Communications to the Editor

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ISOLATION OF THE INTERMEDIATES AND RELATED METABOLITES OF SHIKONIN BIOSYNTHESIS FROM LITHOSPERMUM ERYTHRORHIZON CELL CULTURES

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Compounds biogenetically related to shikonin in both the shikonin-producing and nonproducing cell suspension cultures of Lithospermum erythrorhizon were investigated to compare their secondary metabolism. From the shikonin-producing cultures two intermediates of shikonin biosynthesis, m-geranyl-p-hydroxybenzoic acid and geranylhydroquinone, and two shikonin-related metabolites, shikonofuran E and a new compound named deoxyshikonofuran were isolated. On the other hand, only m-geranyl-p-hydroxybenzoic acid was isolated from the cultures that did not produce shikonin. This suggests that a biosynthetic step leading to geranylhydroquinone from m-geranyl-p-hydroxybenzoic acid through decarboxylation is repressed in these cultures.

KEYWORDS—— Lithospermum erythrorhizon; Boraginaceae; plant tissue culture; biosynthesis; m-geranyl-p-hydroxybenzoic acid; geranylhydroquinone; shikonofuran E; deoxyshikonofuran

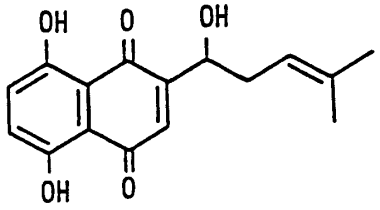
Callus cultures of Lithospermum erythrorhizon Sieb. et Zucc. grown on Linsmaier-Skoog (LS) agar medium[1] in the dark produce red naphthoquinone pigments consisting of shikonin (1) derivatives.[2] Using these callus cultures, Inouye et al.[3] demonstrated by tracer experiments that shikonin is synthesized from L-phenylalanine and two molecules of mevalonic acid via m-geranyl-p-hydroxybenzoic acid and geranylhydroquinone, although these intermediates were not isolated from the callus cultures.

On the other hand, it has been shown that Lithospermum cells grown in LS liquid medium without agar fail to synthesize shikonin derivatives, but they produce large quantities of these pigments in "production medium" M9 containing NO_3 as a sole source of nitrogen.[4-6] Here, we have compared the two cultures chemically to try to find the difference in the biosynthesis processes.

Shikonin-producing cells (400 g dry wt.) which were cultured in M9 medium[6] in the dark at 25°C for 14 days, were extracted with methanol. The ether soluble fraction of methanol extract was subjected successively to Lobar C8 column chromatography (70% acetone), Sephadex LH-20 column chromatography (ethanol), and preparative TLC (SiO_2, chloroform : methanol = 20 : 1) to give the following four prenylated aromatic compounds.

m-Geranyl-p-hydroxybenzoic acid (2): $C_{17}H_{22}O_3$, UV $\lambda_{max}^{methanol}$ nm (log ε) 207 (4.12), 254 (3.92), colorless oil (71mg). The mass spectrum of 2 showed the

parent peak at m/z 274, and its IR absorption band (chloroform) at 1685 cm^{-1} indicated the presence of a carboxyl group in the molecule. The ^{1}H-NMR spectrum of 2 (200 MHz, $CDCl_3$) showed an ABX system which is due to three aromatic protons [δ 7.89 (1H, d, J=2 Hz), 7.88 (1H, dd, J=2, 9 Hz), 6.85 (1H, d, J=9 Hz)], two vinyl protons [δ 5.32 (1H, t, J=7 Hz), 5.07 (1H, br t, J=7 Hz)], benzyl methylene protons [δ 3.41 (2H, d, J=7 Hz)], four methylene protons [δ 2.00-2.20 (4H, m)], and three methyl groups on double bonds [δ 1.78 (3H, s), 1.68 (3H, s), 1.60 (3H, s)]. This suggests that 2 is a hydroxybenzoic acid having a geranyl group as a side chain. The identification of 2 as m-geranyl-p-hydroxybenzoic acid was achieved by direct comparison with an authentic specimen.[3)]



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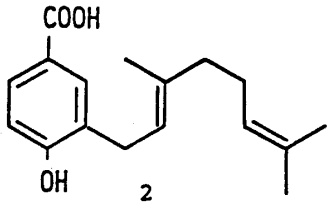

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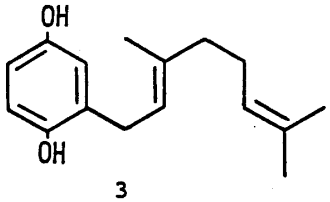

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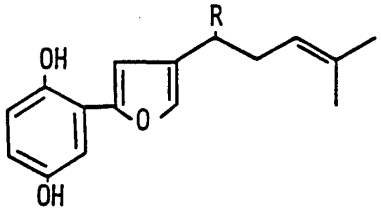

4 : R=OCOCH=C(CH3)CH3

5 : R=H

Geranylhydroquinone (3): $C_{16}H_{22}O_2$, UV $\lambda_{max}^{methanol}$ nm (log ε) 208 (4.05), 293 (3.53), colorless oil (73mg), and its mass spectrum showed m/z 246 as the parent ion. Its ^{1}H-NMR spectrum (200 MHz, $CDCl_3$) showed an ABX pattern in the aromatic region [δ 6.68 (1H, d, J=8 Hz), 6.60 (1H, d, J=3 Hz), 6.57 (1H, dd, J=3, 8 Hz)], two vinyl protons [δ 5.29 (1H, t, J=7 Hz), 5.07 (1H, br t, J=7 Hz)], benzyl methylene protons [δ 3.30 (2H, d, J=7 Hz)], four methylene protons [δ 2.00-2.22 (4H, m)], and three methyl groups on double bonds [δ 1.75 (3H, s), 1.68 (3H, s), 1.60 (3H, s)]. On the basis of these data, 3 was considered to be geranylhydroquinone. The identification was verified by direct comparison with an authentic sample.[3)]

Shikonofuran E (4): $C_{21}H_{24}O_5$, $[\alpha]_D$ -60.8° (C=0.5, $CHCl_3$), UV $\lambda_{max}^{methanol}$ nm (log ε) 214 (4.27), 264 (sh)(3.99), 269 (4.08), 281 (3.99), 323 (3.96), colorless oil (93 mg). The molecular formula was supported by the peak at m/z 356 in the mass spectrum. The spot of 4 on TLC (SiO_2) turned orange on standing overnight. This suggested that 4 had a hydroquinone moiety in the molecule, which was subject to oxidation to give a benzoquinone derivative such as echinofuran.[7)] The ^{1}H-NMR

spectrum of 4 (200 Hz, $CDCl_3$) showed three protons assignable to a 1,3,4-trisubstituted benzene ring [δ 6.99 (1H, d, J=3 Hz), 6.79 (1H, d, J=9 Hz), 6.68 (1H, dd, J=3,9 Hz)], two aromatic protons characteristic of a furan ring [δ 7.44 (1H, s), 6.70 (1H, s)], one proton on a carbon having a hydroxyl group [δ 5.79 (1H, t, J=7 Hz)], a vinyl proton [δ 5.09 (1H, br t, J=7 Hz)], two methylene protons [δ 2.60 (2H, t, J=7 Hz)], and two methyl groups on double bonds [δ 1.68 (3H, s), 1.61 (3H, s)]. In addition, the signals assignable to a β, β-dimethylacryloyloxy group [δ 5.71 (1H, br s), 2.17 (3H, s), 1.90 (3H, s)] were observed. These data were similar to those for shikonofuran E which was isolated by Yoshizaki et al. [8] as a mixture of shikonofuran E and D from a Chinese crude drug "Yingzicao" (L. erythrorhizon). Compound 4 was oxidized with Ag_2O in dry ether to give echinofuran C, the physical data (IR, UV, ^{1}H-NMR, and MS) of which were identical with those described in ref. 9). Furthermore, this compound was identified by direct comparison with an authentic sample of echinofuran C. This is the first time that 4 has been isolated as a pure compound.

Deoxyshikonofuran (5): $C_{16}H_{18}O_3$, UV $\lambda_{max}^{methanol}$ nm (log ε) 207 (4.18), 266 (sh)(4.02), 272 (4.10), 284 (4.39), 324 (3.94), colorless needles (mp 139-141°C, 33mg). Compound 5 showed the same color change on TLC plate as 4, indicating that 5 was a furylhydroquinone derivative similar to 4. The mass spectrum of 5 showed the parent peak at m/z 258, and its ^{1}H-NMR spectrum (200MHz, $CDCl_3$) showed in the aromatic region a signal pattern similar to 4:[δ 6.97 (1H, d, J=3 Hz), 6.83 (1H, d, J=9 Hz), 6.67 (1H, dd, J=3, 9 Hz)] for benzene protons, and [δ 7.27 (1H, s), 6.56 (1H, s)] for furan protons. In addition, one vinyl proton [δ 5.16 (1H, t, J=7 Hz)], two methylene protons [δ 2.48 (2H, t, J=7 Hz), 2.27 (2H, dt, J=7 Hz)] and two methyl groups on double bonds [δ 1.70 (3H, s), 1.61 (3H, s)] were observed. This suggested that 5 was a shikonofuran derivative having no oxygen function on the side chain, which was confirmed by the oxidation of 5 with Ag_2O in dry ether to give the benzoquinone derivative echinofuran B [9] in a quantitative yield.

The methanol extract of the shikonin-free cell cultures (400 g dry wt.) grown in LS medium was fractionated the same as the pigmented cells cultured in M9 medium. However, only 2 (4 mg) was isolated and none of the other three compounds (3, 4, 5) was detected by TLC and HPLC.

This is the first time that compounds 2 and 3, which are considered to be the key intermediates in shikonin biosynthesis,[3] were isolated from Lithospermum cultured cells or the intact plants. It should also be noted that even cells that do not produce shikonin when cultured in LS liquid medium are capable of synthesizing a small amount of the intermediate 2, although the decarboxylation process of 2 seems to be strongly repressed in LS medium as in callus cultures grown on LS agar medium containing 2,4-D instead of indoleacetic acid (IAA).[3]

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