Phytochemistry, 1971, Vol. 10, pp. 3015 to 3020. Pergamon Press. Printed in England.

A SECOND PATHWAY LEADING TO ANTHRAQUINONES IN HIGHER PLANTS*

E. LEISTNER

Institut fur Pflanzenphysiologie der Ruhr Universitat Bochum, West Germany

(*Received* 19 *January* 1971)

Abstract—The biosynthesis of chrysophanol (1,8-dihydroxy-3-methylanthraquinone) and emodin (1,6,8-trihydroxy-3-methylanthraquinone) has been studied in *Rhamnus frangula* and *Rumex alpinus*. Degradation of both anthraquinones after feeding 1-^{14}C-acetate and 2-^{14}C-acetate showed that these compounds are derived from acetate by linear combination. ^{14}C-Shikimate and ^{14}C-mevalonate were not incorporated.

INTRODUCTION

ALIZARIN (I) and purpurincarboxylic acid (II) are anthraquinones which are not hydroxylated in ring A. They occur in *Rubia tinctorum*,[1] a higher plant. Biosynthetically these anthraquinones are derived from shikimate[2] and mevalonate.[3,4] Chrysophanol (III) and emodin (IV) are hydroxylated in ring A and occur not only in higher plants but also in fungi.[1] Fungi are known to form these anthraquinones by linear combination of acetate.[5] It has been reported, however, that in higher plants chrysophanol arises from mevalonate and shikimate.[6]

We have reinvestigated the biosynthesis of these anthraquinones in higher plants. Our results show that higher plants contain two distinct pathways for anthraquinone-biosynthesis.

I Alizarin, R_2, R_3=H
II Purpurincarboxylic acid, R_2=COOH, R_3=OH
III Chrysophanol, R_1=H
IV Emodin, R_1=OH

SCHEME I. TWO TYPES OF ANTHRAQUINONES OCCURRING IN HIGHER PLANTS.

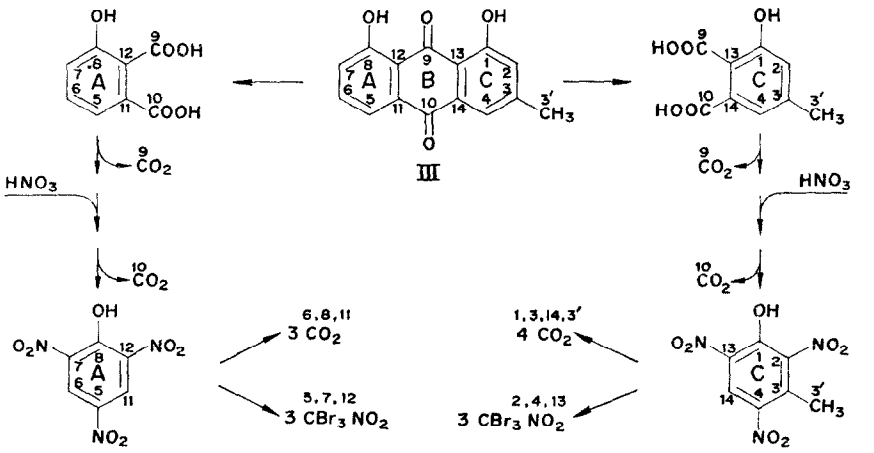
RESULTS

Radioactively labelled potential precursors were administered to intact plants or cuttings (roots, leaves, etc.) (Tables 1 and 2), of four plant species known to contain emodin and chrysophanol. In each case, the plant material was extracted and the anthraquinones purified to constant specific activity by thin-layer and paper-chromatography (Table 3).

In Table 2, experiments are listed which were conducted in order to confirm the reported precursor-product relationship between shikimate or mevalonate and chrysophanol.[6] Leaves and roots of *Rheum* and *Rumex* (Polygonaceae) as well as branches of *Rhamnus* (Rhamnaceae) were incubated with ^{14}C-labelled precursors by various techniques. However,

* A preliminary account of part of this work has been published: E. LEISTNER and M. H. ZENK, *Chem. Commun.* 210 (1969).

[1] R. H. THOMSON, *Naturally Occurring Quinones*, Butterworths, London (1957).
[2] E. LEISTNER and M. H. ZENK, *Z. Naturforsch* **22b**, 865 (1967).
[3] E. LEISTNER and M. H. ZENK, *Tetrahedron Letters* 1395 (1968).
[4] A. R. BURNETT and R. H. THOMSON, *Chem. Commun.* 1125 (1967).
[5] R. H. THOMSON, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 309, Academic Press, London (1965).
[6] A. MEYNAUD, A. VILLE and H. PACHECO, *Compt. Rend.* **266D**, 1783 (1968).



SCHEME II. DEGRADATION OF CHRYSOPHANOL.

in every case negligible incorporation of ^{14}C-shikimate or ^{14}C-mevalonate into emodin or chrysophanol was observed (Table 2). On the other hand 1-^{14}C-acetate and 2-^{14}C-acetate were well incorporated into emodin and chrysophanol in *Rhamnus frangula* and *Rumex alpinus* (Table 1). For example, incorporation of up to 0·35% was observed after feeding acetate 1-^{14}C to *Rumex alpinus*, the emodin isolated had a specific activity of 857,000 dis/min/μM (Table 1).

Kuhn–Roth-oxidation[7] of emodin and chrysophanol yielded acetic acid (Table 4) corresponding to C-3 and C-3′ of the anthraquinones. The specific activity of acetic acid was shown to be slightly higher than the average specific activity of the C_2 units forming the anthraquinones (Table 4). As expected, either the carboxyl or the methyl-group of the Kuhn–Roth-acetate was radioactive after feeding, respectively, acetate-1 or 2-^{14}C.

TABLE 1. RESULTS OF FEEDING OF $^{14}CO_2$, ^{14}C-ACETATE AND ^{14}C-MALONATE TO *Rumex* AND *Rhamnus*

Plant	Feeding technique	Feeding time (hr)	Precursor administered	μM	μC/M	Anthraquinone Emodin(E) Chryso-phanol(C)	Isolated μM	Spec. Activity dis/min/μM	Incorporation (%)
Rumex alpinus	Assimilation of $^{14}CO_2$ by leaves (3·2 g)	2	$^{14}CO_2$	—	—	C	7·16	1,300	—
	Excised leaves (2·9 g) feeding in darkness	24	1-^{14}C-Acetate	5·0	40·0	C	0·99	235,400	0·06
						E	0·36	857,000	0·07
	Excised leaves (6 1 g) feeding in darkness	24	2-^{14}C-Acetate	2·63	38·0	C	3·01	161,100	0·22
						E	0·2	371,500	0 07
	Sliced roots (3·05 g) shaken in water (10 ml) with penicillin (0·5 g)	24	2-^{14}C-Acetate	50·0	1·3	C	26·8	22 0	0 0004
Rhamnus frangula	Excised branches (12 g)	24	1-^{14}C-Acetate	1·25	40·0	C	0·62	18,320	0 01
						E	2·85	2,580	0 007
	Excised branches (15 g)	24	2-^{14}C-Acetate	2·63	38·0	C	2·48	125,000	0·352
						E	2·72	66,900	0·206
	Excised branches (16 g)	24	2-^{14}C-Malonic acid	1·65	12·1	C	1·18	39,350	0·104
						E	1·78	57,980	0·232

Per cent incorporation is: total activity in the anthraquinone divided by total activity fed, × 100.

[7] R. KUHN and H. ROTH, *Ber.* **66**, 1274 (1933).

TABLE 2. RESULTS OF FEEDING OF ^{14}C-LABELLED PRECURSORS OF ALIZARIN TO PLANT MATERIAL CONTAINING A-RING HYDROXYLATED ANTHRAQUINONES

Plant	Feeding technique	Feeding time (hr)	Labelled compound administered		Incorporation of activity (%) into Anthraquinones	
			μM	μC/μM	Emodin	Chrysophanol
Rheum officinale	Excised leaves (1·2 g)	24	1,2-^{14}C-DL-Shikimic acid 0·5	8·7	0	0
	Leaf discs (1·2 g) shaken in water (20 ml)	25	1,2-^{14}C-DL-Shikimic acid 0·62	8·7	0	—
Rheum palmatum	Sliced roots (5·5 g) shaken in water (12 ml)	24	1,2-^{14}C-DL-Shikimic acid 1·15	8·7	0	0
	Intact excised root (9·1 g) fed through cut end	24	1,2-^{14}C-DL-Shikimic acid 0·62	8·7	0	0
Rumex alpinus	Excised leaves (3·0 g)	5·5	1,2-^{14}C-DL-Shikimic acid 0·58	8·7	0·004	0·004
	Excised leaves (2·2 g)	25	2-^{14}C-DL-Mevalonic acid 2·0	5·03	—	0
Rhamnus frangula	Excised branches (18 g)	24	7-^{14}C-D-Shikimic acid 0·4	20	0·001	0·001
	Excised branches (21 g)	24	2-^{14}C-DL-Mevalonic acid 2·11	4·8	0	0

In order to establish the labelling pattern of the whole chrysophanol molecule, a new degradation procedure was devised (Scheme II). Chrysophanol was oxidized by alkaline hydrogen peroxide solution yielding 3-hydroxyphthalic-acid (ring A) and 3-hydroxy-5-methylphthalic acid (ring C) which were separated chromatographically (Table 3). The ratio of the specific activities of ring A and ring C were 0·96 (theory < 1·0) after 1-^{14}C-

TABLE 3. CHROMATOGRAPHIC SYSTEMS USED FOR THE PURIFICATION OF EMODIN AND CHRYSOPHANOL AND THEIR OXIDATION PRODUCTS

Support	Solvents, v/v		Emodin	Chryso phanol	R_f-values 3-hydroxy-phthalic acid	3-Hydroxy-5-Methyl-phthalic acid
Silica Gel GF (Merck)	Benzene Ethyl formate Formic acid	75 24 1	0·58	0·87		
	Benzene Acetic acid	8 2	0·74	0·92	0·50	0·53
	Chloroform Ethylacetate Formic acid	5 4 1	0·80	0·69		
	Toluene Ethyl formate Formic acid	5 4 1			0·40	0·42
Chromatography paper (Schleicher and Schüll 2045b)	Isopropanol Ammonia Water	8 1 1	0·55	0·58	0·22	0·28
	Formic acid Water	2 98			0·78	0·84

TABLE 4. KUHN–ROTH OXIDATIONS AND SCHMITT DEGRADATIONS OF ANTHRAQUINONES

Plant	Radioactive substrate	Isolated anthraquinone and specific activity (dis/min/μM)*	Acetic acid ($C_3 + C_3'$) spec act. (dis/min/μM)		$-COOH$ (C_3) spec. act. (dis/min/μM)	
			Found	Calculated	Found	Calculated
Rhamnus frangula	2-^{14}C-Acetate	Emodin 200·0	33·5	>25·0	2·2	0
Rumex alpinus	2-^{14}C-Acetate	Chrysophanol 191·5	28·4	>23·9	1·2	0
Rumex alpinus	1-^{14}C-Acetate	Chrysophanol 122·5	19·0	>17·5	17·2	17·5

* After dilution with carrier.

acetate and 0·76 (theory < 0·8) after 2-^{14}C-acetate feeding, indicating an equal distribution of the radioactivity from ^{14}C-acetate in the chrysophanolmolecule (cf. Table 5).

Further degradation of ring A and ring C was conducted in a way which permitted determination of the specific activity of individual C-atoms. The main degradation products of chrysophanol are shown in Scheme II and Table 5 shows the results of the degradation. It can be seen that the label enters into alternate carbon atoms in the samples derived from each ^{14}C-acetate feeding. In the sample obtained from the experiment with carboxyl-^{14}C-acetate, C-atoms 6,8 and 11 contained 48·3% of the activity of the whole molecule (theory 42·8%), but only 3·3% (theory 0%) was present in these C-atoms after methyl-^{14}C-acetate feeding. C-atoms 2, 4 and 13 amount for 2·2% activity (theory 0%) after carboxyl-^{14}C-acetate feeding, but for 37·5% (theory 41·1%) after methyl-^{14}C-acetate feeding.

DISCUSSION

Acetate is incorporated into alizarin[8] as well as chrysophanol and emodin (Table 1). However, radioactivity from acetate enters only ring C and the keto-C-atoms of alizarin, whereas radioactivity from ^{14}C-acetate is found to be equally distributed in chrysophanol (Table 5). This indicates a different mode of incorporation of ^{14}C-acetate into structurally different types of anthraquinones. The labelling of alizarin after ^{14}C-acetate-feeding can be

TABLE 5. PER CENT DISTRIBUTION OF RADIOACTIVITY IN CHRYSOPHANOL AFTER 1-^{14}C AND 2-^{14}C-ACETATE FEEDING

	1-^{14}C-Acetate		2-^{14}C-Acetate	
C-atoms	% Calculated	% Found	% Calculated	% Found
9	14·3	13·2	0	2·9
10	0	1·6	12·5	10·8
6, 8, 11	42·8	48·3	0	3·3
5, 7, 12	0	2·3	37·5	30·8
1, 3, 14, 3′	42·8	47·2	12·5	12·5
2, 4, 13	0	2·2	37·5	41·1

[8] E. LEISTNER and M. H. ZENK, *Tetrahedron Letters* 475 (1967).

explained by the finding that ring C of this anthraquinone is derived from mevalonic acid[9] which itself originates from acetate.

The alternate labelling of the chrysophanol molecule after 1-^{14}C and 2-^{14}C-acetate feeding strongly supports the view that acetate units are linked together by way of acetyl-CoA and malonyl-CoA. Cyclization of the polyketide unit most probably gives rise to an anthraquinone precursor which in several steps[5] is transformed to chrysophanol.

As indicated in Table 4, specific activity at C-3 and C-3′ of emodin and chrysophanol is slightly higher than one eighth (2-^{14}C-acetate) or one-seventh (1-^{14}C-acetate) of the total activity. This is consistent with a polyketide route[10]: C-atoms 3 and 3′ are directly derived from the 'starter'–acetyl–CoA molecule, whereas the radioactivity enters the remaining carbon skeleton by way of the malonyl–CoA pool and is thus further diluted.

Although the data presented in Table 2 are only negative, they indicate but do not prove that ^{14}C-shikimate and ^{14}C-mevalonate are not precursors of chrysophanol and emodin. The reported incorporation of ^{14}C-shikimate into chrysophanol[6] in *Rheum rhaponthicum* was actually nonspecific since only 23% (expected 100%) of the activity of the whole molecule was found in hydroxyphthalic acid which represents ring A of chrysophanol,[6] whereas, in alizarin the radioactivity was confined only to ring A after 1,2-^{14}C-shikimate feeding.[3] The possibility cannot be excluded that radioactivity from shikimic acid entered chrysophanol by way of phenylalanine and homogentisic acid. These are possibly catabolized to acetate which is now known to be an immediate precursor of chrysophanol.

A competition experiment which was designed by Meynaud *et al.*[6] to prove that mevalonic acid is a precursor of chrysophanol adds further evidence to the suggestion[11] that competition experiments are not reliable in biosynthetic studies as long as their interpretation is based on incorporation rates only.

The strictly alternate labelling pattern of chrysophanol after 1-^{14}C-and 2-^{14}C-acetate-feeding, the results of the Kuhn–Roth-oxidations and the equal distribution of radioactivity can only be explained by a polyacetate route which operates in *Rumex* as well as *Rhamnus* to produce A-ring hydroxylated anthraquinones like emodin and chrysophanol.

EXPERIMENTAL

Plant material and feeding techniques. The plant material was obtained from the Botanical Garden, Munich, Germany. Leaves and roots of *Rheum* andbr anches of *Rhamnus* were taken from outdoors just prior to feeding. *Rumex* was grown in the greenhouse. Tracers fed to aerial parts of Polyganaeceae (*Rheum, Rumex*) were administered to young expanding leaves. Unless otherwise stated in Table 1 feedings were carried out under normal light conditions. Feeding techniques are given in Tables 1 and 2.

m ^{14}C-*Labelled compounds.* 1-^{14}C-Acetate, 2-^{14}C-acetate, 2-^{14}C-malonic acid, $Ba^{14}CO_3$ and 2-^{14}C-DL-evalonolactone were purchased from the Radiochemical Centre, Amersham, 1,2-^{14}C-DL-shikimic acid from CEA-France. 7-^{14}C-D-shikimic acid was synthesized by Dr. K. H. Scharf, of this laboratory, by a biochemical method.[12]

Isolation and purification of anthraquinones. After feeding ^{14}C-labelled compounds to *Rheum* or *Rumex* the plant material was macerated and refluxed for 1 hr in a mixture of conc. H_2SO_4 (1 ml) and acetone (100 ml). The extraction was repeated twice for shorter times and the filtered extracts combined. A threefold excess of H_2O was added and the anthraquinones extracted repeatedly into benzene. The combined benzene extracts were extracted with 5% KOH. The alkaline extract was acidified and the anthraquinones were extracted into Et_2O. The Et_2O was washed with $(NH_4)_2CO_3$ (5%) and H_2O and evaporated to dryness.

Incubated branches of *Rhamnus frangula* were peeled, the heartwood was broken into pieces and the leaves discarded. Cortex and heartwood were extracted repeatedly with boiling EtOH (80%). The extracts were

[9] E. LEISTNER and M. H. ZENK, unpublished results.
[10] A. J. BIRCH, *Proc. Chem. Soc.* 3 (1962).
[11] M. H. ZENK and E. LEISTNER, *Z. Naturforsch.* **22b**, 460 (1967).
[12] K. H. SCHARF and M. H. ZENK, in preparation.

evaporated, the residue dissolved in N H_2SO_4 and the solution heated to 100°. The anthraquinones were extracted into Et_2O and the Et_2O solution washed with $(NH_4)_2CO_3$ (5%) and H_2O, dried, and evaporated.

The anthraquinones were purified chromatographically (Table 3) and the concentration was determined spectrophotometrically after elution with ethanol. Chrysophanol, E_{434} nm = $6{\cdot}75 \times 10^6$ cm²/mol; emodin E_{428} nm = $2{\cdot}895 \times 10^6$ cm²/mol.

Degradation of chrysophanol to substituted phthalic acids. Purified ^{14}C-chrysophanol was mixed with a solution of carrier material (50 mg) in MeOH. The solution was evaporated; the residue was taken up in N NaOH (12·5 ml). The chrysophanol was oxidized with H_2O_2 according to a procedure employed for the degradation of juglone.[13] After the reaction had been completed the mixture was acidified and the 3-hydroxyphthalic acid and 3-hydroxy-5-methylphthalic acid were extracted into Et_2O. The Et_2O was evaporated and the acids separated chromatographically (Table 3). The acids were eluted with EtOH (80%) and their concentrations were determined spectrophotometrically. 3-Hydroxyphthalic acid (E_{323} nm = $2{\cdot}961 \times 10^6$ cm²/mol), yield 2·9 mg, 8%; 3-Hydroxy-5-methylphthalic acid (E_{323} nm = $4\,50 \times 10^6$ cm²/mol) yield 3·7 mg, 9·5%.

Decarboxylation of phthalic acids. Degradation of 3-hydroxyphthalic acid (C-atoms 5–12 of chrysophanol) was carried out according to Gatenbeck.[14] The acid was decarboxylated yielding C-atom 9 as $BaCO_3$. The *m*-hydroxybenzoic acid (C-atoms 5–8, 10–12) was nitrated and decarboxylated yielding C-atom 10 as $BaCO_3$. The resulting picric acid (C-atoms 5–8, 11 and 12) was submitted to bromopicrin-cleavage, bromopicrin representing C-atom 5, 7 and 12 and $BaCO_3$ representing C-atoms 6, 8 and 11 of chrysophanol.

3-Hydroxy-5-methylphthalic acid (3·7 mg) (ring C) was refluxed in 50% H_2SO_4 (10 ml) for 25 min yielding 3-hydroxy-5-methylbenzoic acid (C-atoms 1–4, 10, 13, 14, 3′). The reaction mixture was diluted with H_2O and the organic acid extracted into Et_2O. 3-Hydroxy-5-methylbenzoic acid was purified by paper chromatography (isopropanol–ammonia–water, 8:1:1, R_f = 0·46 and 2% formic acid, R_f = 0·58) The acid was eluted with EtOH (80%) and the concentration determined spectrophotometrically (E_{300} nm = $2\,868 \times 10^6$ cm²/mol) yield 2·5 mg 72% The identity of the 3-hydroxy-5-methylbenzoic acid was checked by comparison with an authentic sample synthesized according to Meldrum and Perkin.[15] UV-spectra and R_f-values in nine different solvent systems were identical.

Nitration and decarboxylation of 3-hydroxy-5-methylbenzoic acid. To the ^{14}C-3-hydroxy-5-methylbenzoic acid carrier (120 mg) was added and the acid dissolved in conc. H_2SO_4 (4·8 ml), the solution was chilled to 0° and cold 68% HNO_3 (4 ml) added dropwise. The mixture was stirred at room temp. for 2 hr and the collected precipitate washed (H_2O at 0°). The yield of 3-hydroxy-5-methyl-2,4,6-trinitrobenzoic acid was 90 mg, 40%; m.p. 191° (Lit. 180°). The product was chromatographically homogenous (Found: C, 33·38, H, 1·88; N, 14·43; O, 50·16. Calc. for $C_8H_5N_3O_9$. C, 33 45; H, 1·75; N, 14 60; O, 50·2%.) 3-Hydroxy-5-methyl-2,4,6-trinitrobenzoic acid (C-atoms 1–4, 10, 13, 14 and 3′) (90 mg) was decarboxylated by refluxing in glycerol (5 ml) for 25 min. The liberated CO_2 representing C-atom 10 of chrysophanol was flushed with N_2 into a receiving vessel containing $Ba(OH)_2$ (5 ml) $BaCO_3$ yield 58 mg, 94%. The reaction mixture was diluted with a fourfold excess of H_2O and acidified. The 2,4,6-trinitro-*m*-cresol was extracted into Et_2O. The Et_2O was evaporated and the residue crystallized from H_2SO_4 (2N) The yield of 2,4,6-trinitro-*m*-cresol was 62 mg. 81%; m.p. 106° (lit. 106°) The 2,4,6-trinitro-*m*-cresol was compared with an authentic sample prepared according to Birch *et al*[16] The IR and UV spectra and R_f-values, in two different solvents, were identical.

Bromopicrin-cleavage of ring C. Baryta (50 ml containing 5 6% $Ba(OH)_2$ and 10% of $BaCl_2$) was chilled to 0° under N_2 and Br_2 (0·32 ml) added. In this solution, 2,4,6-trinitro-*m*-cresol (50 mg) was suspended and the mixture stirred for 1 hr. H_2O (15 ml) was added and the bromopicrin separated by steam-distillation, and washed with dil. HCl and H_2O. An IR spectrum showed the absence of impurities.[17] The bromopicrin was combusted[18] giving $BaCO_3$ (yield 24 3 mg, 20%) representing C-atoms 2,4 and 13 of chrysophanol. The $BaCO_3$ remaining in the original mixture was collected, washed (H_2O) and the CO_2 liberated with $HClO_4$ (10%) and retrapped in baryta. The $BaCO_3$ (yield 113 mg, 70%) represents C-atom 1,3,3′ and 14 of chrysophanol.

Melting points are uncorrected. Elementary analysis by Dr F. Pascher, Bonn, Germany.

Counting of radioactivity. The radioactivity of the $BaCO_3$ was counted as reported previously.[3]

Acknowledgements—The author is grateful to Miss C. Kalteis and Mrs. M. Fuerbringer for excellent technical assistance. This work has been supported by a grant from the Bundesminister fuer wissenschaftliche Forschung.

[13] E. LEISTNER and M. H. ZENK, *Z. Naturforsch.* **23b,** 259 (1968).
[14] S. GATENBECK, *Acta. Chem. Scand.* **12,** 1985 (1958).
[15] A. N MELDRUM and W. H. PERKIN, *J. Chem. Soc.* **95,** 1889 (1909).
[16] A J. BIRCH, R. A. MASSY-WESTROPP, and C. J. MOYE, *Austral. J. Chem.* **8,** 539 (1955).
[17] A J BIRCH, C. J. MOYE, R. W. RICKARDS, and Z. VANEK, *J. Chem. Soc.* 3586 (1962).
[18] S. ARONOFF, *Techniques of Radiobiochemistry*, p 44, The Iowa State College Press, Ames (1956).