearly identical to that of a 6-hydroxy or methoxy group. Thus, the 5-substitution should have a negligible effect on the chemical shifts of both the *peri*-hydroxyls. The calculated chemical shifts in Table 2 were obtained in this way.

Robustaquinone H (**8**): The VIS spectrum (λ_{max} 412 nm) showed that this anthraquinone only had one *peri*-hydroxyl group. The mass spectrum indicated an M_r of 344, corresponding to $C_{18}H_{16}O_7$. The ^{1}H NMR spectrum displayed the signals typical for a 1-hydroxy substituted ring (Schripsema & Dagnino, 1996) and additionally four signals of methoxyl groups. The other ring was thus fully substituted with methoxyl groups. The structure of this anthraquinone was thus established as 5-hydroxy-1,2,3,4-tetramethoxyanthraquinone (**8**). The calculated chemical shift for the *peri*-hydroxyl of robustaquinone H (Table 2) is higher than the observed value, because the sample of robustaquinone H contained a high concentration of water. Because only a minimal quantity of this compound was isolated and this was lost in the identification process, the spectrum could not be repeated.

Another anthraquinone had an M_r of 300, corresponding to $C_{16}H_{12}O_6$. The VIS spectrum (λ_{max} 430 nm) indicated 1,5 or 1,8 dihydroxy substitution. Comparison of the ^{1}H NMR data with those of copareolatin 6-methyl ether (**9**) (Wijnsma, & Verpoorte, 1986; Koyama, Ogura, & Tagahara, 1993), showed that these compounds were identical.

Table 2. Comparison of observed and calculated chemical shifts of the *peri*-hydroxyl protons of the anthraquinones, isolated from the *C. robusta* cell suspension culture. Calculations were performed as reported by Schripsema & Dagnino (1996)

Compound	Observed	Calculated
Robustaquinone A (**1**)	13.75	13.74
	12.93	12.98
Robustaquinone B (**2**)	13.00	12.91
Robustaquinone C (**3**)	13.00	13.08
Robustaquinone D (**4**)	13.00	12.91
Robustaquinone E (**5**)	13.30	13.28
	12.87	12.91
Robustaquinone F (**6**)	13.00	13.06
	12.67	12.63
	12.32	12.29
Robustaquinone G (**7**)	13.78	13.74
	12.98	12.98
Robustaquinone H (**8**)	12.73	13.06
Copareolatin 6-methyl ether (**9**)	13.28	13.27
	13.00	13.04
1,3,8-Trihydroxy-2-methoxy-anthraquinone (**10**)	12.50	12.50
	12.10	12.09

The last anthraquinone which was isolated had spectral data identical to those of a compound previously isolated from a *Cinchona ledgeriana* suspension culture by Wijnsma et al. (1984). The structure of this compound was revised by Schripsema & Dagnino (1996) as 1,3,8-trihydroxy-2-methoxyanthraquinone (**10**).

All the anthraquinones identified are substituted in both rings. This is remarkable, because in the family Rubiaceae the anthraquinones are considered to be derived from shikimate and mevalonate and originally it was considered to be a characteristic of these anthraquinones that they were substituted in only one ring (Thomson, 1971; Burnett & Thomson, 1968). This led us to question the presumed biosynthesis of anthraquinones

in *Cinchona*. Because the identified anthraquinones are closely related, a biosynthetic relationship between them can be proposed (Fig. 1). In this scheme, it is considered that only the following reactions occur: hydroxylation, *O*-methylation, loss of the C-methyl group and methylenedioxy-formation. The scheme with the smallest number of intermediates (**11**–**19**) is shown. Alternatives with more intermediates are possible, e.g. robustaquinone F (**6**) could be derived from copareolatin 6-methyl ether (**9**). Some of the intermediates in the scheme have been isolated from other Rubiaceae: **15** was reported from *Galium spurium* (Koyama et al., 1993) and **17** from callus cultures of *Cinchona pubescens* (Wijnsma et al., 1986) and *Cinchona ledgeriana* (Wijnsma et al., 1984). From this scheme the conclusion can be drawn that the original precursor of all the isolated anthraquinones might be 1,6,7-trihydroxy-2-methylanthraquinone (**11**). This structure might point to a phenylpropanoid origin of the A-ring. Maybe caffeic acid, reported as a component of *Cinchona* bark in 1882 (Körner, 1882) could be a precursor.

A comparison of the identified structures with those of the anthraquinones isolated from other *Cinchona* species and with those isolated from other Rubiaceae might indicate a different pathway for anthraquinone formation within the family Rubiaceae. A problem encountered in this comparison is the uncertain identification of numerous anthraquinones previously reported from *Cinchona* (Mulder-Krieger et al., 1982; Wijnsma et al., 1984; Robins et al., 1986; Wijnsma et al., 1986). For example, alizarin, alizarin 1-methyl ether and 1,8-dihydroxy anthraquinone were supposed to be present on the basis of a TLC comparison (Mulder-Krieger et al., 1982), but later investigations (Wijnsma et al., 1984; Robins et al., 1986) could not confirm their occurrence. For many other anthraquinones data were reported which were not consistent with the derived structures. This has led already to a number of structure revisions (Schripsema & Dagnino, 1996; Simoneau & Brassard, 1986; this paper), but in other cases the data are insufficient to deduce the correct structure. For example, the very informative chemical shifts of the *peri*-hydroxyl protons were not reported or could not be measured due to the solvent used. The anthraquinones, about which no doubt exists, show a striking similarity to the anthraquinones reported here. A number of the anthraquinones are only substituted in a single ring, such as the 2-, 1,2-, 1,3- and 1,2,3-methyl ethers of anthragallol and 2-hydroxy-1,3,4-trimethoxy anthraquinone. Considering the biosynthetic scheme in Fig. 1, it seems probable that this is a substitution of the A-ring and not of the C-ring, thus turning them into anthraquinones closely related to those reported in this study. Morindone 5-methyl ether and 1,4,6-trihydroxy-5-methoxy-2-methylanthraquinone could be derived from a different ring closure of caffeic acid or another phenylpropanoid could be involved in the biosynthesis.

In other genera of the Rubiaceae alizarin, its methyl ethers, 1-hydroxy-2-methylanthraquinone, lucidin, munjistin, rubiadin, purpurin and pseudopurpurin are very common. All these are only substituted in the C-ring. A relatively small number of anthraquinones substituted in both benzenoid rings has been reported, e.g. morindone and copareolatin 6-methyl ether. They are mainly encountered in the genera *Morinda* and *Galium*. In particular *Galium sinaicum* (Halim et al., 1992; El-Gamal et al., 1995, 1996) contained a large number of anthraquinones which were reported to show much similarity with the *Cinchona* anthraquinones: copareolatin 6-methyl ether and a number of anthraquinones trisubstituted in the A-ring. Particularly noteworthy is the occurrence of several anthraquinones which are monosubstituted in the C-ring with a methyl group. If they were formed in the same way as the *Cinchona* anthraquinones the absence of the hydroxyl at position 1 could be due to a dehydrative mechanism of aromatization instead of a reductive one.

One can thus conclude that the *Cinchona* anthraquinones have a special substitution pattern, which is also found to some extent within the genera *Galium*, *Morinda* and maybe *Coprosma*. Further, it has to be pointed out that only a limited number of experiments has been performed to determine the biosynthetic route to the anthraquinones in Rubiaceae, of which none have been performed on *Cinchona*. Only a more thorough investigation of anthraquinone biosynthesis within Rubiaceae will yield the definitive answers to the questions raised above.

3. Experimental

3.1. General procedures

NMR: Bruker WM-300 (^{1}H at 300 MHz, ^{13}C at 75 MHz) or Bruker MSL-400 (^{1}H at 400 MHz, ^{13}C at 100 MHz); HMBC: Bruker AM-600 (^{1}H at 600 MHz, ^{13}C at 150 MHz). All spectra were recorded in $CDCl_3$ and the chemical shifts are given in δ values (ppm) relative to TMS. MS: Thermospray LC-MS (Finnigan MAT TSQ 70 triple quadrupole mass spectrometer equipped with a Finnigan MAT TSP interface).

3.2. Cell cultures

Cell suspension cultures of *Cinchona robusta* How. were grown in Gamborg B5 medium (Gamborg et al., 1968) containing 2% sucrose, 2 mg/l 2,4-D, 0.2 mg/l kinetin and 50 mg/l cysteine. The cultures were subcultured weekly by adding 15 ml of the suspension to 50 ml of fresh medium. The cultures were grown in the light at 25° on gyratory shakers at 120 rpm. Elicitation was performed by adding an autoclaved *Phytophthora cinnamomi* preparation (80 mg in 4 ml of water) to cultures

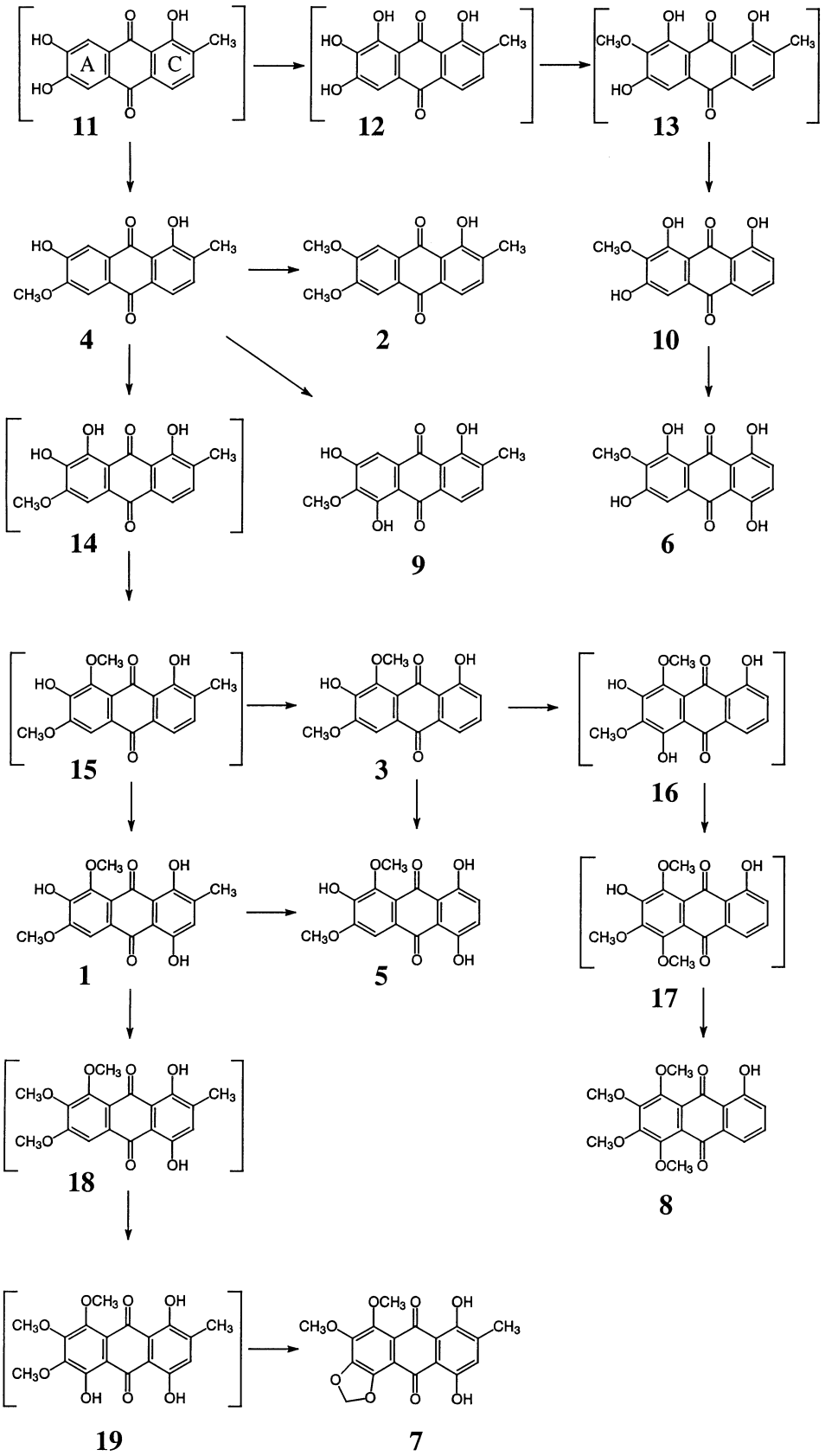

Fig. 1. Proposed biosynthetic relationship of the anthraquinones isolated from *C. robusta* cell suspension culture.

grown for 4 days in 500 ml flasks containing 100 ml of medium. After another 72 h the cultures were harvested and lyophilized. *P. cinnamomi* (ex *Cinchona robusta*) (CBS No. 403.48) was purchased from Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands). The elicitor preparation was prepared as described previously (Ramos-Valdivia, 1997).

3.3. Extraction and isolation

8.5 g of the lyophilized elicited *C. robusta* culture (originating from 180 g of fresh material, obtained from 600 ml of suspension culture) was extracted with CH_2Cl_2 and the extract evaporated under reduced pressure. The anthraquinones were obtained by prep. HPLC using MeCN–HoAc (3%) gradients in combination with a S5 Sperisorb ODS2 column (250 × 4 mm, PhaseSep, Clwyd, U.K.). In a separate experiment the anthraquinones were isolated from the extract of 4.5 g of lyophilized biomass through prep. TLC on silica gel plates (2 mm thick. Silica gel 60 F254) with $CHCl_3$–MeOH–aq. ammonia 25% (85:17:1) as eluent. The anthraquinones were eluted from the collected silica gel with 50% MeOH. From most anthraquinones less than a mg was obtained. From the major anthraquinone, robustaquinone A about 2 mg was obtained.

3.3.1. Robustaquinone A (**1**)

MS *m*/*z* (rel. int.): 331 (100), 316 (97), 298 (74), 271 (57), 243 (15), 242 (15); UV-VIS λ_{max} nm: 310sh, 465; ^{1}H NMR ($CDCl_3$): Table 1; ^{13}C NMR ($CDCl_3$) δ 186.5 (C-9), 185.3 (C-10), 157.3 (C-4), 157.1 (C-1), 154.6 (C-6), 145.6 (C-8), 141.3 (C-2), 135.7 (C-7), 127.7 (C-3), 125.9 (C-11), 120.2 (C-12), 112.4 (C-13), 110.7 (C-14), 110.1 (C-5), 61.7 (2 × OCH_3), 16.7 (Me).

3.3.2. Robustaquinone B (**2**)

MS *m*/*z* (rel. int.): 299 (100), 284 (26), 269 (16), 254 (26), 239 (9); UV-VIS λ_{max} nm: 305sh, 412; ^{1}H NMR ($CDCl_3$): Table 1.

3.3.3. Robustaquinone C (**3**)

MS *m*/*z* (rel. int.): 301 (100), 286 (56), 268 (83), 240 (18), 212 (22); UV-VIS λ_{max} nm: 408. ^{1}H NMR ($CDCl_3$): Table 1.

3.3.4. Robustaquinone D (**4**)

MS *m*/*z* (rel. int.): 285 (100), 270 (44), 257 (14), 239 (29), 225 (17), 211 (29); UV-VIS λ_{max} nm: 412; ^{1}H NMR ($CDCl_3$): Table 1.

3.3.5. Robustaquinone E (**5**)

MS *m*/*z* (rel. int.): 317 (100), 302 (93), 284 (96), 257 (20), 228 (10); UV-VIS λ_{max} nm: 465; ^{1}H NMR ($CDCl_3$): Table 1.

3.3.6. Robustaquinone F (**6**)

MS *m*/*z* (rel. int.): 303 (72), 302 (100), 289 (7), 271 (6), 257 (4), 242 (7); UV-VIS λ_{max} nm: 487; ^{1}H NMR ($CDCl_3$): Table 1..

3.3.7. Robustaquinone G (**7**)

MS *m*/*z* (rel. int.): 359 (100); UV-VIS λ_{max} 310sh, 465; ^{1}H NMR ($CDCl_3$): Table 1.

3.3.8. Robustaquinone H (**8**)

MS *m*/*z* (rel. int.): 345 (100); UV-VIS λ_{max} nm: 412; ^{1}H NMR ($CDCl_3$): Table 1.

3.3.9. Copareolatin 6-methyl ether (**9**)

MS *m*/*z* (rel. int.): 301 (100); UV-VIS λ_{max} nm: 310sh, 430; ^{1}H NMR ($CDCl_3$): Table 1.

3.3.10. 1,3,8-Trihydroxy-2-methoxyanthraquinone (**10**)

MS *m*/*z* (rel. int.): 287 (100); UV-VIS λ_{max} nm: 430; ^{1}H NMR ($CDCl_3$): Table 1.

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