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Biosynthesis of furanochromones in *Pimpinella monoica*

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Abstract. During a search for bioactive compounds from indigenous plants, *Pimpinella monoica* (Umbelliferae) was found to contain furocoumarin, isopimpenellin (3) and five biogenetically related furocoumarins viz khellin (1), visnagin (2), visamminol (4), ammiol (5) and khellol (6). Labelled (1) and (2) were isolated from [1-^{14}C]- and [2-^{14}C]-acetates. Labelling pattern, determined by degradation of biosynthesised compounds, establishes the polyketide origin of their aromatic and pyrone rings while the furan ring originates via an acetate–mevalonate pathway. The plant also utilises glycine and leucine as substrate *via* acetate. Biotransformation of [3-^{3}H]-visnagin to (6) but not to (2) was also observed.

Keywords. Biosynthesis; furochromones; polyketide origin; [3-^{3}H]-visnagin; khellin.

1. Introduction

Furochromones, though a small group of natural products with limited occurrence, have been known for their pharmacological properties such as selective coronary vasodilatory, bronchodilatory and photodynamic activities (Livingstone 1977). Recent reports on their antiatherosclerotic properties and possible use in photo-radiation therapy have brought them into prominence once again (Stevens *et al* 1985). Though considerable work has been carried out on their pharmacology and synthesis (Gammill and Nash 1986; Reed and Moore 1988), only scant attention has been given on their biosynthesis. Based on striking similarities between certain chromones and coumarins such as khellin (1) and isopimpinellin (3) Geissman (Geissman and Hinreiner 1952) suggested that both the classes are derived by the shikimate-pathway. However experiments carried out in *Ammi visnaga* (Chen *et al* 1969) and *Eranthis hiemelis* (Egger 1962) have shown that the aromatic and the pyrone rings of the furochromones are formed from acetate while mevalonate furnishes the carbons of the furan ring. Remarkable co-occurrence of furocoumarin, isopimpinellin (3) and furochromone khellin (1) in the plant *Pimpinella monoica*, prompted us to investigate which of the two pathways (i.e. polyketide vs phenylpropane) is operative for the biosynthesis of furochromones in *P. monoica*. In our on-going programme on biosynthesis of natural products, a detailed investigation on the biosynthesis of khellin (1) and visnagin (2) in the plant, *P. monoica* was undertaken. Fates of acetates and amino-acids in the biosynthesis of (1) and (2) have been studied by extensive degradation of the biosynthesised products and the results are discussed here.

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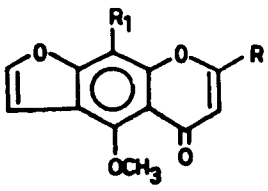
2. Results and discussion

During our search for bioactive compounds, the extracts of the plant, *Pimpinella monoica* Dalz. (N.O. Umbelliferae) showed significant feeding deterrency to the larvae of tobacco caterpillar, *Spodoptera litura* F. Bio-assay directed fractionation led to the isolation of, in addition to previously reported furocoumarin, isopimpinellin (3), five biogenetically related furochromones viz. khellin (1), visnagin (2), visamminol (4), ammiol (5) and khellol (6) (Luthria 1990). Compounds 1–3 and 6 showed significant insect antifeedant activity (figure 1) (Luthria *et al* 1992). It may be noted that so far Egyptian plants belonging to the genus *Ammi* were the major source of furochromones, while those belonging to the genus *Pimpinella* are known to elaborate only furocoumarins and not furochromones. This is the first report of the occurrence of furochromones in *Pimpinella* species. *P. monoica* is the first Indian plant to contain these bioactive compounds in substantial quantities.

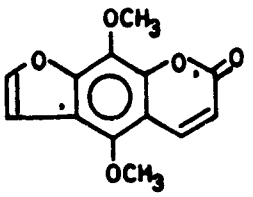
The two major components, khellin (1) and visnagin (2) were selected for biosynthetic investigations. An investigation on the distribution revealed that these were



(1, 2, 5, 6)

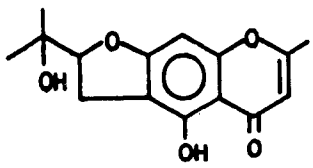
	R	R_1	EC_{50} (ppm)
(1) Khellin	CH_3	OCH_3	107.7
(2) Visnagin	CH_3	H	69.2
(5) Ammiol	CH_2OH	OCH_3	*
(6) Khellol	CH_2OH	H	99.0

* 55% feeding inhibition at 330 ppm



(3)

Isopimpinellin (3) (EC_{50}: 5 ppm)



(4)

Visamminol (4) (30% feeding inhibition at 1000 ppm)

Figure 1. Structures and antifeedant activities of compounds isolated from *P. monoica*.

Table 1. Distribution* of khellin (1) and visnagin (2) in *P. monoica*.

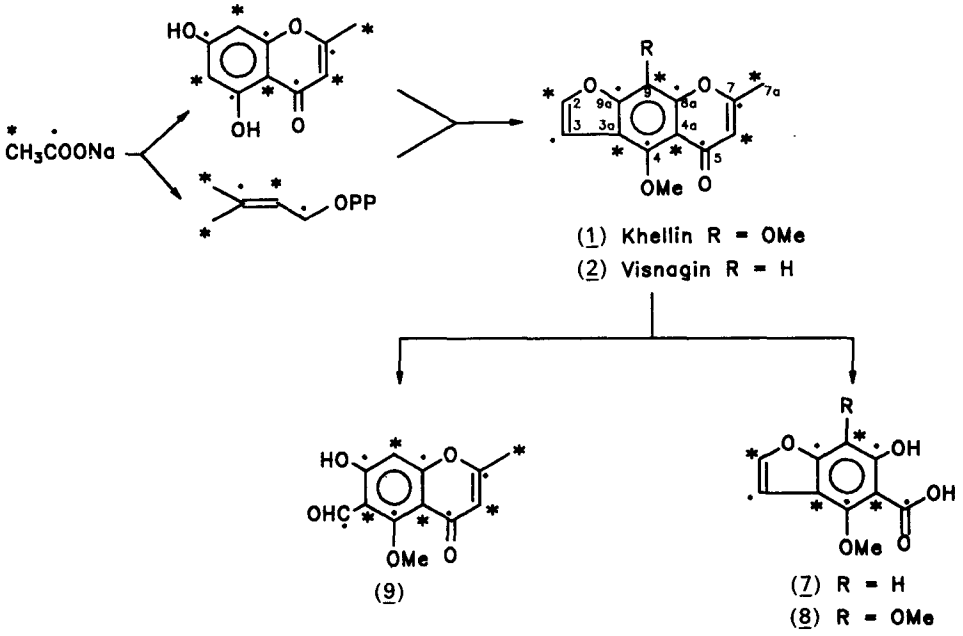
Part of the plant	Khellin (%)	Visnagin (%)
Whole plant	0·09	0·13
Leaves	0·08	0·32
Flowers	0·17	0·54
Mature seeds	0·12	0·12
Stems	0·03	0·04
Roots	0·03	0·02

*% based on weight of fresh plant

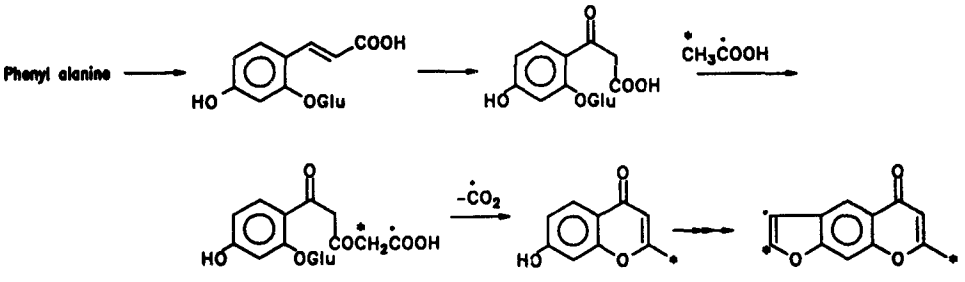
present in all the parts though stem and roots contained very small amounts (table 1). For the biosynthetic experiments, aqueous solutions of the labelled substrates were administered to the plant through the stem. The plants were harvested after 24 hours and (1) and (2) were isolated in pure states from the ether extracts by elaborate preparative TLC followed by recrystallisations.

2.1 *Incorporation of* [1-^{14}C]- *and* [2-^{14}C]-*acetates*

If the polyketide hypothesis is operative for the biosynthesis of furochromones (1) and (2) in *P. monoica*, positions 2, 3a, 4a, 6, 7a and 9 would be labelled from [2-^{14}C]-acetate while positions 3, 4, 5, 7, 8a and 9a would be labelled if [1-^{14}C]-acetate is used as a precursor (scheme 1). However considering the alternate phenylpropane pathway only carbons 2 and 7a are expected to be labelled from [2-^{14}C]-acetate while [1-^{14}C]-acetate would label only carbon-3 (scheme 2). Thus, by judicious use of labelled acetates and degradation of biosynthesised (1) and (2), it should be possible



Scheme 1.



Scheme 2.

to detail the biosynthetic pathway. For this purpose it was essential to develop appropriate chemical degradation procedures for (1) and (2).

The location of the labels in the biosynthesised furochromones was determined by chemical degradations. Two methods for the small-scale degradation of (1) and (2) were developed (Luthria *et al* 1993). The pyrone ring could be cleaved by controlled oxidation with alkaline H_2O_2. Thus 6-hydroxy-4-methoxybenzofuran-5-carboxylic acid (7) and 6-hydroxy-4,7-dimethoxybenzofuran-5-carboxylic acid (8) were obtained by the degradation of (1) and (2) respectively. The degradation products (7) and (8) are devoid of the carbons 6, 7 and 7a of the pyrone nucleus of the parent molecules. The furan ring could be cleaved selectively by cautious chromic acid oxidation of (2) when 6-formyl-7-hydroxy-5-methoxy-2-methylchromone (9) was obtained. The product (9) retains all the carbons of the parent molecule except carbon −2.

Radio-labelled (1) and (2) were isolated from the plants which were fed with labelled acetates (table 2). The degradation product (7) obtained from (2) biosynthesised from [2-^{14}C]-acetate retained 60·3% of the activity of the parent compound, corresponding to loss of closer to two radioactive carbons out 6. The same degradation product (7) from (2) biosynthesised from [1-^{14}C]-acetate showed radioactivity which corresponded to the loss of about one carbon out of six. 7-Hyroxy-6-formyl-2-methylchromone (9) obtained by the cleavage of furan ring in visnagin (2) biosynthesised from sodium-[1-^{14}C]-acetate, retained all the activity while only 83·7% activity (corresponding to the loss of one carbon out of 6) was obtained when sodium -[2-^{14}C]- acetate was used as a substrate. The results obtained are fully consistent with the formation of aromatic and pyrone rings through the polyketide pathway. These results conclusively prove the polyketide origin of (1) and (2) and that the phenylpropane pathway (scheme 2) is not operative in *P. monoica.*

2.2 *Incorporation of leucine and glycine*

Besides the mevalonic acid (MVA) route, other minor pathways are known to operate for the biosynthesis of terpenoids (Banerji and Chintalwar 1984). Incorporation of leucine and related compounds into monoterpenes, sesquiterpenes, steroids and triterpenoids have been reported (Anastasis *et al* 1985). We have found non-specific incorporation of amino acids into the meroterpenoid, bakuchiol in the plant, *Psoralea corylifolia* (Banerji and Chintalwar 1989). Since the furan moieties of (1) and (2) are believed to have originated from the dimethylallyl pyrophosphate (DMAPP), it was of interest to investigate the fate of leucine in their biosynthesis. If the incorporation of leucine is exclusively to the furan rings of (1) and (2) via DMAPP, then the labels from [U-^{14}C]-leucine should be confined exclusively to C-2 and C-3 and the aromatic and pyrone rings should be devoid of any activity.

On feeding L-[U-^{14}C]-leucine to *P. monoica*, significant incorporation of the label into (1) and (2) was observed. Cleavage of the furan ring of the biosynthesised (2) gave (9) which retained 95% of the radioactivity corresponding to a loss closer to one carbon out of 12. A substantial loss of activity (25%) was observed when the pyrone ring of biosynthesised (2) was cleaved to (7). Similar results were obtained by the degradation of (1) to (8). These results show that the labels from L-[U-^{14}C]-leucine are not only confined to furan rings but are distributed throughout the molecules of (1) and (2). The labelling pattern suggests that L-[U-^{14}C]-leucine is metabolised to [1,2-^{14}C]-acetate which is incorporated into (1) and (2) uniformly.

Table 2. Radioactivities in khellin (1), visnagin (2) and degradation products 7–9.

Activity fed (Sp. activity)	Incorporation (%) (1)	(1) ($\times 10^7$) (dpm/mM)	(8) ($\times 10^7$) (dpm/mM)	Incorporation (%) (2)	(2) ($\times 10^7$) (dpm/mM)	(7) (10^7) (dpm/mM)	(9) ($\times 10^7$) (dpm/mM)
Acetate-[1-^{14}C], 50 μCi (60 mCi/mM)	0·007	0·367	0·309	0·02	1·31	0·995	1·3
Acetate-[2-^{14}C], 50 μCi (4·66 mCi/mM)	0·06	5·09	3·19	0·09	8·59	5·18	7·12
L-Leucine-[U-^{14}C], 100 μCi (13·2 mCi/mM)	0·14	2·96	2·10	0·15	1·60	1·19	1·52
L-Leucine-[1-^{14}C], 100 μCi (13, 46 mCi/mM)	Insignificant			Insignificant			
Glycine-[1-^{14}C], 50 μCi (14·4 mCi/mM)	0·01	1·11	0·892	0·05	2·33	1·8	2·26
Glycine-[2-^{14}C], 50 μCi (21·4 mCi/mM)	0·09	3·59	3·02	0·19	5·65	4·29	4·93

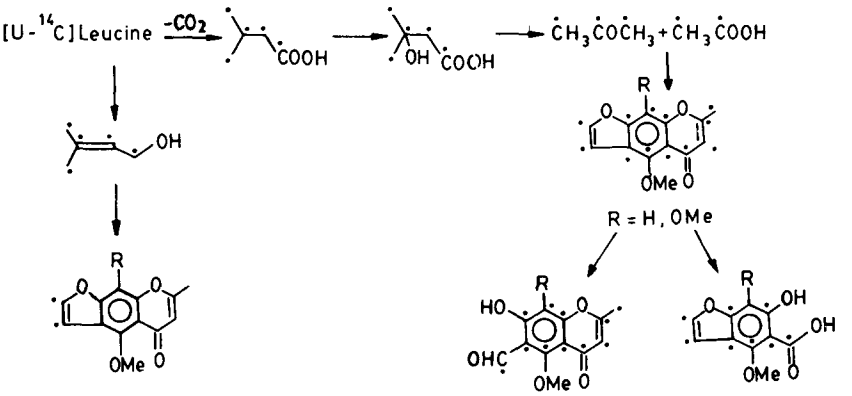
This anomalous incorporation can be explained by the oxidative deamination and decarboxylation of leucine to yield isovaleryl CoA which undergoes further metabolism via β-hydroxyisovaleric acid to acetyl CoA and acetone (Aberhart 1979). Thus, in this process the carboxyl group of leucine is lost. Additional support for incorporation of leucine via [1,2-^{14}C]-acetate could be obtained by using L-[1-^{14}C]-leucine as a substrate. As expected, the activity from this substrate was not incorporated suggesting the loss of the carboxyl carbons of leucine during its incorporation into (1) or (2). Very substantial incorporation of [1-^{14}C]- and [2-^{14}C]-glycine into (1) and (2) was also observed. The distribution of the labels show similarity with that obtained using acetate as substrate (table 2). The results suggest that incorporation of glycine involve deamination to acetate. However, this would need further investigation.

2.3 *Biotransformation of* [3-^{3}*H*]-*visnagin*

Biogenetically, khellin (1) could either be formed from (2) by para-hydroxylation and subsequent O-methylation or it could arise independently (Steck and Brown 1970). According to the former pathway, (2) will be an intermediate in the biosynthesis of (1). [3-^{3}H]-Visnagin required for the biotransformation was prepared by careful opening of the γ-pyrone ring to the corresponding diketone, introducing [3-^{3}H]-label by isotopic exchange with tritiated water and ring closure (Luthria and Banerji – unpublished work). Experiments were carried out by feeding ^{3}H-visnagin (Luthria 1990) to *P. monoica* and furochromones were isolated. Khellin (1) did not show significant activity while khellol (6) and recovered (2) were found to be radioactive (table 3). Thus, (1) does not arise from (2) while (6) is biosynthesised from (2) via

Table 3. Activities of furochromones after fee ding ^{3}H-visnagin.

Compounds isolated	Yield (mg)	Activity (dpm/mM)
Visnagin	92	1'.54 × 10^{8}
Khellin	70	In significant
Khellol	2·3	2·98 × 10^{7}



Scheme 3.

oxidation of C-7 methyl. The biogenetic interrelation between the furochromones in *P. monoica* is shown in scheme 3.

3. Experimental

Pimpinella monoica: Dalz (Umbelliferae) plants commonly known as lady's lace were grown in the experimental field station of BARC. All the experiments were carried out on mature plants (3-month old). Aqueous solutions of the substrates were fed to the stem by the wick method. The plants were harvested after 24 h.

General experimental procedure: All the radiochemicals were procured from the Board of Radiation and Isotope Technology, Bombay. Radioactivity measurements and feeding of substrates were carried out as described earlier (Banerji and Chintalwar 1983).

Isolation of visnagin (2) and khellin (1): Fresh plant material ($\approx$ 80 g) was macerated and extracted with hot ether (4 times). The extracts were pooled, filtered and dried over sodium sulphate. The dried extract was then concentrated to furnish crude extract. This was then subjected to preparative TLC (acetone:$CHCl_3$, 4:96, triple run). The isolated furochromones were purified by crystallization. The radiochemical purity was checked by repeated crystallisations/TLC. Compounds were characterised as described earlier (Luthria *et al* 1993).

6-Formyl-5-methoxy-7-hydroxy-2-methyl chromone (9), 6-hydroxy-4,7-dimethoxybenzofuran-5-carboxylic acid (8) and 6-hydroxy-4-methoxybenzofuran-6-carboxylic acid (7) were prepared as described earlier (Luthria *et al* 1993).

4. Conclusions

Thus it has been conclusively established that the furochromones khellin (1) and visnagin (2) are biosynthesised via polyketide and acetate-mevalonate pathways.

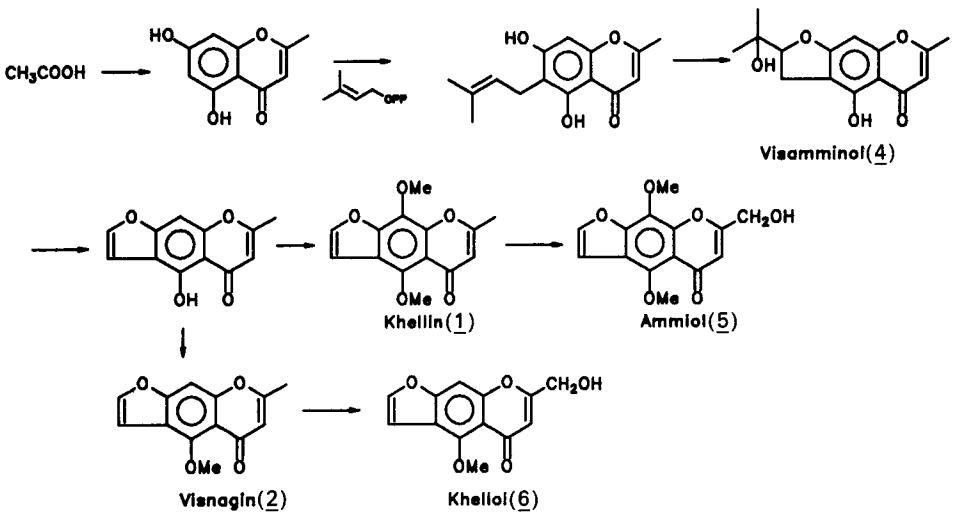


Figure 2. Biogenetic interrelations between furochromes from *P. monoica*.

Anomalous incorporations of glycine and leucine into (1) and (2), possibly via metabolism to acetate have been observed. Visnagin (2) is the precursor for (6) but not for (1). The biogenetic interrelations between the furochromones in *P. monoica* are shown in figure 2.

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