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Shikonin: A Geranyl Diphosphate-Derived Plant Hemiterpenoid Formed *via* the Mevalonate Pathway

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Abstract: Feeding of [1-^{13}C]glucose to cell cultures of *Lithospermum erythrorhizon* revealed a labelling pattern in the isoprenoid part of shikonin consistent with a biosynthesis *via* the mevalonate pathway. This is in contrast to other plant monoterpenoids.

In the past, the mevalonate pathway had been accepted as the universal biosynthetic route to isopentenyl diphosphate (IPP), the common precursor of terpenoids.[1] Recent studies with ^{2}H- and ^{13}C-labelled precursors, however, revealed that many isoprenoids are formed by a mevalonate-independent pathway, i.e. from pyruvate and glyceraldehyde 3-phosphate *via* 1-deoxy-D-xylulose-5-phosphate.[2] The existence of this pathway has been demonstrated in plants[2b-g], in green algae[2g-h] and in bacteria[2i-j]. E. g. isoprene in *Hordeum vulgare, Daucus carota* and *Lemna gibba*,[2b] monoterpenes in *Mentha x piperita*[2c] and *Conocephalum conicum*,[2d] and diterpenes in *Taxus chinensis*[2e] and *Marrubium vulgare*[2f] are synthesized *via* this non-mevalonate pathway, whereas the biosynthesis of the sesquiterpenes in *Ricciocarpos natans*[2d] *and Heteroscyphus planus*[3] proceeds *via* the classic mevalonate pathway. At present it is hypothesized that in plants the non-mevalonate pathway is located in the plastids and is involved in the formation of mono-, di- and tetraterpenes, whereas sesquiterpenes and triterpenes are formed in cytosol *via* the mevalonate pathway.[2a]

Shikonin (**1**, Fig. 1), a red pigment from *Lithospermum erythrorhizon*, is formed from 4-hydroxybenzoic acid (4HB) and the monoterpenoid precursor geranyl diphosphate (Fig. 1).[4] Radioactive feeding experiments have demonstrated the incorporation of ^{14}C-mevalonate into shikonin[5] and its enantiomer alkanin.[6] The involvement of the classic mevalonate pathway in the biosynthesis of shikonin was questioned, however, by Sankawa *et al.*[7] who reported that ^{14}C-mevalonate was not incorporated into shikonin. All recent studies with ^{2}H- and ^{13}C- labelled precursors in plants have shown that mono- and diterpenoid compounds are synthesized in plastids *via* the non-mevalonate pathway.[2a,c-f] If geranyl diphosphate for the biosynthesis of shikonin were formed from mevalonate, shikonin would represent the first compound of monoterpenoid origin in plants to be biosynthesized by the classic isoprenoid pathway. We therefore decided to investigate the origin of the carbon skeleton of shikonin by a feeding experiment with ^{13}C-labelled glucose.

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Shikonin and its derivatives were produced by culturing *Lithospermum erythrorhizon* cells in a modified M9 medium,[8] containing 3 % sucrose (76 % of total carbon source) and 1% [1-^{13}C]glucose (99 atom- % ^{13}C, 24 % of total carbon source). The produced shikonin was isolated, after hydrolysis of its esters, by HPLC[9] and subjected to NMR analysis.[11] In order to quantify the ^{13}C enrichment, ^{13}C NMR spectra of the isolated labelled shikonin and of an unlabelled reference sample were taken under identical conditions. The absolute ^{13}C enrichments of selected carbon atoms, e.g. of C-11, C-13, C-15 and C-16, were obtained from satellite signals in ^{1}H NMR. The relative enrichments of the other carbons were then referenced to these carbons.[13] The results were further confirmed by measurement of the satellite signals in ^{13}C NMR due to ^{13}C-^{13}C coupling.

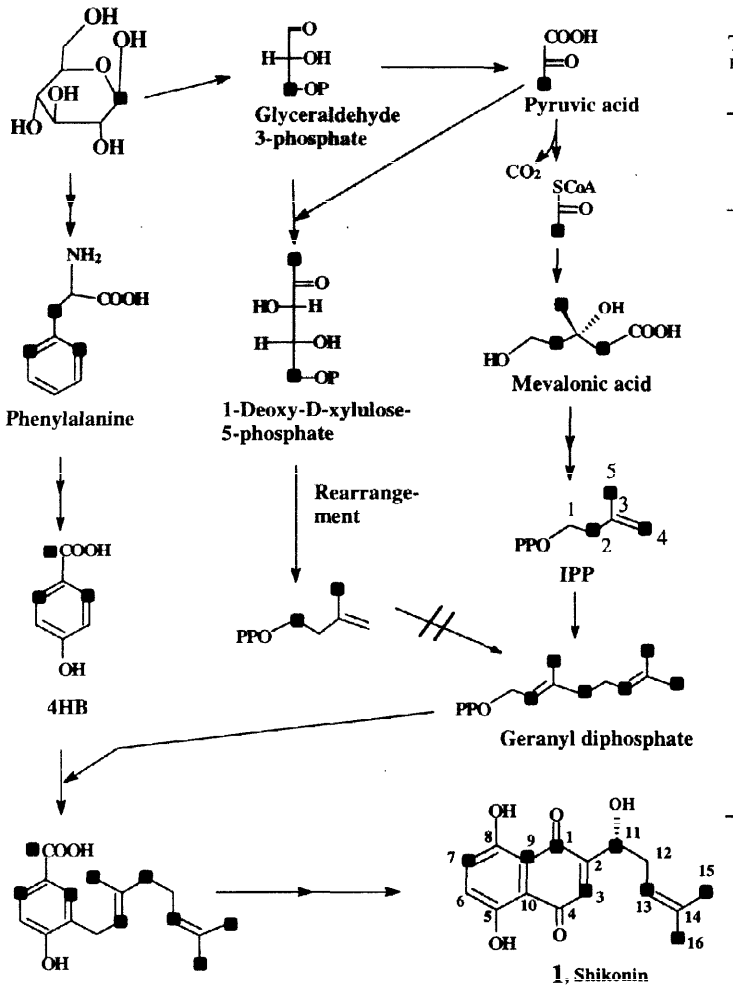

Fig. 1: Incorporation of [1-^{13}C]glucose into shikonin

Table 1: ^{13}C Enrichment in shikonin after feeding of [1-^{13}C]glucose (99 atom- % ^{13}C)

C-Atom (see Fig. 1)	Chemical Shifts (δppm, $CDCl_3$)	Enrichment (%)	Corresponding C-Atom of IPP
1	179.82	10.3	C-5
2	151.46	2.4	
3	131.92	10.5	C-2
4	180.59	2.6	
5	165.03	0.8	
6	132.45	approx. 1.1	
7	132.45	approx. 9.8	
8	165.66	0.0	
9	112.10	15.0	
10	111.62	1.1	
11	68.45	12.6	C-4
12	35.74	2.9	
13	118.51	11.1	C-2
14	137.41	2.1	
15	18.11	11.5	C-5
16	25.96	10.7	C-4

With [1-^{13}C]glucose as carbon source, enrichments at C-2, C-4 and C-5 of IPP are expected from a mevalonate pathway, in contrast to enrichments at C-1 and C-5 of IPP from a non-mevalonate pathway *via*

glyceraldehyde 3-phosphate and pyruvate (Fig. 1). Results obtained from labelled shikonin were in perfect accordance with a biosynthesis *via* the mevalonate pathway. Between 10 and 13 % enrichments were observed at C-1, C-3, C-11, C-13, C-15 and C-16 of shikonin (Fig. 1, Table 1), which correspond to the carbon atoms 2, 4 and 5 of IPP. The ^{13}C enrichment of the remaining carbons of the isoprenoid portion of the shikonin skeleton was on average 2.5 %, which may be explained by a complex metabolic turnover of [1-^{13}C]glucose during the 14 days feeding period. Similar effects have been observed in feeding experiments in other plants.[2d,e]

Enrichment at position 7 could only be estimated, since the ^{13}C signal of this carbon, as well as its ^{13}C-^{13}C coupling peaks, overlapped with those of C-6, and the satellite peaks of H-7 in ^{1}H NMR overlapped with those of H-3. The observed unequal enrichment at C-7 and C-9 is unexpected due to their origin from equivalent positions of a symmetric intermediate (Fig. 1). It cannot be excluded, however, that this finding is due to the inaccuracy in the estimate of the enrichment at C-7 rather than to a real difference between C-7 and C-9.

The presence of sucrose in the medium was necessary for the production of shikonin. Since [1-^{13}C]glucose (99 atom- %) constituted 24 % of the total carbon in the medium, ^{13}C abundance at C-3 of the resulting triose phosphates is expected to be approx. 12.9 % (including the natural abundance of ^{13}C in unlabelled carbons), or less due to loss of label in the oxidative pentose phosphate cycle. The 15 % enrichment measured for C-9, and confirmed by the ^{13}C-^{13}C coupling peaks in the signal of C-1, may therefore indicate a preferential use of glucose *versus* sucrose for the biosynthesis of shikonin under the experimental conditions employed.

The results of this study prove that geranyl diphosphate for the biosynthesis of shikonin is formed *via* the mevalonate pathway. This is in contrast to all other plant mono- and diterpenoid compounds studied so far. This surprising result may be explained, however, by a different intracellular localization of shikonin biosynthesis compared to mono- and diterpene formation. Whereas the latter compounds are formed in the plastids,[14] shikonin is formed at the endoplasmic reticulum.[15] Cell fractionation studies have indicated that the geranyl diphosphate synthase involved in shikonin biosynthesis is localized in the cytosol rather than in the plastids.[16] Therefore, shikonin may be formed from the cytosolic pool of isopentenyl diphosphate, which is known to derive from mevalonate. The finding that shikonin biosynthesis is strongly inhibited by the HMG-CoA reductase inhibitor mevinolin[17] provides additional support for this hypothesis.

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9. For the feeding experiment, M9 medium was supplemented with 1% [1-^{13}C]glucose (99 atom- % ^{13}C, Omicron Biochemicals, South Bend, IN, USA). Four 100 ml Erlenmeyer flasks, each containing 30 ml medium and 3 ml liquid paraffin, were inoculated with 2 g each of *Lithospermum erythrorhizon* cells strain TOM, a derivative of M18,[10] which had been cultured in ordinary M9 medium at 25°C on a reciprocal shaker (170 strokes /min) in the dark for 5 days. The cells were cultured under the same condition for further 14 days. The paraffin layer was diluted with 120 ml n-hexane, and the medium was extracted with another 120 ml n-hexane. The combined organic phases were extracted with 100 ml 0.5 N NaOH. After washing with 2 x 100 ml n-hexane and incubation at room temperature for 10 min (hydrolysis), the aqueous phase was acidified with 5N HCl to pH 3 and extracted with 2 x 120 ml n-hexane. After evaporation of the solvent, the residue (shikonin) was dissolved in methanol and further purified by HPLC using a RP-18 column (250 x 4 mm, 5 μ) and a gradient of methanol in 1% aqueous HCOOH as solvent system. 5.5 mg labelled shikonin were obtained and subjected to NMR analysis.
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11. The NMR spectra were recorded on a Bruker AC 250 spectrometer in $CDCl_3$. The chemical shifts of isolated substance were in accordance with the published data,[12] but the ^{13}C-^{13}C coupling observed for C-8 in our feeding experiment suggested a reversion of the assignment of the signals of C-8 and C-5.
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