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BIOSYNTHESIS OF MORINDONE AND ALIZARIN IN INTACT PLANTS AND CELL SUSPENSION CULTURES OF *MORINDA CITRIFOLIA*

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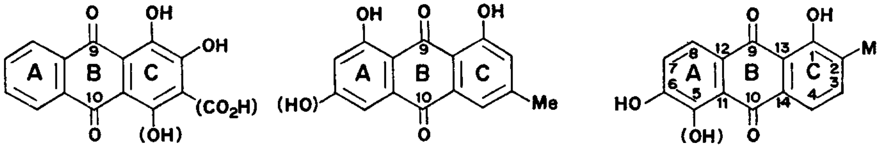
Key Word Index—*Morinda citrifolia*, Rubiaceae; cell suspension culture; quinones; biosynthesis, morindone, alizarin.

Abstract—Chrysophanol (1,8-dihydroxy-3-methylanthraquinone) is an acetate-derived anthraquinone which is substituted in both benzenoid rings. Morindone (1,5,6-trihydroxy-2-methylanthraquinone) is also substiuted in both rings but is shown to be derived from shikimic acid via *o*-succinoylbenzoic acid.

INTRODUCTION

TWO DIFFERENT pathways for anthraquinone biosynthesis in lower and higher plants are known: emodin (I) and islandicin in lower plants[1] and emodin and chrysophanol (II) in higher plants[2] have been shown to be derived from acetic acid. These anthraquinones are substituted in both ring A and ring C (Scheme 1).



Alizarin (III)
(Purpurin carboxylic acid IV)

Chrysophanol (II)
(Emodin I)

Soranjidiol (IX)
(Morindone VIII)

SCHEME 1. ANTHRAQUINONES WITH DIFFERENT SUBSTITUTION-PATTERN.

Alizarin (III) and purpurin carboxylic acid (IV) from *Rubia tinctorum* L. (Rubiaceae), however, are derived from shikimic acid (V), glutamic acid and mevalonic acid (VI) by way of *o*-succinoylbenzoic acid (VII) and an unknown naphthalenic intermediate.[3,4] Nineteen anthraquinones have been isolated from *Rubia tinctorum* all of which are substituted in ring C only.[5] *Morinda citrifolia* L. is another anthraquinone containing plant of the Rubiaceae. Nine of its anthraquinones, including alizarin, are also substituted in ring C only.[5] The most abundant anthraquinone in this plant, however, morindone (VIII; 1,5,6-trihydroxy-2-methylanthraquinone) as well as a minor constituent soranjidiol (IX; 1,6-dihydroxy-2-methylanthraquinone) are hydroxylated in both rings A and C. There is no obvious way to distinguish between the two pathways judging from the substitution pattern. Therefore,

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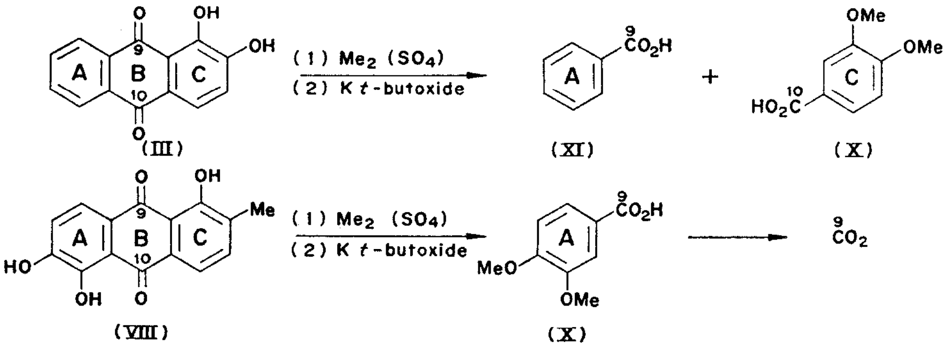
[1] GATENBECK, S. (1960) *Acta Chem. Scand.* **14**, 296.
[2] LEISTNER, E. (1971) *Phytochemistry* **10**, 3015.
[3] LEISTNER, E. (1973) *Phytochemistry* **12**, 337.
[4] LEISTNER E. and ZENK, M. H. (1967) *Z. Naturforsch.* **22b**, 865.
[5] THOMSON, R. H. (1971) *Naturally Occurring Quinones*, 2nd Edn, Academic Press, London.

experiments were carried out to see whether the anthraquinones in *Morinda* are derived from acetate, from *o*-succinoylbenzoic and mevalonic acid, or from both pathways.

RESULTS

The potential precursors in question were applied to intact 1-year-old plants by the wick technique[6] for 48 hr (Table 1). Alternatively, precursors were fed to cell suspension cultures (Table 2). The sterile aqueous solutions of the labelled precursors were added to the cultures during the onset of the logarithmic phase of quinone production.[7] After 3–5 days the plant material was filtered off and washed with water. The plant material obtained either from the intact plant (roots) or from the suspension culture was extracted with boiling ethanol (80%). Morindone (VIII) was isolated under identical conditions from both types of plant material. Alizarin was not detected in 1-year-old plants but occurred in plant suspension cultures. Carrier material was added to the isolated alizarin and morindone and the anthraquinones were recrystallized from acetic acid. The quinones were methylated[3] and again recrystallized from acetic acid. They were then submitted to degradation with K *t*-butoxide.[8] The main degradation product of morindone (VIII) was veratric acid (X) (ring A plus C-9) which was decarboxylated (Scheme 2). Alizarin was split into benzoic acid (XI) (ring A plus C-9) and veratric acid (X) (ring C plus C-10)[3] (Scheme 2).



SCHEME 2. DEGRADATION OF ALIZARIN AND MORINDONE.

The amount of incorporation of each precursor is listed in Table 1 (intact plant) and Table 2 (cell suspension culture). No incorporation was obtained with mevalonic acid. Incorporation with acetic acid was also negligible. Highest incorporation was observed with 7-^{14}C-D-shikimic acid and *o*-(succinoyl 4-^{14}C) benzoic acid carboxyl-^{14}C. Alizarin and morindone isolated after these feedings were degraded. Specific incorporation of activity from carboxyl-^{14}C-D-shikimic acid into C-9 of alizarin and morindone was observed (Table 3). Specific incorporation of *o*-(succinoyl-4-^{14}C) benzoic acid carboxyl-^{14}C into both morindone and alizarin was indicated by the drop of the specific activity to approximately 50% in the case of veratric acid from both anthraquinones and in the case of benzoic acid from alizarin (Table 3).

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[6] GEAR, J. R. and SPENSER, I. D. (1965) *Can. J. Chem.* **41**, 783.

[7] ZENK, M. H. forthcoming publication.

[8] DAVIES, D. G. and HODGE, P. (1971) *J. Chem. Soc.* C, 3158.

DISCUSSION

GLC analysis of the anthraquinones produced by cell-suspension cultures of *Morinda citrifolia* indicated that damnacanthal (1-methoxy-3-hydroxy-anthraquinone-2-aldehyde) is most abundant, whereas alizarin (III) and morindone (VIII) are produced in minor amounts. In contrast, morindone is the most abundant anthraquinone in intact plants. Incorporation into morindone, however, is fairly similar with both feeding techniques (Tables 1 and 2).

Since incorporations of shikimic acid and *o*-succinoyl-benzoic acid are relatively low, two parallel feeding experiments were carried out with U-^{14}C-D-glucose (Table 1). Since incorporation of this common precursor was in both cases much less than that of shikimic acid and *o*-succinoylbenzoic acid (Table 1) the low incorporation of these precursors is attributed to a slow morindone (alizarin) production or a high morindone (alizarin) turnover.

TABLE 1. RESULTS OF FEEDING ^{14}C COMPOUNDS TO INTACT PLANTS OF *Morinda citrifolia*

Labelled compounds administered*	Total and specific activity fed μCi	μCi/μM	Morindone purified μmol	Total activity (dpm)	Incorporation (%)
2-^{14}C-Acetic acid	50	10·4	0·290	717	0·0006
2-^{14}C-DL-Mevalonic acid	50	5·8	0·710	110	0·0001
U-^{14}C-D-Glucose	50	3·0	0·275	403	0·0004
7-^{14}C-D-Shikimic acid	10	25·9	1·135	1982	0·0089
o-(Succinoyl-4-^{14}C)-benzoic acid carboxyl-^{14}C	3·1	3·1	1·120	6541	0·0949

* By the wick technique; feeding time 48 hr.

The specific incorporation of shikimic acid and *o*-succinoylbenzoic acid leaves no doubt that morindone and alizarin in *Morinda citrifolia* are formed by the same biosynthetic pathway as alizarin is in *Rubia tinctorum*.[3]

TABLE 2. RESULTS OF FEEDING ^{14}C COMPOUNDS TO PLANT SUSPENSION CULTURES OF *Morinda citrifolia*

Labelled compounds administered	Total and specific activity fed μCi	μCi/μM	Anthraquinones μmol	Total activity (dpm)	Incorporation (%)
1-^{14}C-Acetic acid	40	24·2	m 0·080	106	0·0001
2-^{14}C-Acetic acid	40	10·4	m 0·045	1806	0·0024
2-^{14}C-DL-Mevalonic acid	40	48·2	m 0·065	166	0·0002
7-^{14}C-D-Shikimic acid	4·9	0·41	m 0·025	2275	0·0209
			a 0·255	4254	0·0391
† 7-^{14}C-D-Shikimic acid	4·0	29·5	m 0·53	6730	0·0758
o-(Succinoyl-4^{14}C)-benzoic acid carboxyl^{14}C	1·6	0·4	m 0·265	5804	0·1665
			a 0·313	2374	0·0669

Feeding time 5 days or 3 days† respectively. m—morindone, a—alizarin.

It is assumed that shikimic acid is transformed to chorismic acid[10] prior to incorporation into quinones. α-Ketoglutarate and chorismate are supposed to combine to give *o*-succinoylbenzoic acid[10] which cyclizes to give a naphthalene of unknown structure.

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[9] SCHARF, K. H. and ZENK, M. H. (1971) *J. Labelled Compounds* **7**, 525.

[10] DANSETTE, P. and AZERAD, R. (1970) *Biochem. Biophys. Res. Commun.* **40**, 1090.

This naphthalene could be 1,4-dihydroxy-2-naphthoic acid[11] which is linked to γ,γ-dimethylallyl pyrophosphate, derived in turn from mevalonic acid. Decarboxylation and ring closure would lead to an anthraquinone which on further transformations would yield alizarin and morindone. Such a biosynthetic scheme suggests that activity from 2-^{14}C DL-mevalonic acid should enter into morindone. The β-carbon of the morindone side chain should be derived from C-2 of mevalonic acid as is the β-carbon of purpurin carboxylic acid.[12,13]

TABLE 3. DEGRADATION OF MORINDONE TRIMETHYL ETHER AND ALIZARIN DIMETHYL ETHER WITH K *t*-BUTOXIDE

Precursor	Morindone trimethyl ether (dpm/μM, %)	Veratric acid (dpm/μM, %)	CO_2 *ex*-veratric acid CO_2 = C atom 9	
7-^{14}C-D-Shikimic acid	24·8 (100)	24·5 (98·8)	—	a
	46·8 (100)	43·6 (93·2)	41·2 (88·2)	b
o-(Succinyl-4^{14}C)	92·6 (100)	44·5 (48·1)	—	a
benzoic-acid carboxyl-^{14}C	15·2 (100)	6·9 (45·3)	—	b

Precursor	Alizarin dimethyl ether (dpm/μM, %)	Benzoic acid (dpm/μM, %)	Veratric acid (dpm/μM, %)	
7-^{14}-C-D-Shikimic acid	8·7 (100)	6·4 (73·6)	1·5 (17·2)	b
o-(Succinyl 4-^{14}C) benzoic acid carboxyl-^{14}C	4·1 (100)	1·94 (47·3)	2·01 (49·2)	b

a—intact plant, b—cell suspension culture.

No incorporation, however, was obtained with mevalonic acid in repeated experiments. It is assumed that mevalonic acid entered into the cells but was not further metabolized. Uptake of the mevalonic acid is suggested by the recovery of most of the activity in the alcohol extract and minor activity in the medium (Table 4).

TABLE 4. ACTIVITY FED TO AND RECOVERED FROM CELL-SUSPENSION CULTURES

Labelled compound administered	Total and specific activity fed		Activity recovered (%)			
	μCi	μCi/μM	In dried extracted tissue	In the alcohol extract	In the culture medium	Total activity recovered (%)
1-^{14}C-Acetic acid	40	24·2	5·6	5·0	1·6	12·2
2-^{14}C-Acetic acid	40	10·4	11·4	6·4	2·0	19·8
2-^{14}C-DL-Mevalonic acid	40	48·2	2·9	72·3	2·5	77·7
7-^{14}C-D-Shikimic acid	4·9	0·4	2·1	34·7	2·8	39·6
o-(Succinoyl-4-^{14}C)-benzoic acid carboxyl-^{14}C	1·6	0·4	2·5	82·3	9·4	94·2

Most of the activity after *o*-succinoylbenzoic acid feeding was also recovered in the alcohol. Since *o*-succinoylbenzoic acid was incorporated into the quinones it was obviously not inactivated. Rather, it is assumed that the products of *o*-succinoylbenzoic acid are inert as opposed to shikimic and acetic acid (Table 4).

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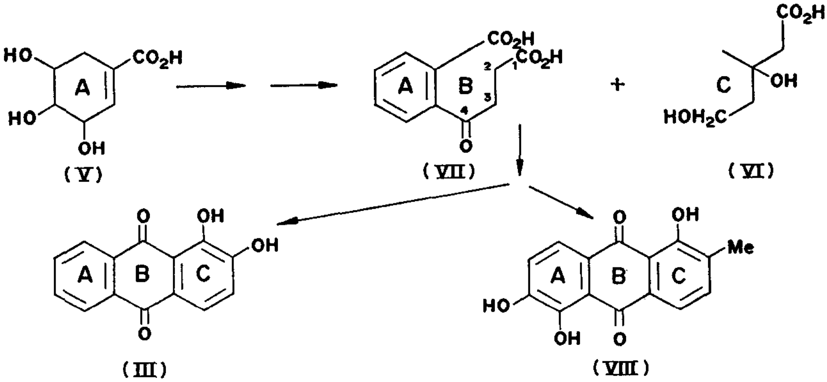
[11] ROBINS, D. J., CAMPBELL, J. M. and BENTLEY, R. (1970) *Biochem. Biophys. Res. Commun.* **39**, 1081.

[12] BURNETT, A. R. and THOMSON, R. H. (1967) *Chem. Commun.* 1125.

[13] LEISTNER, E. and ZENK, M. H. (1968) *Tetrahedron Letters* 1395.

Since ring C of those anthraquinones which are derived from *o*-succinoylbenzoic acid originates from mevalonic acid,[3,12,13] there can be little doubt, that ring C of morindone is also derived from this precursor. The position of the β-methyl-carbon attached to ring C indicates that C-5 of mevalonic acid is attached to C-14 of the morindone skeleton which would correspond to C-3 of the hypothetical 1,4-dihydroxy-2-naphthoic acid. Such a mechanism has been shown to occur in the case of alizarin biosynthesis[3] in *Rubia tinctorum*. The minute activity in morindone after 1-^{14}C-acetate feeding and the somewhat higher incorporation of 2-^{14}C-acetate (Table 2) has also been observed during incorporation experiments with alizarin.[3] Some of the activity from 1-^{14}C and 2-^{14}C acetate is incorporated into ring C via mevalonic acid, whereas activity from 2-^{14}C-acetate also enters C-13 and/or C-14 of the anthraquinone skeleton by way of α-keto-glutaric acid.

The specific incorporation of *o*-succinoylbenzoic acid into morindone shows that the hydroxy groups attached to ring A are not derived from the hydroxy groups of shikimic acid (V) (Scheme 3). The hydroxy groups are introduced at a later stage of the biosynthetic pathway. The same applies to juglone (5-hydroxy-1,4-naphthaquinone). *o*-Succinoylbenzoic acid has also been incorporated into juglone[10] indicating that the hydroxy group is not derived from shikimic acid.



SCHEME 3. BIOSYNTHETIC FORMATION OF RINGS A, B AND C OF ANTHRAQUINONES.

The biosynthesis of morindone and alizarin in sterile cell-suspension culture shows that no microorganisms are involved in anthraquinone biosynthesis in *Morinda citrifolia*.

EXPERIMENTAL

Plant material and feeding technique. Plants of *Morinda citrifolia* L. were grown from seeds in the greenhouse. 1-yr-old plants were used for application of labelled precursors by the wick technique.[6] After 6–8 hr the tracer solution (0·5 ml) had been taken up. The wick was kept in contact with H_2O throughout the incubation time (48 hr). Labelled compounds were also added to cell-suspension cultures 14–19 days after inoculation when the cells started enhanced anthraquinone production.[7] The feeding was stopped 3–5 days after addition of labelled material.

Isolation and purification. When intact plants were fed, only the roots were worked up. The shoots did not contain any morindone or morindin (i.e. morindone glycoside). The roots were sliced and extracted with boiling EtOH as was the filtered and washed plant material from cell-suspension cultures. Morindone and alizarin were isolated as the glycosides on Whatman No. 3 paper (*n*-BuOH–HOAc–H_2O, 2:1:1). A band (R_f 0·5–0·9) containing morindin and alizarin glycoside was cut out and eluted. The eluate was evaporated and the residue hydrolyzed in H_2SO_4 (2%) for 8 hr at 100°C. Anthraquinones were extracted from the hydrolyzate with Et_2O. The Et_2O was washed, dried and evaporated. The residue was chromatographed on silica gel plates. The silica gel was mixed with tartaric acid.[3] Morindone R_f 0·59, alizarin R_f 0·55. The anthraquinones were rechromatographed on cellulose-plates (tetrahydrofuran -EtOAc–H_2O, 35:6:47) R_f morin- done 0·55, alizarin 0·75). Finally, the morindone was passed over a column (1 × 12 cm) of Sephadex LH20. The anthraquinone was eluted with (70%) MeOH.

Degradation of alizarin and morindone. The isolated anthraquinones were mixed with 30 mg (morindone) or 50 mg (alizarin) carrier material and recrystallized from HOAc. The anthraquinones were methylated[3] (morindone trimethylether m.p. 276–279°; alizarin dimethylether m.p. 216–8°) and submitted to cleavage with K *t*-butoxide.[3] The products of degradation were isolated and purified as described previously.[3]

Decarboxylation of veratric acid. Veratric acid (100 μM) was dissolved in quinoline (5 ml), copper powder (18·2 mg) was added and the reaction mixture heated to 230–60° the CO_2 was swept with a stream of N_2 into an aq., saturated solution of $Ba(OH)_2$. The $BaCo_3$ formed was collected (yield 50%) and the activity measured in a scintillation counter in a suspension of 'liquifluor' and thixotropic gel powder.

GLC of anthraquinones. Plant material taken from cell suspension cultures was washed and extracted with EtOH (80%) until the plant material was colourless. The EtOH was filtered, evaporated to dryness and the residue dissolved in M HCl. The glycosides were hydrolyzed for 5 hr at 100°. The aglycones were extracted continuously into Et_2O for 9·5 hr. The Et_2O was evaporated to dryness, the residue dried and bis-trimethylsilylacetamide (BSA) (0·1 ml) as well as dry dioxan (0·1 ml) were added. After 10 min the fractions were used for GLC (Varian Aerograph 1860–3, equipped with a flame ionization detector; column: 122 cm × 3·2 mm o.d.s.s., 5% QF-1 on Chromosorb WAW, DMGS, mesh 80–100, N_2 24 psi at 240°; inlet and detector temp. 240°; initial column temp. 150°, increasing (after 5 min) to 170° at 4° per min and to 244° at 6° per min). The following retention times, identical with those of authentic specimens, were observed; alizarin 21·3 min; morindone 23·9 min; damnacanthal 27·3 min.

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