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THE PRODUCTION OF ANTHRAQUINONES BY CELL SUSPENSION CULTURES OF *CINCHONA LEDGERIANA**

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Key Word Index—*Cinchona ledgeriana*; Rubiaceae; suspension culture; anthraquinone; media modifications.

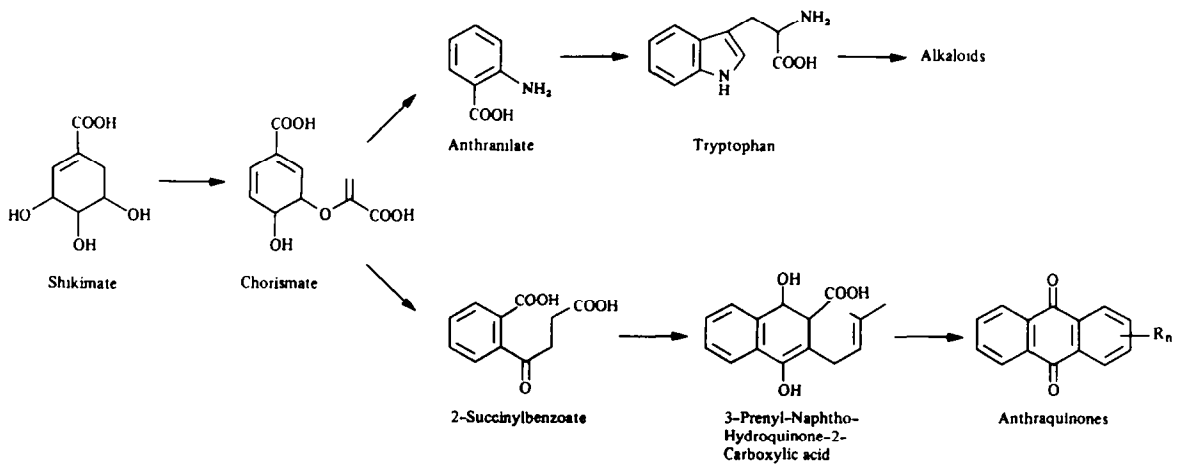
Abstract—In a suspension culture of *Cinchona ledgeriana* a range of anthraquinones accumulate. Fifteen aglycone components of this fraction have been identified, six of which (anthragallol-1,2,3-trimethyl ether, 1,4,5-trihydroxy-2-methylanthraquinone, 5-hydroxy-2-methylanthraquinone, 1,5-dimethoxy-2,3-methylenedioxyanthraquinone, 2,4,6-trihydroxy-1,3-dimethoxyanthraquinone and 1,2,5,6-tetramethoxyanthraquinone) have not previously been found in this species. The time course of anthraquinone production follows neither the growth of the cells nor alkaloid synthesis. The anthraquinones are exported from the cells, with about 80% of the aglycones present normally being recovered from the medium. High levels in the medium are found to inhibit growth of the culture. The final level accumulated is shown to be dependent on a number of nutritional factors, the best yield being obtained when cells are grown with the growth regulators IBA (0.5 mg/l) and ZR (0.1 mg/l) present. Effectors of the shikimic acid pathway such as tryptophan and glyphosate are found to inhibit anthraquinone accumulation.

INTRODUCTION

Anthraquinones are commonly found in plants of the Rubiaceae [1] but it is only recently that they have been isolated [2] and characterized [3–6] from species of the genus *Cinchona*. Verpoorte and coworkers showed that callus cultures of both *C. ledgeriana* Moens [3, 4] and *C. pubescens* Vahl [5, 6] synthesize a range of anthraquinones which accumulate in the cells. The extent to which accumulation in callus occurs can be manipulated by nutritional factors [7]. The biosynthesis of anthraquinones has been reported for a number of other Rubiaceous plants in culture, notably suspension cultures of *Morinda citrifolia* [8, 9], *Galium mollugo* [10] and *Rubia cordifolia* [11]. Cell cultures have proved particularly valuable in studies to determine the pathway of anthraquinone biosynthesis [12]. Using, for example, *Galium mollugo* (Rubiaceae) [13] and *Streptocarpus dunnii* (Gesneriaceae) [14] cultures, the route from shikimate via chorismic acid, with prenylation to form the A-ring, has been clearly established and a number of intermediates identified (Scheme 1) [12]. In all these examples the biosynthesis of anthraquinones can be strongly influenced by exogenous physiological and environmental factors [7, 8, 11, 15–17].

*Part 3 in the series "Cell Suspension Cultures of *Cinchona ledgeriana*." For part 2 see ref. [19].

Abbreviations: 2,4-dichlorophenoxyacetic acid, 2,4-D; benzyladenine, BA; 4-chlorophenoxyacetic acid, 4CPA; 2-methyl-4-chlorophenoxyacetic acid, MCPA; naphthalene acetic acid, NAA; indole acetic acid, IAA; isopentenyl adenosine, IPA; zeatin riboside, ZR; indoylbutyric acid, IBA.



Scheme 1. Biosynthetic relationship of alkaloids and anthraquinones in *Cinchona*.

During our investigation of the capacity of suspension cultures of *C. ledgeriana* to synthesize quinoline alkaloids [18, 19] we have found that a wide range of anthraquinones are also made. In *Cinchona*, as with other Rubiaceae [12], the anthraquinones are probably made from chorismic acid, which is also a precursor of the alkaloids (Scheme 1). The close biosynthetic relationship of these two groups of products in *Cinchona* thus made it pertinent to investigate the manipulation of both groups of secondary products and examine the relative flux of metabolites into the two pathways.

RESULTS AND DISCUSSION

Structural identification of the anthraquinones

Callus cultures of *C. ledgeriana* have been shown [4] to synthesize at least 16 anthraquinones with a wide range of substitution patterns. In suspension culture, 25 components within the anthraquinone fraction can be separated by analytical HPLC, although a number of these are present in only trace amounts. Using preparative HPLC, 15 anthraquinones have been purified (Fig. 1) in sufficient quantity for structural identification by ^{1}H NMR and MS analysis. Nine of these compounds show spectral characteristics identical to anthraquinones isolated previously from *C. ledgeriana* callus [4]. These are purpurin [20], 1-hydroxy-2-hydroxymethylanthraquinone [20], 1-hydroxy-2-methylanthraquinone [21] and anthragallol-1,3-dimethylether [4, 20], all known previously from other Rubiaceous species, and 1,3-dihydroxy-4-methoxyanthraquinone, 1,3-dihydroxy-2,5-dimethoxyanthraquinone, 1,6-dihydroxy-5-methoxy-2-methylanthraquinone, 2-hydroxy-1,3,4-trimethoxyanthraquinone and 1,4-dimethoxy-2,3-methylenedioxyanthraquinone [4]. The other seven anthraquinones isolated from callus [4] were not identified in the suspension cultures, but may be represented by some of the minor, unidentified, components. Of the six further anthraquinones isolated only one, anthragallol-1,2,3-trimethyl ether, is already known [22, 23]. The five new structures are as follows.

1,4,5-*Trihydroxy-2-methylanthraquinone.* Fractions 13 and 14 (Fig. 1) contained a dark orange compound with an $[M]^+$ at *m/z* 270. In the ^{1}H NMR spectrum a singlet at δ2.35 is present due to a methyl group which, on biogenic grounds [1] and comparison with other structures [21, 23, 24], is likely to be at the C-2 position. The absence of any resonance in the 3.8–4.2 region rules out any methyl ether substituents, while the presence of a singlet aromatic proton at δ7.11 in conjunction with the methyl resonance at δ2.35 is consistent with a 1,4-dihydroxy-2-methyl substitution pattern in the A-ring [24] but not with a 1,3-dihydroxy-2-methyl pattern as in rubiadin [9, 25]. The presence of major fragments in the MS at *m/z* 241 and 213 (−29, CHO followed by −28, CO) confirms the presence of two peri-positioned hydroxyl groups [26]. The aromatic region of the ^{1}H NMR spectrum shows resonances at δ7.76, 7.6 and 7.22, consistent with a C-5 or C-8 hydroxyl group. By comparison with other anthraquinones from *C. ledgeriana* having a single hydroxyl substituent in the C-ring [4] it is most likely that this compound is substituted at the C-5, making it 1,4,5-trihydroxy-2-methylanthraquinone.

1,5-*Dimethoxy-2,3-methylenedioxyanthraquinone.* Two anthraquinones eluted in fraction 14 (Fig. 1) giving $[M]^+$ at *m/z* 312 and 270 respectively. On the basis of the spectra from fraction 13 the minor mass ion at *m/z* 270 was assigned to 1,4,5-trihydroxy-2-methylanthraquinone, the only product in fraction 13. Thus, spectral data from the mass ion at *m/z* 312 could be assigned by subtraction of the fraction 13 data from the spectra. In the ^{1}H NMR

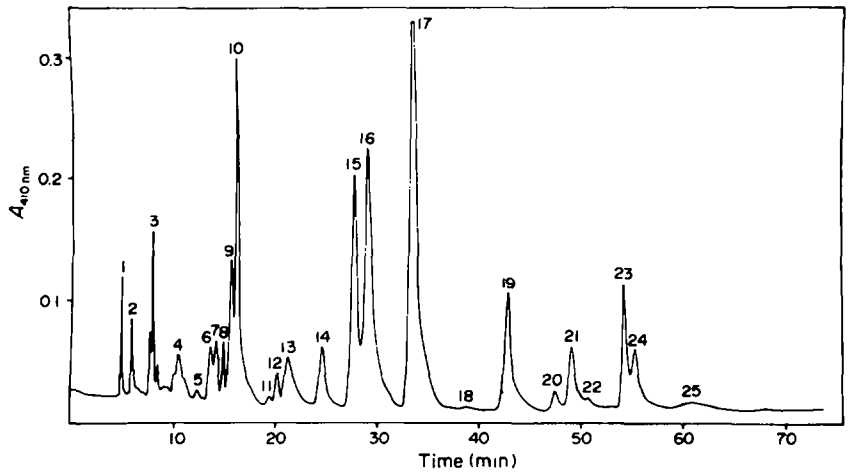


Fig. 1. Preparative HPLC elution profile obtained by separating the anthraquinones using the conditions described in the Experimental. Peaks were identified by ^{1}H NMR and MS as follows: 1, 1-hydroxy-2-methylanthraquinone; 2–6, unidentified; 7, purpurin; 8, anthragallol-1,2,3-trimethyl ether; 9, unidentified; 10, purpurin-1-methyl ether; 11, unidentified; 12, 1,6-dihydroxy-5-methoxy-2-methylanthraquinone; 13, 1,4,5-trihydroxy-2-methylanthraquinone; 14, 1,5-dimethoxy-2,3-methylenedioxyanthraquinone; 15a, 1,2,5,6-tetramethoxyanthraquinone; 15b, 2,4,6-trihydroxy-1,3-dimethoxyanthraquinone; 16, 1,4-dimethoxy-2,3-methylenedioxyanthraquinone + 1-hydroxy-2-hydroxymethylanthraquinone; 17, 1,3-dihydroxy-2,5-dimethoxyanthraquinone; 18, 5-hydroxy-2-methylanthraquinone; 19, anthragallol-1,3-dimethyl ether; 20–22, unidentified; 23, 2-hydroxy-1,3,4-trimethoxyanthraquinone; 24 and 25, unidentified.

spectrum a two proton singlet at δ6.17 demonstrates the presence of a 2,3-methylenedioxy structure, as found in ledgerquinone [4] and not a 1,2-methylenedioxy as in morindaparvin A, which resonates at δ6.32 [27]. In the region δ3.8–4.1 two singlets at δ4.04 and 4.05 are resolved, indicating two methoxyl residues with different degrees of shielding. Thus the compound is not ledgerquinone [4], which elutes in fraction 16, and in which the two methoxyl residues both resonate at δ4.04. Furthermore, the C-ring shows a pattern of coupling consistent with a C-5 or C-8 monosubstituted system. A singlet in the aromatic region at δ8.21 represents the C-4 proton. These assignments are confirmed by the MS in which major fragments are found at *m/z* 297 (−Me), 294 ($-H_2O$) and 269 (−Me−CO), a pattern consistent with the presence of a methoxyl substituent in the peri position. By comparison with other anthraquinones from *Cinchona* it is probable that the C-ring is substituted at the C-5 position, making this compound 1,5-dimethoxy-2,3-methylenedioxyanthraquinone.

1,2,5,6-*Tetramethoxyanthraquinone.* This anthraquinone was isolated by further separation of fraction 15 by HPLC using the solvent composition for the initial conditions running isocratically (see Experimental). In the ^{1}H NMR spectrum there are two strong resonances at δ3.97 and 4.03 and no resonances in the regions δ2.1–2.6 or δ4.5–5.0, indicating the absence of either a methyl or an hydroxymethyl substituent. Coupled with an $[M]^+$ measured at *m/z* 328, it is concluded that this compound is a tetra-methoxyl substituted anthraquinone. In the MS, prominent ions are present at *m/z* 373 (−Me) and 298 (−Me−Me) indicating the presence of at least two peri-methoxyl groups. The aromatic region of the ^{1}H NMR spectrum is simple, showing two overlapping doublets at δ8.147 and 8.151 and a doublet at δ7.69. These may be assigned to the C-4, C-8 and C-3 plus C-5 respectively, by comparison with the C-ring substitution of 5,6-dimethoxy-1-hydroxy-2-hydroxymethylanthraquinone [4], resulting in the conclusion that this compound is 1,2,5,6-tetramethoxyanthraquinone.

2,4,6-*Trihydroxy*-1,3-*dimethoxyanthraquinone.* This red anthraquinone eluted in fraction 15b, also obtained by the further separation of fraction 15, and gave an $[M]^+$ at *m/z* 316. The ^{1}H NMR spectrum showed there to be two non-equivalent methoxyl groups with resonances at δ3.98 and 4.16. The presence in the MS of a prominent ion at *m/z* 298 ($-H_2O$) indicates that one of these occupies a peri position, while a major fragment at *m/z* 301 (−Me) indicates a distal methoxyl group. The aromatic region of the ^{1}H NMR spectrum is simple, showing only resonances at δ7.75 (*s*), 7.24 (*d*) and 8.12 (*d*). This is not consistent with a C-ring monosubstituted at the C-5 or C-8 position, as in 1,3,5-trihydroxy-2-methoxyanthraquinone [4] but is compatible with a C-6 or C-7 hydroxyl derivative [20]. The absence of other aromatic resonances indicates a fully substituted A-ring. The MS shows only a single carbonyl elimination from the B-ring (*m/z* 287, −COH) indicative of the presence of only a single carbonyl with a peri hydroxyl substituent, confirming the assignment of a methoxyl group to the C-1 or C-4 position. Thus it is concluded that this compound is 2,4,6-trihydroxy-1,3-dimethoxyanthraquinone.

5-*Hydroxy*-2-*methylanthraquinone.* This anthraquinone eluted in fraction 18 (Fig. 1) and shows an $[M]^+$ at *m/z* 238. In the ^{1}H NMR there is a singlet at δ2.51 which, by comparison with such compounds as 5,8-hydroxy-2-methylanthraquinone [21], may be assigned to the C-2 in an otherwise unsubstituted A-ring. This structure for the A-ring is confirmed by the presence in the aromatic region of a singlet at δ8.08 (C-1) and doublets showing *ortho* coupling at δ8.25 and 7.5, as in this compound [21]. In the MS there is a major fragment ion at *m/z* 210 due to a peri-positioned hydroxyl group [26]. In the ^{1}H NMR, aromatic resonances at δ7.74, 7.61 and 7.24 are consistent with this assignment and have the coupling pattern typical of a mono-substituted C-ring containing a C-5 substituent [4]. Thus, the structure 5-hydroxy-2-methylanthraquinone is assigned to this compound.

Pattern of growth and production in liquid suspension culture

A typical time-course for production on a 2,4-D/BA medium is shown in Fig. 2. Increase in pigment concentration does not occur until late in the growth cycle, at which period a 5-fold increase is found. In this pattern *C. ledgeriana* differs markedly from suspension cultures of *Morinda citrifolia* [8] in which growth and anthraquinone synthesis are closely linked but mimics other Rubiaceous species like *Galium mollugo* [10] and *Rubia cordifolia* [11] in which pigment production starts or accelerates in late-growth phase when grown in batch culture. In continuous cultures of *G. mollugo* anthraquinone accumulation is very dependent on external factors [28], indicating that particular conditions may be required for inducing anthraquinone biosynthesis. In callus culture also, *C. ledgeriana* produces anthraquinones most satisfactorily at slow growth rates, though the overall production is low [7].

The anthraquinones, like the alkaloids [18, 19, 29] are readily exported from the cells to the medium, about 80–85% of the aglycones typically being found extracellularly. About half of the intracellular anthraquinones appear to be conjugated but the form of conjugation has not been determined. Conjugated anthraquinones do not appear in the medium. The amount of anthraquinone exported usually exceeds the solubility of the products in the medium and a wash line of product precipitated on the surface of the flask usually forms. The ability to export anthraquinones probably explains the much higher syn-

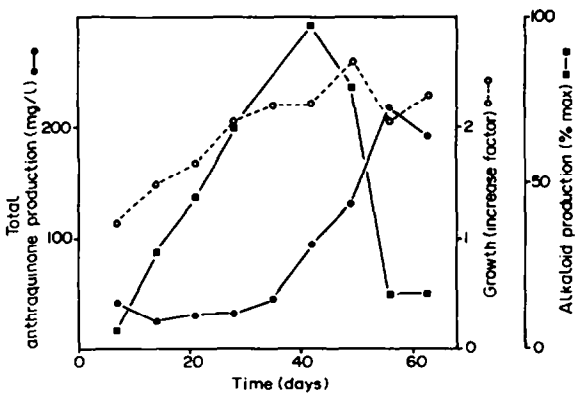


Fig. 2. Time course of cell growth (○), anthraquinone production (●) and alkaloid production (■) on a medium containing 2,4-D (0.5 mg/l) and BA (0.1 mg/l). Duplicate samples were taken as shown and anthraquinones and alkaloids [18] extracted.

Table 1. Effect of added anthraquinones on cell growth and alkaloid production

Anthraquinone concentration* (mg/ml)	Cell growth (% control)	Alkaloid production (% control)
0.005	115	85.3
0.01	115	122.3
0.02	113	63.4
0.1	71	33.0

*The crude anthraquinones (fraction A) were dissolved in chloroform and the appropriate volumes pipetted into clean flasks. The solvent was removed with nitrogen gas and 50 ml medium added to the flasks which was then autoclaved. The aqueous solution became saturated at 0.1 mg/ml (2.5 mg/flask).

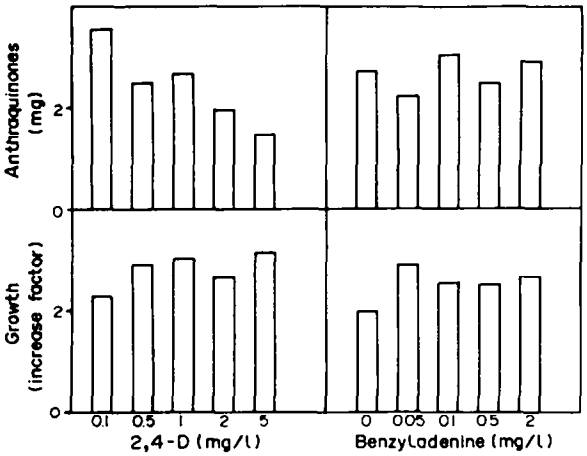


Fig 3. The effect on anthraquinone production and growth of varying the concentration of 2,4-D (at 0.1 mg/l BA) and BA (at 0.5 mg/l 2,4-D). Each bar represents the mean for that parameter determined in duplicate at 28 days and followed over three successive sub-cultures.

thetic capacity of the suspension cultures compared to callus [7], although, surprisingly, *M. citrifolia*, which accumulates very high concentrations of anthraquinones within the cells [8], and *R. cordifolia* [11] do not export anthraquinones to the medium except for very late in the cell cycle, when the loss of pigment is probably due to lysis.

Some deposition of anthraquinone on the surface of the cell aggregates also occurs and, using another line of cells which normally accumulates much less anthraquinone, FRI-2A6 (to be described elsewhere), it was possible to demonstrate that high levels of these compounds in the medium have a phytostatic effect. Cells of FRI-2A6 were sub-cultured into medium in which anthraquinones were dissolved (Table 1). At low levels, cell growth and alkaloid synthesis was comparable to the control, but the presence of anthraquinones at 0.1 mg/ml had a more marked effect on total alkaloid production (70% inhibition) than on growth (30% inhibition). Whether this indicates some level of control of metabolism prior to the bifurcation of the pathway to form these two groups of products or is an indirect effect was not established.

Influence of plant growth regulators

It has previously been shown for many secondary products, including anthraquinones [8], that the auxin analogue 2,4-D suppresses their synthesis. The effect of the concentration of 2,4-D and BA on production were therefore examined (Fig. 3). Varying the 2,4-D concentration at a constant 0.5 mg/l BA had a marked influence on anthraquinone synthesis and, as with *M. citrifolia* [8] and *R. cordifolia* [11], a low level of 2,4-D (0.1 mg/l) led to slightly improved production. Cell growth was, however, promoted to a lesser extent by these low levels of 2,4-D, as found for callus, where 1–10 mg/l auxins was required [7]. In contrast, varying the BA concentration at a fixed 1.0 mg/l 2,4-D showed no effect on pigment production (Fig. 3), though growth was decreased in the absence of added cytokinin.

Replacing 2,4-D and BA with other combinations of growth regulators was found much more effective as a means of promoting anthraquinone synthesis (Table 2). Substituting 2,4-D with NAA (2 mg/l) or IAA (2 mg/l) led to a 2-fold enhancement of anthraquinone production while substituting BA with ZR (0.5 mg/l) caused a further small improvement. Thus, again as with *M. citrifolia* [8] and *R. cordifolia* [11], NAA was found to be the best auxin for anthraquinone production, as with, for example, nicotine production by tobacco cultures [30, 31]. NAA, and to a lesser extent IAA, led to extensive root formation in the cultures and it may be that differentiation to form this type of tissue is beneficial for anthraquinone production. Although no organisation was observed in the *M. citrifolia* cultures, Zenk *et al.* [8] showed the roots of this species to be the only tissue accumulating these products in the intact plant. Similarly, it is in the roots of

Table 2. Effect of different hormone combinations on anthraquinone production

	Anthraquinone production (mg/l/day)				
	Auxin (mg/l)				
Cytokinin (0.5 mg/l)	2,4-D (0.5)	MCPA (0.5)	4CPA (0.5)	NAA (2.0)	IAA (2.0)
Kinetin	0.81	0.83	1.16	0.83	0.67
BA	0.49	0.32	0.90	1.17	0.91
ZR	0.66	0.42	0.99	1.35	0.97
IPA	0.34	0.76	0.82	0.91	0.85

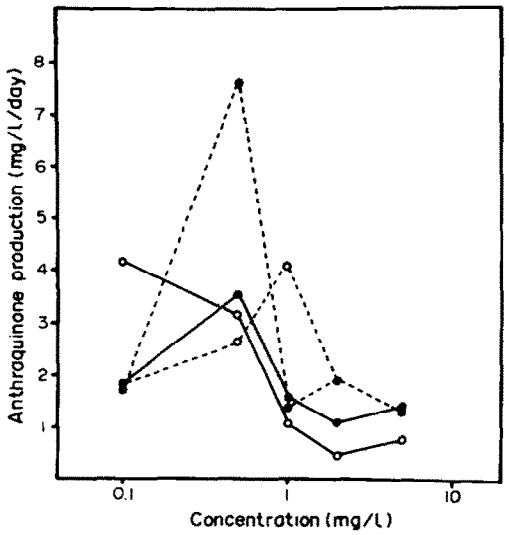


Fig 4. The effect on anthraquinone production determined 28 days after subculture of varying the concentration of IBA at a constant 0.1 mg/l ZR (●– – –●); IAA at a constant 0.1 mg/l ZR (O– – –O); ZR at a constant 2.0 mg/l IAA (●——●); ZR at a constant 1.0 mg/l IBA (O——O). All values represent the results of duplicate samples. All s.e. within ± 10%.

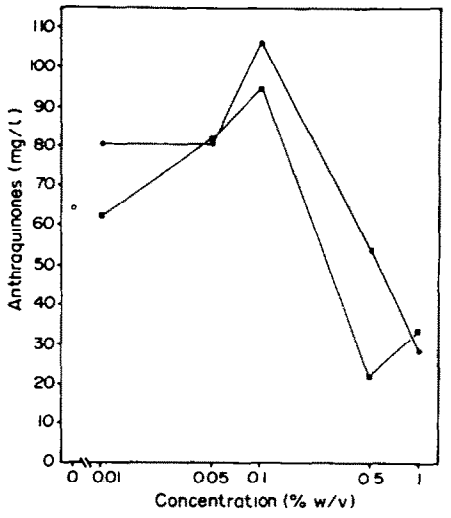


Fig 5. The effect on the accumulation of anthraquinones at 28 days of adding an amino acid supplement (N,Z-amine; Difco) at 7 (●) or 14 (■) days after sub-culture.

R. cordifolia that anthraquinones accumulate [25]. The effect of different auxin types on anthraquinone synthesis by callus cultures of *C. ledgeriana* has not been studied. Due to the extensive root formation caused by NAA the growth rate decreased markedly after one generation, leading to a lower total yield. With IAA or IBA, however, growth was less suppressed and these auxins proved the best promoters of overall anthraquinone accumulation.

The stimulation of anthraquinone synthesis found using these non-phenoxyacetic acid based auxin analogues showed marked sensitivity to the concentration exhibited. From Fig. 4 it can be seen that, with the optimal cytokinin, ZR, present varying the level of IAA or IBA led to peaks of productivity at 1.0 mg/l and 0.5 mg/l respectively, the best combination being IBA (0.5 mg/l) with ZR (0.1 mg/l), in which cultures made 8 mg/l/day as averaged over a 28-day period. The enhancement may be due to stimulation earlier in the growth cycle, rather than to derepression of the pathway as the data in Fig. 1 show that production on the 2,4-D/BA medium does not occur until further into the growth cycle. Thus, although the final levels achieved on both media are comparable, the stimulation of production appears to occur earlier with the IAA/ZR medium.

Effect of nutritional factors

Anthraquinone production by *C. ledgeriana* suspension cultures is remarkably insensitive to varying the nutritional factors in the medium, as was also found for callus cultures [7]. Substituting Murashige and Skoog [32] salts for B5 [33] salts led to a small stimulation (110%) in the first sub-culture and this was maintained in the second passage, although cells grew poorly (42%). In cultures growing on a stimulatory medium (2.0 mg/l IAA; 0.1 mg/l ZR) altering neither the sucrose (range 27–270 mM) nor the inorganic phosphate (range 1–50 mM) concentration caused any significant change in the level of anthraquinone production. A similar insensitivity to such factors was found with *R. cordifolia* cultures [11].

Adding an organic-N source (N,Z-amine; Difco) to the B5 medium [33] did, however, stimulate anthraquinone production (Fig. 5), but to a less marked extent than the hormonal changes. The increased yield was maximal around 0.1% (w/v) in both the 2,4-D/BA and IAA/ZR media. Stimulation was only found over a narrow range and levels in excess of 0.1% (w/v) were inhibitory to production. This observation closely follows that reported for *M. citrifolia* where 0.3% (w/v) N,Z-amine stimulated production but 0.4% (w/v) proved inhibitory [8].

Effectors of the pathway of biosynthesis

In an attempt to stimulate the diversion of products from the shikimic acid pathway towards anthraquinones, the effects of feeding anthranilic acid and tryptophan were examined. Anthranilic acid, even at low concentration (5×10^{-4} M), proved toxic to the cells, and was not satisfactory as a precursor. Feeding tryptophan to cultures either at 7 or 14 days after sub-culture suppressed anthraquinone production by 20–60% over the range 0.1–5 mM. In part this was due to the inhibitory effect of higher concentrations on growth, the productivity per gram fresh weight showing a less marked change. Tryptophan has also been found to inhibit anthraquinone production in *M. citrifolia*, where 2×10^{-5} M caused 50% inhibition of production [15, 17] though, in contrast to *C. ledgeriana*, growth was not inhibited below 10 mM [15]. The amino acid is presumably exerting this effect through feed-back inhibition of the common pathway.

The inhibitor of the shikimic acid pathway *N*-phosphonomethylglycine (glyphosate) [34, 35] also decreased anthraquinone production (Fig. 6). When present at 1 mM concentration this inhibitor had a marked effect on growth, which was alleviated by an aromatic amino acid supplement in the medium of L-tyrosine, L-phenylalanine and L-tryptophan each at 0.2 mM. In contrast, 1 mM glyphosate caused about a 50% decrease in anthraquinone synthesis which could not be reversed

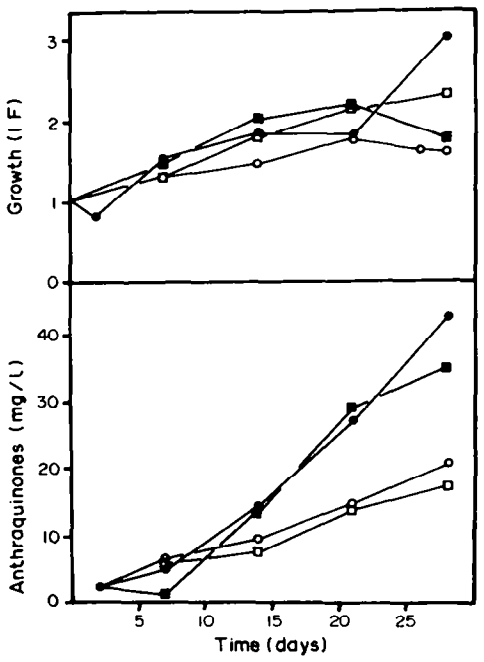


Fig. 6. Time courses for growth and the production of anthraquinones on a 2,4-D/BA medium (0.5 and 0.1 mg/l respectively) in the presence of (○) 1 mM glyphosate; (■) an aromatic amino acid supplement containing L-tryptophan, L-tyrosine and L-phenylalanine each at 0.2 mM; (□) 1 mM glyphosate plus the aromatic amino acid supplement; (●) control.

by the amino acid supplement. These results concur with the effects observed with *G. mollugo* [34]. Such inhibitory effects may be valuable in manipulating the balance of the flux of metabolites between the alkaloid and anthraquinone pathways. At the level of L-tryptophan used, however, no effect on alkaloid synthesis was observed.

Conclusions

Anthraquinones appear to be readily synthesized by members of the Rubiaceae in cultures, both liquid and callus, and *C. ledgeriana* is no exception. This species has now been shown to synthesize a wide range of anthraquinones in both callus [4] and suspension culture [present work]. Eight of the products identified are the same chemical species in both culture types but six anthraquinones have been isolated from suspension culture having a novel structure, while seven of the structures found in callus have not been identified from liquid suspension culture. A number of minor components present in the extract from suspension cultures have not been identified (Fig. 1) and these may well represent some of the further products known from callus. Thus, it appears that, at least broadly, the range of anthraquinones made is similar but the quantitative balance is altered considerably. These differences may be due to the present study having examined only the anthraquinones accumulated in the medium as the extent to which they are transported may vary depending on the substitution patterns of the different products.

The composition of the medium, in particular the plant growth regulators present, has been found to exert major effects on anthraquinone synthesis in a number of species [7, 8, 11, 15–17]. Thus, in *C. ledgeriana* callus it was found that the level of auxins affected production though cytokinins appeared less effective regulators. The role of these components of the medium has been rigorously investigated using suspension cultures and it has now been shown that both the concentration of growth regulators and the combination of auxin and cytokinin used can be critical. A similar result was found with this line of cells for quinoline alkaloid production, though optimal production of alkaloids occurred at different levels of growth regulators [18, 19].

Other factors in the medium were found to have less influence on anthraquinone production, though as with *Morinda citrifolia* [8], a supply of amino acids (N,Z-amine) was beneficial. This does not appear to have been due to the addition of L-tryptophan which, even at low levels, was inhibitory to growth when administered separately.

The relative insensitivity of anthraquinone production to factors in the medium is in marked contrast to alkaloid biosynthesis [18, 19; unpublished results]. The high accumulation rates determined for anthraquinones (up to 8 mg/l/day) indicate that the pathway (Scheme 1) leading to these products via chorismate is expressed under a wide range of conditions. Experiments are currently being conducted to investigate whether the two groups of products share common precursor pools or whether their biosynthetic pathways operate separately.

Currently, however, it has not been possible to determine whether the differential effects of plant growth regulators and other metabolites on alkaloid accumulation reflect separate control of the expression of the pathways at the genetic level or effects on the regulatory control of the operation of the pathways. It may, therefore, also be possible to control the relative production of the two groups of products using suitable combinations of plant growth regulators or inhibitors specific to the anthraquinone pathway which do not affect the flux through the common pathway, thus improving overall alkaloid biosynthesis.

The quantitative yield of anthraquinones obtained from suspension culture is very much greater than from callus [7]. This may reflect the ability of the cultures to export the product to the medium, thus maintaining a cellular concentration which does not inhibit further synthesis. The insolubility of anthraquinones in water, leading to their deposition as a ring of precipitated material adhering to the culture vessel, may help to maintain a concentration gradient from the cells into the medium. Thus, rates of production of 8 mg/l/day have been attained. In a further communication [36] we report that a considerable further stimulation of both production and export can be achieved by the continuous removal of anthraquinones from the medium using polymeric adsorbents.

EXPERIMENTAL

General procedures. ^{1}H NMR (300 MHz) on a Bruker CXP spectrometer in $CDCl_3$ with TMS internal standard; MS on a Kratos 50 spectrometer under EI conditions; solvents of analytical grade, re-distilled before use; chemicals of 'Analar' grade. N,Z-Amine (casein acid hydrolysate) was obtained from Difco Laboratories, Detroit, U.S.A. and glyphosate (84% purity) was the generous gift of Monsanto Europe S.A. (Louvain-La-Neuve, Belgium).

Cell cultures. These experiments were conducted using suspension cultures of *C. ledgeriana* cell line FRI-CLla, the establishment and maintenance of which is described in Robins *et al.* [18]. Cells were routinely sub-cultured every three weeks into medium containing B5 salts [33] supplemented with 2,4-D (1 mg/l) and BA (0.1 mg/l) and grown at 26 ± 1° under low light (600–1000 lx) in a 16 hr light/8 hr dark cycle on orbital shakers (90–100 rpm). Additives to the medium were either dissolved in medium prior to autoclaving or administered from a filter-sterilized soln. Samples were harvested by vacuum filtration and stored at −20° until required.

Extraction procedures. (a) Cells: *ca* 5 g tissue was homogenized (Ultra Turrax) in 10 ml 0.2 M H_2SO_4 and 10 ml $CHCl_3$. A further 5 ml $CHCl_3$ was used to rinse the Turrax probe. After 90 min at room temp. debris was removed by filtration *in vacuo* and phases separated by centrifugation. The $CHCl_3$ phase was retained and, in some cases, the aq. phase washed with a further 5–10 ml $CHCl_3$. Solvent was removed from the pooled $CHCl_3$ phase by evaporation *in vacuo* and the residue dissolved in 3 or 6 ml MeOH.

(b) Medium: *ca* 50 ml medium was acidified with 10 ml 0.2 M H_2SO_4 and vigorously shaken with 20 ml $CHCl_3$. After 90 min at room temp. phases were separated and the $CHCl_3$ treated as in (a).

For the purposes of routine quantitation this extract was used and the A_{480} determined in neutral and alkaline conditions.

Purification of anthraquinones. To 7 l. spent medium collected from routine sub-culturing of stock FRI-CLla cells was added 30 ml conc. H_2SO_4 and 1 l. $CHCl_3$. The mixture was agitated for 60 min, the $CHCl_3$ removed and the aq. phase extracted with a further 1 l. $CHCl_3$. After removal of $CHCl_3$ *in vacuo* the pooled residues were taken into 15 ml MeOH and left at −17° for 48 hr to precipitate out lipids. The ppt was removed by centrifugation, washed twice with ice-cold MeOH and the MeOH supernatants pooled. Solvent was removed *in vacuo* and the residue dissolved in toluene–MeOH–EtOAc (50:50:1) in which it was extremely soluble. After drying with N_2 and *in vacuo* a yield of 0.35 g crude anthraquinones was obtained (fraction A).

Separation of anthraquinones. (a) TLC: a solvent was developed suitable for transfer to HPLC using toluene–EtOAc–HOAc (97:30:2) and silica gel 0.25 mm plates (Merck) which gave 18 identifiable components ranging in R_f from 0.03 to 0.75. In daylight these were mostly yellow and many showed enhanced intensity and a shift towards orange on alkalination.

(b) Analytical HPLC was conducted on fraction A using a Partisil-5 column (4.6 × 25 mm) eluted at 25° with toluene–EtOAc–HOAc (100:5:1) flowing at 1.5 ml/min with a detector at 280 or 410 nm. Under these conditions the five minor components which on TLC had an $R_f < 0.2$ were not detected.

(c) Prep. HPLC was performed on fraction A using a Partisil-5 M9 column (9.4 × 25 mm) eluted with a non-linear gradient. Initial conditions were toluene–EtOAc–HOAc (100:1:1) and the chromatogram run for 25 min in these conditions. Then, using a Waters gradient maker set on Curve 8, a non-linear gradient was run over 20 min to a final solvent composition of toluene–EtOAc–HOAc (100:5:1) and elution continued for a further 30 min. With this system 25 components were resolved and fractionated from repeated injections. Solvent was removed *in vacuo* and the mass and ^{1}H NMR spectra obtained.

A typical prep. HPLC elution profile is shown in Fig. 1. Fractions 1, 7, 8, 10 and 12 correspond to known anthraquinones (see text) and fractions 2–6, 9 and 11 are unidentified.

1,4,5-*Trihydroxy*-2-*methylanthraquinone* (**13**). MS *m/z* (rel. int.): 270 $[M]^+$ (30.5), 255 $[M-Me]^+$ (2.1), 241 $[M-CHO]^+$ (1.6), 213 $[M-CHO-CO]^+$ (1.8), 185 (1.0), 167 (22.3), 149 (61.8); ^{1}H NMR (300 MHz, $CDCl_3$): δ2.35 (*s*, Me), 7.11 (*s*, H-3), 7.25 (*d*, *J* = 8 Hz, H-6), 7.68 (*t*, *J* = 8 Hz, H-7), 8.24 (*d*, *J* = 8.1 Hz, H-8).

1,5-*Dimethoxy*-2,3-*methylenedioxyanthraquinone* (**14**). MS *m/z* (rel. int.): 312 $[M]^+$ (70), 297 $[M-Me]^+$ (12.5), 294 $[M-H_2O]^+$ (5.4), 254 $[M-Me-CO]^+$ (100); ^{1}H NMR (300 MHz, $CDCl_3$): δ4.04 (*s*, OMe, C-5), 4.05 (*s*, OMe, C-1), 6.17 (*s*, OCH_2O), 7.25 (*d*, *J* = 8.5 Hz, H-6), 7.6 (*t*, H-7), 8.13 (*d*, *J* = 8.5 Hz, H-8), 8.21 (*s*, H-4).

1,2,5,6-*Tetramethoxyanthraquinone* (**15a**). MS *m/z* (rel. int.): 328 $[M]^+$ (7.8), 313 $[M-Me]^+$ (3.8), 299 $[M-CHO]^+$ (1.2), 298 $[M-2\times Me]^+$ (1.2), 280 (2.6), 254 (3.3), 167 (29.6), 149 (78.8); ^{1}H NMR (300 MHz, $CDCl_3$): δ3.97 (*s*, 2 × OMe), 4.03 (*s*, 2 × OMe), 7.25 (*d*, *J* = 9.5 Hz, H-3 + H-7), 8.147 and 8.151 (*d*, *J* = 8.5 Hz, H-4 + H-8).

2,4,6-*Trihydroxy*-1,3-*dimethoxyanthraquinone* (**15b**). MS *m/z* (rel. int.): 316 $[M]^+$ (40.6), 301 $[M-Me]^+$ (13.3), 298 $[M-H_2O]^+$ (18.8), 287 $[M-CHO]^+$ (4.5), 270 $[M-H_2O-CO]^+$ (15.4), 137 (14.2), 95 (20.1), 69 (48), 44 (100); ^{1}H NMR (300 MHz, $CDCl_3$): δ3.97 (*s*, OMe), 4.12 (*s*, OMe), 7.24 (*d*, *J* = 9 Hz, H-7), 7.76 (*s*, H-5), 8.12 (*d*, *J* = 8.5 Hz, H-8).

Fractions 16 and 17 correspond to known anthraquinones (see text).

5-*Hydroxy*-2-*methylanthraquinone* (**18**). MS *m/z* (rel. int.): 238 $[M]^+$ (100), 223 $[M-Me]^+$ (40), 210 $[M-CO]^+$ (36.4), 209 $[M-CHO]^+$ (22), 195 $[M-Me-CO]^+$ (19), 181 $[M-CHO-CO]^+$ (48), 152 (40), 139 (47), 114 (43); ^{1}H NMR (300 MHz, $CDCl_3$): δ2.51 (*s*, Me), 7.24 (*d*, *J* = 8 Hz, H-6), 7.51 (*d*, *J* = 8 Hz, H-3), 7.61 (*t*, H-7), 7.74 (*d*, *J* = 8 Hz, H-8), 8.08 (*s* with *meta* coupling *J* = 1 Hz, H-1), 8.25 (*d*, *J* = 8 Hz, H-4).

Fractions 19 and 23 correspond to known anthraquinones (see text) and fractions 20–22, 24 and 25 remain unidentified.

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