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ANTHRAQUINONES IN CALLUS CULTURES OF *CINCHONA PUBESCENS*

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Key Word Index—*Cinchona pubescens*; Rubiaceae; callus cultures; anthraquinones; ^{1}H NMR.

Abstract—From callus cultures of *Cinchona pubescens* seven known anthraquinones, alizarin-2-methylether, anthragallol-1,2-dimethylether, purpurin, purpurin-1-methylether, 1-hydroxy-2-hydroxymethylanthraquinone, 2-hydroxy-1,3,4-trimethoxyanthraquinone and 2,5-(or 3,5-)dihydroxy-1,3,4-(or -1,2,4-)trimethoxyanthraquinone, and five new anthraquinones, 2-hydroxy-1,3,4,6-(or -1,3,4,7-)tetramethoxyanthraquinone, 1,6-(or 1,7-)dihydroxy-2-methylanthraquinone, 5-hydroxy-purpurin-1-methylether, 4,6-(or 4,7)-dihydroxy-2,7-(or -2,6-)dimethoxyanthraquinone and 6,7-dihydroxy-1-methoxy-2-methylanthraquinone have been isolated.

INTRODUCTION

Many genera within the Rubiaceae contain considerable amounts of anthraquinones, both free and as glycosides [1, 2]. Also in tissue culture several Rubiaceae species produce large amounts of anthraquinones, ranking among the highest producing cell lines known today [3]. Many species produce even more anthraquinones on a dry weight basis in tissue culture than does the parent plant [3]. Despite the enormous amount of literature that has been published on the chemical constituents of *Cinchona* sp. until now there seems to have appeared only one report indicating that the genus *Cinchona* might also contain anthraquinones [4].

During the course of our investigations in the biosynthesis of the *Cinchona* alkaloids and the production thereof in tissue culture systems of *Cinchona* sp. we observed that in callus and cell suspension cultures of both *Cinchona ledgeriana* and *C. pubescens* quite large amounts of coloured material were present. A more close examination showed that these compounds were anthraquinones. In previous publications we have reported on the identification of anthraquinones from callus cultures of *C. ledgeriana* [5, 6] and on a preliminary study on the anthraquinones from *C. pubescens* callus cultures [7].

In the present study we report on a more detailed examination of an anthraquinone extract obtained from callus cultures of *C. pubescens* Vahl. Twelve anthraquinones were isolated by means of preparative TLC in sufficient amounts to allow their identification by means of spectroscopic methods. Seven of them are known compounds, five have not been reported before.

RESULTS

Twelve compounds were isolated in sufficient amounts to allow their identification by means of spectroscopic methods. Seven of them were known compounds, which were identified by comparison of their spectral data with those reported in the literature and partly by co-TLC with reference compounds obtained from *C. ledgeriana* [6]. These were: purpurin [6, 8], purpurin-1-methylether [6], anthragallol-1,2-dimethylether [6], 1-hydroxy-2-hydroxymethylanthraquinone [6, 8, 9], 2-hydroxy-1,3,4-trimethoxyanthraquinone [6], 2,5-(or 3,5-)dihydroxy-1,3,4-(or -1,2,4-)trimethoxyanthraquinone [6] and alizarin-2-methylether [10].

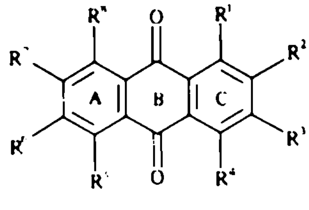
Structures of the new anthraquinones

2-Hydroxy-1,3,4,6-(or -1,3,4,7-)tetramethoxyanthraquinone (**8**). This anthraquinone has an $[M]^+$ at m/z 344. Its ^{1}H NMR spectrum shows four three-proton singlets at δ3.91, 3.94, 4.04 and 4.06 due to four methoxyl groups, two α-positioned to the carbonyl groups (δ4.04, 4.06) [2] and two β-positioned to the carbonyl groups (δ3.91, 3.94) [2]. Also the ^{1}H NMR spectrum shows signals due to three aromatic protons at *ca* δ7.5. From these signals, a double doublet ($J = 8.0$ Hz, $J = 1.5$ Hz) at δ7.26, a doublet showing *meta*-coupling ($J = 1.5$ Hz) and a doublet with *ortho*-coupling ($J = 8.0$ Hz) at δ7.66 and 7.72 respectively, the substitution pattern of ring A can be deduced, i.e. one substituent at C-6. The UV spectrum (MeOH–OH$^-$) shows a maximum at 476 nm indicating that the hydroxyl group is in the 2-position. From these spectral data it can be concluded that this compound is either 2-hydroxy-1,3,4,6-tetramethoxyanthraquinone or 6-hydroxy-1,2,3,4-tetramethoxyanthraquinone. If this compound has the latter structure then the chemical shifts of both the 1- and 4-methoxyl groups and of both the 2- and 3-methoxyl groups would be essentially the same because of the symmetry of ring C. Since the chemical shifts differ by 0.02 and 0.03 ppm respectively it is most probable that this compound is either 2-hydroxy-1,3,4,6-tetramethoxyanthraquinone or 2-hydroxy-1,3,4,7-tetramethoxyanthraquinone, between which structures it is almost impossible to distinguish [2].

1,6-(or 1,7-)Dihydroxy-2-methylanthraquinone (**9**). The $[M]^+$ of this compound is found at m/z 254. Its ^{1}H NMR spectrum shows a three-proton singlet at δ2.38 due to a methyl group. In the aromatic region two doublets with an *ortho*-coupling ($J = 7.5$ Hz) are found and again the pattern which is indicative of an A-ring substituted in the 6- or 7-position (cf. compound **8**). The methyl group is always in the 2-position on the basis of the biosynthetic pathway leading to the anthraquinones in the Rubiaceae [11, 12]. From the M_r of this compound it is concluded that two hydroxyl groups are present in the molecule. On the basis of biogenetic reasoning and ^{1}H NMR spectral data it is concluded that the hydroxyl groups must be in the 1- and the 6- or 7-positions. This is in good agreement with the maximum in the UV-VIS spectrum recorded in MeOH-OH$^-$ at 510 nm [2]. With the spectral data available it is not possible to distinguish between the two possible structures, viz. 1,6-dihydroxy-2-methylanthraquinone (soranjidiol) or 1,7-dihydroxy-2-methylanthraquinone. Since no reference sample of soranjidiol was available both possibilities are left open for this compound.

1-*Methoxy-2,4,5-trihydroxyanthraquinone* (**10**). This compound has an $[M]^+$ at m/z 286. Its ^{1}H NMR spectrum shows a three-proton singlet at δ4.04 due to an α-positioned methoxyl group, a one-proton singlet at δ7.30 and the pattern characteristic of an A-ring substituted in the α-position. The MS shows a peak of rather high relative intensity due to the loss of water from the molecular ion, indicating that the methoxyl group is in the α-position [13]. From the M_r it is concluded that three hydroxyl groups must be present in the molecule. The shift of the proton in the substituted C-ring is more likely to be due to a proton at C-3 than at C-4 and shows reasonably good agreement with the shift of the proton at C-3 in purpurin-1-methylether (compound **2**). In the IR spectrum two absorption bands are present between 1600 and 1700 cm^{-1} indicating that only one carbonyl group is chelated [1]. On the basis of these spectral data it is concluded that this compound is 1-methoxy-2,4,5-trihydroxyanthraquinone or 5-hydroxypurpurin-1-methylether.

1,6-(*or* 1,7-)*Dihydroxy*-3,7-(*or* -3,6-)*dimethoxyanthraquinone* (**11**). The ^{1}H NMR spectrum of this compound having an $[M]^+$ at m/z 300 shows signals due to two methoxyl groups at δ3.94 and 3.99. In the aromatic region signals due to four aromatic protons are observed. Two signals show *meta*-coupling ($J = 1.5$ Hz) indicating *meta* substitution, the other two protons give rise to two singlets at δ7.56 and 7.64. Since the two methoxyl groups only account for half of the substituents and the $[M]^+$ is found at m/z 300 two hydroxyl groups must be present in the molecule. From the maximum (486 nm) in the UV spectrum recorded in MeOH-OH$^-$ it can be concluded that the hydroxyl groups are present in the 1- and 3-positions or in the 1- and 6- or 7-positions



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
Purpurin **1**	OH	OH	H	OH	H	H	H	H
Purpurin-1-methylether **2**	OMe	OH	H	OH	H	H	H	H
Anthragallol-1,2-dimethylether **3**	OMe	OMe	OH	H	H	H	H	H
1-Hydroxy-2-hydroxymethyl A* **4**	OH	CH_2OH	H	H	H	H	H	H
2-Hydroxy-1,3,4-trimethoxy A **5**	OMe	OH	OMe	OMe	H	H	H	H
2,5-(or 3,5-) Dihydroxy-1,3,4 (or-1,2,4-) trimethoxy A **6**	OMe	OH or OMe	OMe or OH	OMe	OH	H	H	H
Alizarin-2-methylether **7**	OH	OMe	H	H	H	H	H	H
2-Hydroxy-1,3,4,6-(or-1,3,4,7-) tetramethoxy A **8**	OMe	OH	OMe	OMe	H	OMe or H	H or OMe	H
1,6-(or 1,7-) Dihydroxy-2-methyl A **9**	OH	Me	H	H	H	OH or H	H or OH	H
1-Methoxy-2,4,5-trihydroxy A **10**	OMe	OH	H	OH	OH	H	H	H
1,6-(or 1,7-) Dihydroxy-3,7-(or-3,6-)dimethoxy A **11**	OH	H	OMe	H	H	OH or OMe	OMe or OH	H
6,7-Dihydroxy-1-methoxy-2-methyl A **12**	OMe	Me	H	H	H	OH	OH	H

*A = Anthraquinone

Table 1. R_f values and colours of the anthraquinones isolated from *C. pubescens* callus cultures

Com-	R_f value		Colour		
pound	S1*	S2	Daylight	UV 366 nm	NH_3 vapour
1	0.00	0.00	red	red-purple	red-purple
2	0.82	0.64	orange	red-brown	orange
3	0.31	0.28	dark-yellow	brown	orange
4	0.58	0.33	yellow	orange	red-orange
5	0.23	0.28	yellow	red-brown	orange
6	0.19	0.24	orange	orange	red
7	0.75	0.57	yellow	orange	orange
8	0.31	0.32	yellow-orange	brown	orange
9	0.45	0.38	yellow	brown	red-orange
10	0.75	0.65	yellow-brown	orange	red
11	0.82	0.71	orange	orange-brown	red
12	0.38	0.24	dark-yellow	brown	red

*S1, Silica gel 60 (precoated TLC plate), $CHCl_3$–MeOH–NH_3 25% (85:14:1) saturated, developing distance 15 cm. S2, Silica gel 60 (precoated TLC plate), toluene–MeOH (9:1) saturated, developing distance 15 cm.

[2, 14]. If the two hydroxyl groups are in the 1- and 3-positions then the two methoxyl groups would be in positions 6 and 7 which would cause a smaller difference in chemical shift between the two singlets due to the methoxyl groups than the 0.05 ppm which is observed. In anthragallol-2,3-dimethylether, for example, the chemical shift for both methoxyl groups is 4.01 ppm [15] and in anthragallol-1,2,3-trimethylether the difference in chemical shift between the 2- and 3-methoxyl groups is only 0.01 ppm [16]. Another fact against the *ortho* positioning of the two methyl groups is the presence of a relatively large $[M - H_2O]^+$ peak in the MS at *m/z* 282 (20%). This peak is a result of the loss of water from the molecular ion caused by the *ortho* positioning of a methoxyl and a hydroxyl group [17]. On the basis of this spectral data it is concluded that this anthraquinone is either 1,6-dihydroxy-3,7-dimethoxyanthraquinone or 1,7-dihydroxy-3,6-dimethoxyanthraquinone. The spectral data did not allow us to discriminate between these two possible structures.

6,7-Dihydroxy-1-methoxy-2-methylanthraquinone (**12**). This compound has an $[M]^+$ at *m/z* 284. Its ^1H NMR spectrum shows a three-proton singlet at δ2.37 due to a methyl group, a three-proton singlet at δ4.09 due to a methoxyl group and in the aromatic region signals due to four protons. Two of these signals appear as singlets while the other two appear as doublets showing an *ortho*-coupling (J = 7.5 Hz). The signal due to the methoxyl group is found at δ4.09 indicating that this methoxyl group is positioned α to a carbonyl group [2]. The methyl group is on biogenetic grounds in the 2-position. In the MS a small peak due to the loss of water from the molecular ion is observed at *m/z* 266 (1%) indicating that the methoxyl group which is in the α-position is not positioned *ortho* to a hydroxyl group because then the $[M - H_2O]^+$ peak would be much larger (cf. compound **11**). On the basis of these spectral data it is concluded that this compound is 6,7-dihydroxy-1-methoxy-2-methylanthraquinone.

DISCUSSION

Of the seven known anthraquinones isolated in this study six have also been isolated from calli of *C. ledgeriana* [6], with the exception of alizarin-2-methylether.

In a previous study on the alkaloids and anthraquinones from callus material of *C. pubescens* [7] alizarin, alizarin-1-methylether, 1,8-dihydroxyanthraquinone, rubiadin and 1-hydroxy-2-hydroxymethylanthraquinone were identified by means of TLC with reference compounds. In the present study, however, the presence of the first four of these compounds could not be demonstrated. This could be due either to the nature of the extract under investigation [6] or to the method of identification. The most striking difference in anthraquinone composition between calli of *C. ledgeriana* and *C. pubescens* grown under identical conditions is that the main compound isolated from *C. ledgeriana* calli, rubiadin, is not found in the callus of *C. pubescens*.

Although it is believed generally that these two *Cinchona* species have been crossed several times to improve alkaloid yield and that the original germplasm being either *C. ledgeriana* or *C. pubescens* is not found anymore nowadays it is striking to notice that such qualitative differences in anthraquinone composition exist between the tissue cultures of these species, whereas their alkaloid profiles only show quantitative differences both at the tissue culture level and at the plant level.

EXPERIMENTAL

Biological material. Callus cultures of *C. pubescens* were grown on medium H at 28° with a 12 hr day period.

Extraction. Ground, freeze dried, calli were mixed with 10% aq. $NaHCO_3$ soln and extracted with $CHCl_3$. After evaporation of the solvent the residue was redissolved in Et_2O. The ethereal soln was extracted with 1 M NaOH, which was then acidified with HCl and extracted with Et_2O. This gave the final extract containing almost no other compounds than anthraquinones.

Prep. TLC. The plates used and the solvent systems employed were the same as in ref. [6]. After detection in daylight, the various bands were scraped off and the anthraquinones eluted with mixtures of $CHCl_3$ and MeOH, varying from 90% $CHCl_3$ for the most non-polar compounds to 90% MeOH for the most polar ones. Analytical TLC was performed using the same TLC systems as in ref. [6] in combination with ready made TLC plates (Merck Kieselgel 60 F 254). Anthraquinones were detected by their colours in daylight, their fluorescence in UV light (366 nm) and after exposure to NH_3 vapour.

EIMS were recorded on a Kratos M9/50 instrument using a direct inlet system and an ionizing energy of 70 eV. 200 MHz ^{1}H NMR spectra were recorded on a Jeol JNM apparatus operating in the Fourier-transform mode. 300 MHz ^{1}H NMR spectra were recorded on a Bruker WM 300 instrument operating in the Fourier transform mode. Chemical shifts are given in δ-values (ppm) relative to TMS. UV-VIS spectra were recorded on a Cary 115 spectrophotometer. IR spectra were recorded as KBr disc.

Purpurin (**1**). The spectroscopic data agrees well with those in the literature [6, 8].

Purpurin-1-methylether (**2**). MS *m/z* (rel. int.): 270 $[M]^+$ (100), 252 $[M-H_2O]^+$ (60), 241 $[M-HCO]^+$ (10), 227 (55), 199 (25), 196 (28); ^{1}H NMR (300 MHz, $CDCl_3$ CD_3OD): δ4.10 (3H, *s*, OMe), 7.40 (H, *s*, H-3), 7.78 (2H, *m*, H-6, H-7), 8.27 (2H, *m*, H-5, H-8); UV-VIS λ_{max}^{MeOH} nm: 240, 243, 283, 313 (sh), 415, 474 (sh).

Anthragallol-1,2-dimethylether (**3**). The spectroscopic data agrees well with those reported in the lit. [6].

1-Hydroxy-2-hydroxymethylanthraquinone (**4**). The spectroscopic data agrees well with those in the lit. [6, 8, 9].

2-Hydroxy-1,3,4-trimethoxyanthraquinone (**5**). The spectroscopic data agrees well with those in the lit. [6], and the chromatographic behaviour (TLC) correlates well with a reference compound.

2,5-(or 3,5-)Dihydroxy-1,3,4-(or -1,2,4-)trimethoxyanthraquinone (**6**). The spectroscopic data correlates very well with those from ref. [6] and the chromatographic behaviour (TLC) is the same as that of a reference compound.

Alizarin-2-methylether (**7**). The spectroscopic data agrees well with those reported in the lit. [10].

2-Hydroxy-1,3,4,6-tetramethoxyanthraquinone (**8**). MS *m/z* (rel. int.): 344 $[M]^+$ (100), 329 $[M-Me]^+$ (50), 326 $[M-H_2O]^+$ (3), 301 (13), 283 (10), 200 (10); ^{1}H NMR (200 MHz, DMSO-d_6): δ3.91 (3H, *s*, OMe-6), 3.94 (3H, *s*, OMe-3), 4.04 (3H, *s*, OMe-1), 4.06 (3H, *s*, OMe-4), 7.26 (1H, *dd*, $J = 8.0$ Hz, $J = 1.5$ Hz, H-7), 7.66 (1H, *d*, $J = 1.5$ Hz, H-5), 7.72 (1H, *d*, $J = 8.0$ Hz, H-8); UV-VIS λ_{max}^{MeOH} nm: 224, 259, 290 (sh.), 402 UV-VIS $\lambda_{max}^{MeOH-OH}$ nm: 224, 259, 476. $C_{18}H_{16}O_7$: calc. 344.0896, found 344.0906.

1,6-(or 1,7-)Dihydroxy-2-methylanthraquinone (**9**). MS *m/z* (rel. int.): 254 $[M]^+$ (63), 239 $[M-Me]^+$ (6), 226 $[M-CO]^+$ (10), 225 $[M-HCO]^+$ (10), 208 (13); ^{1}H NMR (300 MHz, $CDCl_3$-CD_3OD): δ2.38 (3H, *s*, Me-2), 7.50 (1H, *d*, $J = 7.5$ Hz, H-3), 7.57 (1H, *d*, $J = 2.0$ Hz, H-5), 7.63 (1H, *dd*, $J = 7.0$ Hz, $J = 2.0$ Hz, H-7), 7.71 (1H, *d*, $J = 7.5$ Hz, H-4), 8.23 (1H, *d*, $J = 7.0$, H-8); UV-VIS λ_{max}^{MeOH} nm: 213 (sh), 260, 290 (sh), 417 (sh), 437, 464 (sh), 473 (sh); UV-VIS $\lambda_{max}^{MeOH-OH}$ nm: 224, 305, 510. $C_{15}H_{10}O_4$: calc. 254.0579, found 254.0575.

1-Methoxy-2,4,5-trihydroxyanthraquinone (**10**). MS *m/z* (rel. int.): 286 $[M]^+$ (70), 271 $[M-Me]^+$ (7), 268 $[M-H_2O]^+$ (58), 258 $[M-CO]^+$ (5), 257 $[M-HCO]^+$ (11), 243 (40), 240 (17), 215 (19), 212 (15); ^{1}H NMR (200 MHz, $CDCl_3$-CD_3OD): δ4.04 (3H, *s*, OMe), 7.23 (1H, *dd*, $J = 1.2$ Hz, H-6)*, 7.30 (1H, *s*, H-3), 7.64 (1H, *t*, $J = 7.5$ Hz, H-7), 7.80 (1H, *dd*, $J = 7.5$ Hz, $J = 1.2$ Hz, H-8); UV-VIS λ_{max}^{MeOH} nm: 247, 278, 314 (sh), 428 UV-VIS $\lambda_{max}^{MeOH\ OH^-}$ nm: 249, 310, 522; IR ν_{max}^{KBr} cm^{-1}: 3420, 2920, 1672, 1630, 1270. $C_{15}H_{10}O_6$: calc. 286.0477, found 286.0473. (* Part of the doublet is not observed because the $CHCl_3$ signal is superimposed on it).

1,6(or 1,7-)Dihydroxy-3,7-(or -3,6)dimethoxyanthraquinone (**11**). MS *m/z* (rel. int.): 300 $[M]^+$ (100), 285 $[M-Me]^+$ (78), 282 $[M-H_2O]^+$ (20), 271 $[M-HCO]^+$ (8), 257 (17), 186 (16); ^{1}H NMR (200 MHz, DMSO-d_6): δ3.94 (3H, *s*, OMe-6 or OMe-7), 3.99 (3H, *s*, OMe-3), 7.27 (1H, *d*, $J = 1.5$ Hz, H-2), 7.56 (1H, *s*, H-5 or H-8), 7.64 (1H, *s*, H-8 or H-5), 7.67 (1H, *d*, $J = 1.5$ Hz, H-4); UV-VIS λ_{max}^{MeOH} nm: 212, 245, 308, 410; UV-VIS $\lambda_{max}^{MeOH-OH^-}$ nm: 240, 310, 486. $C_{16}H_{12}O_6$: calc. 300.0634, found 300.0629.

6,7-Dihydroxy-1-methoxy-2-methylanthraquinone (**12**). MS *m/z* (rel. int.): 284 $[M]^+$ (100), 269 $[M-Me]^+$ (4), 266 $[M-H_2O]^+$ (1), 256 $[M-CO]^+$ (3), 255 $[M-HCO]^+$ (8); ^{1}H NMR (300 MHz, $CDCl_3$ CD_3OD): δ2.37 (3H, *s*, Me-2), 4.09 (3H, *s*, OMe-1), 7.50 (1H, *d*, $J = 7.5$ Hz, H-3), 7.70 (1H, *s*, H-5 or H-8), 7.72 (H, *s*, H-8 or H-5), 7.72 (H, *d*, $J = 7.5$ Hz, H-4); UV-VIS λ_{max}^{MeOH} nm: 215 (sh), 282, 406; UV-VIS $\lambda_{max}^{MeOH-OH}$ nm: 247, 312, 507. $C_{16}H_{12}O_5$: calc. 284.0685, found 284.0680.

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