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THE HOMOGENTISATE RING-CLEAVAGE PATHWAY IN THE BIOSYNTHESIS OF ACETATE-DERIVED NAPHTHOQUINONES OF THE DROSERACEAE*

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Key Word Index—*Drosophyllum lusitanicum*, Droseraceae, plumbagin, 2-methyl-5-hydroxy-1,4-naphthoquinone, biosynthesis, cell cultures, homogentisate pathway, ring-cleavage

Abstract—Photosynthesis experiments with $^{14}CO_2$ established that of 16 Droseraceae species tested *Drosophyllum lusitanicum* incorporated the highest amount of label into plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) Tyrosine-[β-^{14}C] fed to *Drosophyllum* was shown to label plumbagin efficiently (20% incorporation) Extensive chemical degradation of the labeled naphthoquinone showed, however, that the incorporation of tyrosine was indirect, the label being distributed throughout the molecule It was established that plumbagin and the closely related 7-methyljuglone are biosynthesized via the acetate–polymalonate pathway Tyrosine is broken down to acetate in this tissue via the homogentisate pathway, which was demonstrated by feeding and incorporation of label into plumbagin of intermediates such as homogentisate-[^{14}C], maleyl- and fumarylacetoacetate-[^{14}C] Simultaneous application of tyrosine-[β-^{14}C] and α,α'-bipyridyl, an inhibitor of the homogentisate oxigenase, led to an accumulation of homogentisate-[^{14}C] within the tissue The degradation of tyrosine to acetate by *Drosophyllum* is not due to epiphytic bacteria since ring cleavage of tyrosine and formation of plumbagin from breakdown products occurred both within sterile grown plants and sterile cell suspension cultures In tissue kept in darkness, plumbagin undergoes a slow turnover with a half life of about 400 hr

INTRODUCTION

THREE different pathways are presently known in higher plants for the formation of the naphthoquinone carbon skeleton. Naphthoquinones are formed either by direct incorporation of shikimate[1] via *o*-succinylbenzoic acid,[2] by the toluhydroquinone[3] pathway, or by the *p*-hydroxybenzoate–mevalonate pathway.[4,5] The naphthoquinones plumbagin (**1**) and 7-methyljuglone (**2**) both occur in *Drosera*[1] and can be used as chemotaxonomic markers in this genus.[6] The possible biosynthetic routes to plumbagin in *Drosera* offered some interesting alternatives. It could be visualized as either a *C*-methylation[7] of juglone (**3**) in a manner reminiscent of vitamin K_2 biosynthesis,[8] or as a demethylation and hydroxylation of shikimate[1] via *o*-succinylbenzoic acid[2], by the toluhydroquinone[3] pathway, or by the two possibilities, feeding experiments with ^{14}C-labeled precursors were carried out. If

* A preliminary account of part of this work has been published in *Tetrahedron Letters* **1971**, 3009

[1] THOMSON, R H (1971) *Naturally Occurring Quinones*, Academic Press, London

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

[3] BOLKART, K H and ZENK, M H (1969) *Z Pflanzenphysiol* **61**, 356

[4] SCHMID, H V and ZENK, M H (1971) *Tetrahedron Letters* **44**, 4155

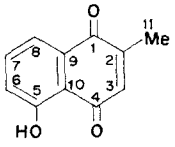
[5] ZENK, M H (1972) *Hoppe-Seyler's Z Physiol Chem* **353**, 123

[6] ZENK, M H, FURBRINGER, M and STEGLICH, W (1969) *Phytochemistry* **8**, 2199

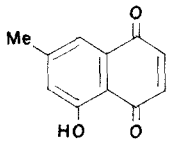
[7] TEUSCHER, E (1970) *Pharmakognosie*, Vol II, pp 245–246, Akademie, Berlin

[8] JACKMAN, L M, O'BRAIN, J G, COX, G B and GIBSON, F (1967) *Biochim Biophys Acta* **141**, 1

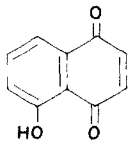
plumbagin is formed via juglone, shikimate-[7-^{14}C] should be incorporated[9] and the 2-methyl group would probably be derived from methionine-S-[^{14}Me] On the other hand, if the biosynthesis proceeds via the intermediate formation of chimaphilin, tyrosine-[β-^{14}C] should be incorporated while mevalonate-[2-^{14}C] should not, since this carbon atom represents the C-7-methyl group of chimaphilin which would be lost during the transformation of chimaphilin to plumbagin



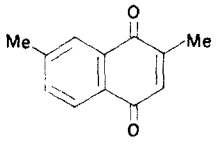
Plumbagin (1)



7-Methyljuglone (2)



Juglone (3)



Chimaphilin (4)

RESULTS

For the selection of the most suitable experimental material, it was necessary to survey the biosynthetic capacity of different *Drosera* species for the formation of the quinones plumbagin and 7-methyljuglone Detached shoots or leaves of plants were exposed to $^{14}CO_2$ and photosynthesis was allowed to proceed for a period of 24 hr. The naphthoquinones were isolated by ether extraction and subsequently purified to constant specific activity by procedures already published [6] As shown in Table 1, $^{14}CO_2$ labels the naphthoquinones present in these plants and the incorporation varies from as little as 0·02% in *D binata* to about 5% in young unrolling leaves of *D lusitanicum* This latter plant was therefore chosen for all subsequent experiments on the biosynthesis of plumbagin while *D capensis* was found to be more suited for the studies on the biosynthesis of 7-methyljuglone Plumbagin occurs in *Drosophyllum* as the free quinone in the form of long needlelike crystals No naphthohydroquinone glucoside could be detected The yield of plumbagin from this plant ranged from 1·8 to 3·3 μmol per leaf (13–27 μmol/g fr wt) The extent of

TABLE 1 INCORPORATION OF $^{14}CO_2$ (SP ACT 1·14 × 10^7 dpm/μmol, TOTAL ACT 1·1 × 10^8 dpm) INTO PLUMBAGIN OR 7-METHYLJUGLONE IN YOUNG SHOOTS OR LEAVES OF DIFFERENT SPECIES OF *Drosera* AFTER 24 HR PHOTOSYNTHESIS (11 000 lx)

Species	Plant organ	Quinone found	Incorp (%)	Sp act (dpm/μmol)	Dilution
Drosera aliciae Hamet	Shoot	7-Methyljuglone	0·56	1·0 × 10^6	11
D auriculata Planch	Shoot	Plumbagin	0·16	3·2 × 10^5	35
D binata Labill	Shoot	Plumbagin	0·02	2·7 × 10^4	422
D burkeana Planch	Shoot	7-Methyljuglone	0·27	1·2 × 10^6	9
D capensis L	Shoot	7-Methyljuglone	0·99	1·9 × 10^6	60
D capillaris Poir	Shoot	Plumbagin	0·10	2·7 × 10^5	42
D dichotoma Smith	Shoot	Plumbagin	0·06	1·4 × 10^4	814
D erythrorhiza Planch	Shoot	Plumbagin	0·13	1·1 × 10^5	103
D intermedia Hayne	Shoot	Plumbagin	0·23	1·5 × 10^5	76
D microphylla Endl	Shoot	Plumbagin	0·04	7·2 × 10^5	15
D rotundifolia L	Shoot	Plumbagin	1·05	2·6 × 10^6	4
D spathulata Labill	Shoot	7-Methyljuglone	0·91	8·3 × 10^5	13
D stolonifera Endl	Shoot	Plumbagin	0·05	5·3 × 10^4	215
D tracii Macfarlane	Leaves	7-Methyljuglone	0·16	1·1 × 10^5	103
D whitakeri Planch	Shoot	Plumbagin	0·17	4·6 × 10^4	247
Drosophyllum lusitanicum Link	Leaves	Plumbagin	4·81	4·5 × 10^5	25

[9] LEISTNER, E and ZINK, M H (1968) *Z. Naturforsch.* **23b**, 259

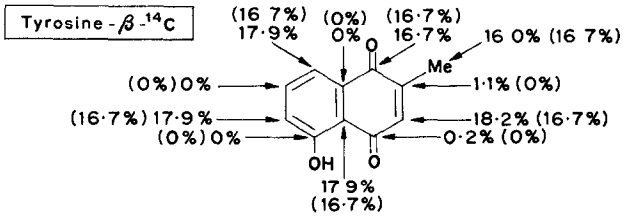
incorporation of labeled precursors into the naphthoquinone was strikingly dependent on the season of the year. Usually, the highest incorporations were obtained during the spring and summer months.

In order to establish the pathway for the biosynthesis of plumbagin a series of ^{14}C-labeled potential precursors was fed to young *Drosophyllum* leaves and the degree of incorporation was measured. Neither shikimate-[7-^{14}C] nor methionine-[^{14}Me] were incorporated to any significant extent (Table 2), thus excluding the possibility that plumbagin is formed by methylation of juglone (Scheme 1a).

TABLE 2 INCORPORATION OF POTENTIAL ^{14}C-LABELED PRECURSORS INTO PLUMBAGIN IN YOUNG LEAVES OF *Drosophyllum lusitanicum* DURING 24 HR IN THE DARK

Precursor applied	(μmol)	Total act. (dpm)	Plumbagin Incorp (%)	Plumbagin Sp act (dpm/μmol)	Dilution
D-Shikimic acid-[7-^{14}C]	0 5	8 4 × 10^6	0 04	2 7 × 10^2	63396
L-Methionine-[^{14}Me]	0 4	5 5 × 10^6	0 06	3 9 × 10^2	35714
Cinnamic acid-[U-^{14}C]	0 4	7 7 × 10^6	0 12	1 8 × 10^4	1064
Cinnamic acid-[ring-U-^{14}C]	0 6	4 6 × 10^6	0 31	1 0 × 10^3	7591
p-Coumaric acid-[2-^{14}C]	1 3	2 0 × 10^6	0 14	8 1 × 10^2	1897
DL-Phenylalanine-[β-^{14}C]	1 0	1 1 × 10^7	0 13	1 3 × 10^4	866
DL-Phenylalanine-[ring-1-^{14}C]	0 4	1 7 × 10^7	0 02	1 8 × 10^3	23352
DL-Tyrosine-[β-^{14}C]	0 7	1 1 × 10^7	20 10	1 4 × 10^5	116
DL-Mevalonic acid-[2-^{14}C]	0 4	1 1 × 10^7	0 04	3 8 × 10^2	72944

None of the differently labeled cinnamic acids, nor phenylalanine, was significantly incorporated, which excludes the *p*-hydroxybenzoic acid pathway.[4] However, DL-tyrosine-[β-^{14}C] showed a remarkably high incorporation (20%), quite unusual for tracer experiments in higher plants. This finding was in agreement with the proposal that plumbagin might be formed via chimaphilin or a similar pathway involving incorporation of tyrosine with the retention of the β-C-atom as the methyl group of plumbagin. However, mevalonic acid-[2-^{14}C] was not incorporated into plumbagin, as might be expected in the above scheme. To examine these results more critically, highly labeled plumbagin was isolated from *Drosophyllum* leaves after application of DL-tyrosine-[β-^{14}C] for 24 hr and subjected



STRUCTURE Distribution pattern of radioactivity within the plumbagin molecule after feeding DL-tyrosine-[β-^{14}C] to *Drosophyllum* leaves Numbers in brackets indicate percentage of label expected if the acetate–polymalonate pathway is operative

to extensive chemical degradation. Kuhn–Roth oxidation was used to determine the radioactivity in carbon atoms C-2 and C-11 and oxidation with alkaline hydrogen peroxide to give 3-hydroxyphthalic acid, which was further degraded according to known procedures.[9] If the toluhydroquinone or a similar pathway is involved in the biosynthesis of plumbagin, all the radioactivity should reside in the methyl carbon atom (C-11) after a tyrosine-[β-^{14}C] feeding experiment.

TABLE 3 DISTRIBUTION OF RADIOACTIVITY IN CHEMICAL DEGRADATION PRODUCTS OF PLUMBAGIN (SP ACT BEFORE DILUTION 1.56×10^8 dpm/mmol, DILUTION 1 47 8, TOTAL ACT 5.0×10^5 dpm) SYNTHESIZED FROM DL-TYROSINE-[β-^{14}C] IN YOUNG LEAVES OF *Drosophyllum lusitanicum*

Degradation product	(dpm/mmol)	DL-Tyrosine-[β-^{14}C] as precursor	
		Theory (%)	Found (%)
Plumbagin (=C-1–C-11)	3.26×10^6	100.0	100.0
C-2 (Kuhn-Roth)	3.60×10^4	0.0	1.1
C-11 (Kuhn Roth)	5.22×10^5	16.7	16.0
3-Hydroxyphthalic acid (=C-1, 4–10)	2.11×10^6	66.6	64.7
C-3 (by difference)	--	16.7	18.2
CO_2 ex 3-Hydroxyphthalic acid (=C-4)	6.00×10^3	0.0	0.2
m-Hydroxybenzoic acid (=C-1, 5–10)	2.10×10^6	66.6	64.5
m-Hydroxybenzoic acid after dilution (1 350)	6.00×10^3	66.6	64.5
CO_2 ex *m*-Hydroxybenzoic acid (=C-1)	1.00×10^3	16.7	16.7
Picric acid (=C-5–10)	5.00×10^3	50.0	53.8
Picric acid after dilution (1 5)	1.00×10^3	50.0	53.8
CO_2 ex picric acid (=C-5, 7, 9)	0	0.0	0.0
CBr_3NO_2 $C_6H_{12}N_4$ (=C-6 8, 10)	1.00×10^3	50.0	53.8

The distribution pattern of the radioactivity within the plumbagin molecule is shown in Table 3. Much to our surprise, C-11 of the plumbagin labeled from tyrosine-[β-^{14}C], contained not 100% but only 16.3% of the total radioactivity in the molecule, almost exactly 1/6. This suggested that the label is distributed on alternative carbon atoms in the molecule and further degradation showed that this is indeed the case. Carbon atoms 1, 3, 6, 8, 10 and 11 of the plumbagin were labeled while carbon atoms 2, 4, 5, 7 and 9 were devoid of radioactivity. This distribution pattern as shown in the structure is reminiscent of the acetate-polymalonate pathway, which is used for the formation of the fungal naphthoquinones mollisin[10] and javanicin.[11] The labeling pattern from tyrosine-[β-^{14}C] in plumbagin indicated that the homogentisate ring-cleavage pathway might be operative in *Drosophyllum*. In this case the β-^{14}C-atom of tyrosine would become C-2 of homogentisate, and after ring-cleavage of this intermediate, C-2 of acetoacetate and finally the methyl group of acetate, if this pathway functions in plants as in animals and microorganisms.[12] To test this hypothesis differently labeled acetate, malonate and acetogenic amino acids were fed to *Drosophyllum*. Indeed, acetate-[1-^{14}C] and [2-^{14}C] as well as malonate-[2-^{14}C] and propionate-[2-^{14}C] are excellent precursors of plumbagin in this tissue (Table 4). Plumbagin derived from an acetate-[1-^{14}C] feeding experiment was subjected to extensive chemical degradation. It was found, that only carbon atoms 2, 4, 5, 7 and 9 were labeled and that between 18 and 21% (theory 20%) of total radioactivity was located in each of these carbon atoms. Plumbagin which was labeled from acetate-[2-^{14}C] gave

[10] BENTLEY, R and GATTENBECK, S (1965) *Biochem* **4**, 1150
[11] GATTENBECK S and BENTLEY, R (1965) *Biochem J* **94**, 478
[12] MEISTER, A (1965) *Biochemistry of the Amino Acids*, Vol II, pp 884–928 Academic Press New York

exactly the same labeling pattern as found after tyrosine-[β-^{14}C] feeding. If tyrosine is being degraded via the homogentisic acid pathway, radioactivity from tyrosine-[1-^{14}C] should not be incorporated into plumbagin since the intermediate *p*-hydroxyphenylpyruvic acid is decarboxylated, releasing the labeled carbon atom as CO_2. This is, in fact, the case (Table 4). The small amount of radioactivity which is found in plumbagin is most likely due to refixation of the $^{14}CO_2$ evolved from the original carboxyl group of tyrosine. A far higher incorporation is found with DL-tyrosine-[α-^{14}C], L-tyrosine-[ring-U-^{14}C] and D-tyrosine-[β-^{14}C]. These results lend much support to the proposal that homogentisic acid is an intermediate between tyrosine and acetate. The Kuhn–Roth degradation of the labeled molecules in this study give results consistent with this assumption. The results also indicate (Table 4) that L-leucine and L-isoleucine are likewise converted to acetate and incorporated into plumbagin. These amino acids are therefore acetogenic in higher plants as well

TABLE 4 INCORPORATION OF POTENTIAL ^{14}C-LABELED ACETOGENIC PRECURSORS INTO PLUMBAGIN IN YOUNG LEAVES OF *Drosophyllum lusitanicum* AFTER 24 HR IN THE DARK

		Plumbagin					
						^{14}C in C-2 + C-11 by Kuhn–Roth degrad	
Precursor applied	(μmol)	Total act	Incorp (%)	Sp act (dpm/μmol)	Dilution	Found (%)	Theory (%)
Acetate-[1-^{14}C]	1 3	1 1 × 10^8	18 90	1 63 × 10^6	519	18 4	20 0
Acetate-[2-^{14}C]	0 9	1 1 × 10^8	19 90	1 82 × 10^6	672	15 7	16 7
Malonate-[2-^{14}C]	0 4	1 1 × 10^7	19 10	4 84 × 10^5	57	—	—
Propionate-[2-^{14}C]	0 5	1 1 × 10^7	14 75	2 58 × 10^5	85	—	—
L-Leucine-[U-^{14}C]	0 5	1 1 × 10^7	3 10	3 54 × 10^4	622	18 6	18 2
L-Isoleucine-[U-^{14}C]	0 5	1 1 × 10^7	2 19	2 49 × 10^4	884	18 4	18 2
DL-Tyrosine-[1-^{14}C]	2 1	1 1 × 10^7	0 02	6 74 × 10^2	7772	1 6	0 0
DL-Tyrosine-[α-^{14}C]	0 5	1 1 × 10^7	13 80	9 71 × 10^4	227	19 4	20 0
D-Tyrosine-[β-^{14}C]	0 5	9 8 × 10^6	16 00	1 34 × 10^4	146	17 1	16 7
DL-Tyrosine-[ring-U-^{14}C]	0 5	2 6 × 10^6	5 75	9 33 × 10^3	557	18 2	18 2

Feeding experiments with acetate-[2-^{14}C] (2 μmol) in the presence of unlabeled malonate (12 μmol) showed that C-2 and C-11 of plumbagin had a significantly higher radioactivity (22%) than in control experiments (Table 4); the reverse was true if malonate-[2-^{14}C] (2 μmol) was fed to differentiated plants in the presence of unlabeled acetate (12 μmol), in this case C-2 and C-11 were less radioactive (10%) than in the control experiments (Table 4). This suggests, that acetate (acetyl-CoA) serves as a starter unit for carbon atoms 2 and 11 during the biosynthesis of plumbagin.

In order to further test the hypothesis that the homogentisate ring-cleavage pathway is operative in *Drosophyllum* a number of suspected intermediates labeled with ^{14}C were prepared from DL-tyrosine-[β-^{14}C] using cell free preparations from rat liver.[13] All intermediates were rigorously purified by preparative paper chromatography. The results of feeding these compounds to *Drosophyllum* are shown in Table 5.

p-Hydroxyphenylpyruvic acid-[3-^{14}C] is surprisingly poorly incorporated (5%) into plumbagin as compared to the more remote precursor tyrosine. However, the low value may reflect the relative lability of this compound or a divergence in metabolism. Homo-

[13] KNOX, W E (1955) *Methods in Enzymologie* (COLOWICK, S P and KAPLAN, N O, eds), Vol II, pp 287–300, Academic Press, New York

TABLE 5 INCORPORATION OF POTENTIAL ^{14}C-LABELLED PRECURSORS INTO PLUMBAGIN IN YOUNG LEAVES OF *Drosophyllum lusitanicum* DURING 24 HR IN THE DARK

Precursor applied	Plumbagin					^{14}C in C-2 + C-11 by Kuhn Roth degrad	
	(μmol)	Total act (dpm)	Incorp (%)	Sp act (dpm/μmol)	Dilution	Found (%)	Theory (%)
p-Hydroxyphenyl-pyruvic acid-[3-^{14}C]	1 9	$7\,7 \times 10^5$	5 60	$1\,7 \times 10^3$	238		-
Homogentisic acid-[2-^{14}C]	0 5	$6\,4 \times 10^5$	20 90	$2\,1 \times 10^4$	609	16 8	16 7
4-Maleylacetoacetic-acid-[2-^{14}C]	0 2	$1\,0 \times 10^5$	29 10	$1\,9 \times 10^3$	263	17 2	16 7
4-Fumarylacetoacetic-acid-[2-^{14}C]	0 2	$1\,0 \times 10^5$	30 90	$2\,0 \times 10^3$	250	16 5	16 7
Maleic acid-[2,3-^{14}C]	0 5	$1\,1 \times 10^7$	5 50	$1\,4 \times 10^4$	1571		
Fumaric acid-[2,3-^{14}C]	2 2	$1\,1 \times 10^7$	4 70	$1\,7 \times 10^4$	809	-	
Fumaric acid-[1 4-^{14}C]	0 8	$9\,0 \times 10^6$	0 05	$2\,6 \times 10^2$	42939		
Ethylacetoacetic acid-[3-^{14}C]	0 9	$8\,8 \times 10^6$	8 20	$3\,8 \times 10^4$	231		-

gentisic acid-2-^{14}C, the central intermediate in the pathway, showed an incorporation which was about equal to that of tyrosine and was surpassed only by those of the aliphatic intermediates 4-maleyl- and 4-fumarylacetoacetic acid which should be formed after the oxidative cleavage of the aromatic ring of homogentisic acid. For higher plants 30% is a rather high rate of incorporation and one is forced to assume that both isomers are directly involved in the degradation of tyrosine to acetate. Maleic and fumaric acid-[2,3-^{14}C] when fed to *Drosophyllum* leaves showed only 1/6 of the extent of incorporation of the foregoing compounds Maleic acid could be formed possibly from maleylacetoacetic acid, while fumaric acid is most likely formed according to the classical homogentisic acid ring-cleavage pathway. The low incorporation could be a consequence of the compartmentation of either the tyrosine degradation or the plumbagin synthesis Fumaric acid-[1,4-^{14}C] is only slightly incorporated into the naphthoquinone. This is consistent with the assumption of an intact citric-acid cycle and a conversion of either malic or oxalacetic acid to pyruvic (or phosphoenolpyruvic) acid which is decarboxylated to acetyl-CoA The label from fumaric acid-[1,4-^{14}C] is thereby entirely lost, while that of fumaric acid-[2,3-^{14}C] is retained and yields uniformly-labeled plumbagin. Finally, acetoacetic acid-[3-^{14}C] (as its ethyl ester) is incorporated into plumbagin thus suggesting that tyrosine degradation in this higher plant tissue operates the same way as it is known from animals and microorganisms. In similar experiments, the same pathway for the biosynthesis of 7-methyljuglone was established.

In order to confirm this hypothesis we attempted to trap any homogentisic acid formed from DL-tyrosine-[β-^{14}C] within the leaf tissue Only a small amount of the free acid (1·8% of the total activity of the extract) was found however, after chromatography of an alcoholic extract of the labeled leaf In order to increase the pool of free homogentisic acid, use was made of the fact that ring-cleavage of homogentisic acid is carried out by an iron-containing enzyme which is inhibited *in vitro* by α,α'-bipyridyl [14] Preliminary experiments showed that *Drosophyllum* leaves were able to tolerate 0 01 M α,α'-bipyridyl for at least

[14] SUDA, M , TAKEDA, Y SUJISHI, K and TANAKA, T (1951) *J Biochem* **38**, 297

24 hr. Leaves were therefore allowed to take up 0·01 M α,α′-bipyridyl for 6 hr and were then transferred to a solution of DL-tyrosine-[β-^{14}C] in 0·01 M α,α′-bipyridyl. These leaves, and those of a control experiment fed only tyrosine-[β-^{14}C], were allowed to metabolize for 24 hr. The results (Table 6) show that in the presence of the chelating agent incorporation of label into plumbagin was reduced to 1/10 of the control value, which is in accordance with the operation of the homogentisic acid pathway in *Drosophyllum*. A control experiment in which leaves were allowed to photosynthesize $^{14}CO_2$ in the presence and absence of α,α′-bipyridyl showed that the incorporation of label from $^{14}CO_2$ into plumbagin was insignificantly reduced. This experiment also shows that the endogenous precursor of plumbagin is not tyrosine but rather acetate or acetyl-CoA formed directly from carbohydrate metabolism

TABLE 6 SPECIFIC INHIBITION OF TYROSINE INCORPORATION INTO PLUMBAGIN IN *Drosophyllum lusitanicum* BY TREATMENT WITH α,α′-BIPYRIDYL (10^{-2} M) DURING 24 HR IN THE DARK

Precursor applied	(μmol)	Tot act. (dpm)	Plumbagin Incorp. (%)	Sp act (dpm/μmol)	Dilution
DL-Tyrosine-[β-^{14}C]	0·5	$9·2 \times 10^6$	19·3	$8·1 \times 10^4$	226
DL-Tyrosine-[β-^{14}C] + α,α′-bipyridyl	0·5	$9·2 \times 10^6$	2·5	$1·5 \times 10^4$	1220
$^{14}CO_2$	9·8	$1·1 \times 10^8$	3·1	$4·7 \times 10^5$	24
$^{14}CO_2$ + α,α′-bipyridyl	9·8	$1·1 \times 10^8$	2·2	$2·6 \times 10^5$	43

Leaves fed tyrosine-[β-^{14}C] in the presence of α,α′-bipyridyl were extracted after 24 hr with 80% ethanol and the extract was chromatographed. Chromatography showed substantial amounts of free homogentisic acid to be present (13·5% of the total radioactivity). The labeled homogentisic acid was isolated and rigorously purified by recrystallization with authentic homogentisic acid, formation of derivatives [2,5-dimethoxyhomogentisic acid methyl ester (sp. act. 330 dpm/μmol) which was converted to 2,5-dimethyoxyhomogentisic acid (sp. act. 310 dpm/μmol)] and radiogas chromatography Thus, homogentisic acid is formed from tyrosine in this tissue when its normal catabolism is blocked by α,α′-bipyridyl, and that under these conditions formation of plumbagin from tyrosine is also severely depressed.

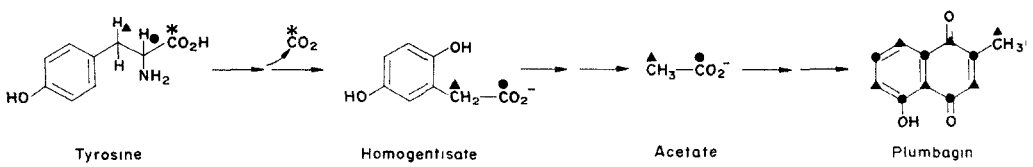
It is noteworthy that plumbagin shows a slow turnover in plants kept in the dark. Pulse feeding of tyrosine-[β-^{14}C] for 1·5 hr to *Drosophyllum* leaves which were kept subsequently in the dark showed that plumbagin had a half life of about 400 hr under these conditions.

One major uncertainty in these experiments is that they were conducted with nonsterile plants. Thus it could not be excluded that epiphytic bacteria living on *Drosophyllum* plants were causing the breakdown of labeled tyrosine and its catabolites, although the high percentage of incorporation of tyrosine into plumbagin made this possibility unlikely. In order to exclude this possibility completely, *Drosophyllum* plants were grown in test tubes on an inorganic mineral salt agar medium, starting with sterilized seeds. Two-month-old plants were checked for their sterility by incubating portions of leaves on nutrient agar (Merck II). There was no indication of any contamination. The shoot was then excised under sterile conditions and allowed to metabolize under rigorously sterile conditions a sterile solution of DL-tyrosine-[β-^{14}C] (5·0 μCi, 0·5 μmol). After 24 hr the plant had respired 1·1% of the radioactivity as CO_2 and 4·8% was incorporated into plumbagin (sp.

act 92000 dpm/μmol, dilution. 239) only 1/5 of the incorporation found with nonsterile plants. We attribute the lower incorporation to the poor physiological condition of the test tube grown sterile plant Clearly, however, sterile tissues of higher plants are able to convert tyrosine via acetate to plumbagin In order to substantiate this, tissue cultures of *D lusitanicum* were established Callus grown on solid agar was transferred to a liquid medium and after about 14 transfers and selection for naphthoquinone production a good growing dedifferentiated suspension culture was obtained which produced plumbagin (*ca* 7 μmol/g fr wt). The culture was routinely checked for contamination. Using this sterile suspension culture, the experiments with labeled tyrosine and its catabolites were repeated, with qualitatively identical results (Table 7) The extent of incorporation of these precursors into plumbagin is lower than in the case of the differentiated tissue, but the reason for this may be the lower metabolic capacity of the undifferentiated tissue. The dilution factors were comparable to the dilution factors obtained with the intact leaf tissue

TABLE 7 INCORPORATION OF POTENTIAL ^{14}C-LABELED PRECURSORS INTO PLUMBAGIN IN CELL SUSPENSION CULTURES OF *Drosophyllum lusitanicum*

Precursor applied	Plumbagin			
	(μmol)	Total act (dpm)	Incorp (%)	Sp act (dpm/μmol)
DL-Tyrosine-[β-^{14}C]	3 6	$3\,6 \times 10^{7}$	0 51	$4\,94 \times 10^{3}$
D-Tyrosine-[β-^{14}C]	3 6	$9\,8 \times 10^{6}$	0 45	$1\,30 \times 10^{4}$
p-Hydroxyphenyl-pyruvic acid-[3-^{14}C]	4 7	$3\,9 \times 10^{5}$	1 54	$4\,19 \times 10^{2}$
Homogentisic acid-[2-^{14}C]	3 5	$1\,2 \times 10^{6}$	0 79	$2\,06 \times 10^{3}$
4-Maleylacetoacetic acid-[2-^{14}C]	3 7	$1\,7 \times 10^{5}$	2 01	$4\,35 \times 10^{3}$
4-Fumarylacetoacetic acid-[2-^{14}C]	3 7	$1\,7 \times 10^{5}$	1 48	$2\,22 \times 10^{3}$
Acetate-[1-^{14}C]	4 1	$5\,5 \times 10^{7}$	0 97	$5\,82 \times 10^{4}$
Acetate-[2-^{14}C]	4 0	$1\,4 \times 10^{7}$	1 65	$7\,51 \times 10^{4}$
Maleic acid-[2,3-^{14}C]	3 9	$1\,1 \times 10^{7}$	0 43	$2\,91 \times 10^{3}$
Fumaric acid-[2,3-^{14}C]	5 6	$1\,1 \times 10^{7}$	0 98	$3\,38 \times 10^{3}$
Ethylacetoacetic acid-[3-^{14}C]	4 0	$8\,7 \times 10^{6}$	1 29	$1\,21 \times 10^{4}$



SCHEME 1 BIOSYNTHETIC PATHWAY FOR THE INCORPORATION OF TYROSINE INTO PLUMBAGIN

Thus it has been established that higher plants possess a catabolic pathway for the breakdown of tyrosine to acetate via homogentisate (Scheme 1). As judged from precursor feeding experiments this catabolic route is identical to the homogentisate ring-cleavage pathway known to occur in a number of animals and microorganisms. Rigorous proof, however, requires the isolation of enzymes of this pathway from sterile grown plant material, which has been successful and will be published elsewhere

DISCUSSION

In 1959 Zaprometov[15] drew attention to the fact that plants are able to degrade phenolic compounds and that obviously plants, like microorganisms and animals, possess pathways to cleave the benzene ring. This suggestion was confirmed in recent years especially by feeding ring-[^{14}C] labeled aromatic compounds to sterile plant tissue and monitoring the respired $^{14}CO_2$ which served as an indicator for ring degradation (literature in [16]). Up to now, however, no complete pathway was demonstrated in higher plants by which the ring-cleavage of the aromatic nucleus is achieved. In this paper, it has been shown that *D. lusitanicum* possesses the homogentisic acid ring-cleavage pathway for tyrosine degradation. Plants grown under sterile and nonsterile conditions as well as in cell suspension cultures are able to degrade tyrosine to acetate. By means of precursor feeding experiments, it was shown that homogentisate and its aliphatic ring-cleavage products are converted to a large extent to acetate. The acetate molecules thus formed are incorporated into the naphthoquinones plumbagin and 7-methyljuglone according to the acetate–polymalonate pathway, a hexaacetyl chain being involved as a likely intermediate. The high efficiency of the conversion of the carbon atoms of tyrosine into plumbagin can presently only be explained by the assumption that degradation of tyrosine and biosynthesis of plumbagin are highly compartmentalized within the plant cell. Despite the high rate of conversion (up to 20%), only a small percentage of the labeled carbon atoms of tyrosine was liberated as $^{14}CO_2$; this demonstrates that measurement of $^{14}CO_2$ set free from labeled aromatic precursors, as an indicator for ring-cleavage can give misleading results and that this method should only be used in a qualitative manner.

One can speculate that there might be an ecological function for the occurrence of this pathway in carnivorous plants. *Drosophyllum* as other Droseraceae occurs on notoriously nitrogen-poor soil. Perhaps the carnivorous habit has developed to supplement the nitrogen supply of these plants by proteolytic breakdown of protein of their prey.[17] The nitrogen of the amino acids obtained from the insect is most likely removed and used for synthetic reactions within the plant. The carbon skeleton of the amino acid is subsequently broken down to acetate by the plant and reused for biosynthetic processes such as naphthoquinone formation. This hypothesis is supported by the fact, that in *Drosophyllum* acetogenic amino acids like tyrosine, leucine and isoleucine are readily degraded to acetate. The naphthoquinone plumbagin is toxic to certain microorganisms[18,19] and it is possible that the relatively water-soluble plumbagin formed by the Droseraceae serves an ecological function by preventing excessive microbial growth on the trapped insects, thereby excluding competition for the valuable nitrogen

EXPERIMENTAL

Plant material Plants were grown in a greenhouse at 18° day and 15° night temp at a relative humidity of 70 ± 15% Young, not yet completely unrolled leaves (length 7 ± 1 cm) of *Drosophyllum lusitanicum* were used for feeding experiments Three individual leaves were used for incubations which were carried out at 21° ± 1° and in complete darkness In order to enhance uptake of the tracer solution the leaves were exposed to a continuous stream of air Sterile plants were grown from surface sterilized seeds in test tubes on a mineral salt

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medium[20] at room temperature and 4100 lx Suspension cultures of *Drosophyllum* were prepared from callus tissue which was grown in essentially the same medium as that developed by Gamborg *et al*.,[21] strains were selected for high naphthoquinone production

Radioactive compounds Isotopic compounds were purchased from commercial sources D-shikimic acid-[7-^{14}C] and L-tyrosine-[ring-U-^{14}C] were synthesized in this laboratory [22,23] *p*-Hydroxyphenylpyruvic acid-[3-^{14}C] and homogentisic acid-[2-^{14}C] were prepared from DL-tyrosine-[β-^{14}C],[24] whereas maleylacetoacetic acid-[2-^{14}C] and fumarylacetoacetic acid-[2-^{14}C] were prepared from homogentisic acid-[2-^{14}C] [13,25] Derivatives of homogentisic acid were prepared and radiogas chromatography was performed according to Bondurant *et al*.[26]

Isolation and purification of naphthoquinones. Plant material was extracted exhaustively with Et_2O The naphthoquinones in the ether extract were purified by preparative TLC on Silica gel G plates in C_6H_6 (plumbagin R_f 0 47 and 7-methyljuglone R_f 0 38), cyclohexane–Et_2O (4 1) containing 3 drops HOAc per 100 ml (R_f 0 5 and 0 37) and toluene-Me_2CO (19.1) (R_f 0 79 and 0 70) Sp act of the naphthoquinones stayed constant from the second TLC step on Concentrations of plumbagin were determined spectrophotometrically in MeOH at 425 nm [$\epsilon = 3\,98 \times 10^6$ cm^2/mol] and 7-methyljuglone at 414 nm [$\epsilon = 3\,83 \times 10^6$ cm^2/mol]

Degradation of plumbagin Labeled plumbagin was diluted with unlabeled carrier material (total 150 μmol) and subjected to an alkaline H_2O_2 oxidation[9] which yielded 3-hydroxyphthalic acid The phthalic acid was degraded further according to known procedures [9]

Bromopicrin was isolated and counted as its hexamethylene tetramine derivative [27] Kuhn–Roth oxidation and degradation of acetate was performed according to standard procedures [28]

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