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FORMATION AND SECRETION OF A NEW BROWN BENZOQUINONE BY HAIRY ROOT CULTURES OF *LITHOSPERMUM ERYTHRORHIZON*

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; hairy root cultures; secretion; shikonin; benzoquinone; echinofuran; hydroxyechinofuran B.

Abstract—Hairy roots of *Lithospermum erythrorhizon* in Murashige-Skoog medium produce a new brown benzoquinone derivative instead of red naphthoquinone (shikonin) derivatives as the main secondary metabolites. The structure of the new quinone was elucidated to be 2-[4-(*E*-4-hydroxymethyl-3-pentenyl)-furan-2-yl]-1,4-benzoquinone (called hydroxyechinofuran B) based on spectroscopic data. Its biosynthesis may be via geranylhydroquinone, a key biosynthetic intermediate of shikonin.

INTRODUCTION

Hairy root cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) in a hormone- and ammonium ion-free liquid medium are capable of synthesizing large amounts of red naphthoquinone pigments [1], shikonin (**1**) derivatives, which possess antibacterial activities and have been used for silk staining and as an external remedy for burns, wounds, haemorrhoids and skin ulcers. Fujita *et al.* [2] reported that the type of nitrogen sources in the medium greatly influenced the production of shikonin derivatives in the undifferentiated cell cultures; in particular ammonium ion conspicuously inhibited the production of the pigment. Recently, we found that the addition of a small amount of ammonium ion as NH_4NO_3 into the medium made the hairy root cultures start to produce a new abnormal brown compound (**2**), given the trivial name hydroxyechinofuran B, accompanied by reduction of the growth of the hairy roots and the formation of shikonin derivatives. Hydroxyechinofuran B was secreted into the medium. This paper reports on the isolation and characterization of the new brown compound.

RESULTS AND DISCUSSION

The culture medium was extracted with ether and the new quinone isolated by chromatography. Hydroxyechinofuran B (**2**), $C_{16}H_{16}O_4$, gave UV–VIS absorption maxima ($CHCl_3$) at 260 and 465 nm (log ε 4.13 and 3.55, respectively), and IR bands at 2925, 1655 (br), 1600, 1290, 990 and 900 cm^{-1}. These data as well as ^1H NMR data showing signals at δ 6.74 (1H, *s*), 6.75 (1H, *s*) and 7.01 (1H, br. *s*) suggested that **2** was a monosubstituted benzoquinone, and NMR signals at δ 7.35 and 7.36 (1H each, *s*) indicated the presence of a 2,4-disubstituted furan ring. The presence of the furan-2-yl benzoquinone group was supported by the same pattern of spectroscopic data as observed in those of echinofuran B (**3**) isolated from *Lithospermum* cell suspension cultures grown on activated C-supplemented medium [3] and of echinofuran from *Echium lycopsis* callus cultures [4].

To clarify the structure of the quinone moiety, **2** was converted to its leucoacetate by reductive acetylation with Zn-Ac_2O-pyridine. The ^1H NMR spectrum showed, in the aromatic proton region, an ABX pattern (δ 6.99, *dd*, J = 2.9 and 8.8 Hz; 7.11, *d*, J = 8.8 Hz; 7.51, *d*, J = 2.9 Hz) typical for a mono-substituted 1,4-quinol diacetate as in the case of the leucodiacetates of echinofuran [4], and echinofurans B and C [3], indicating that **2** was a monosubstituted benzoquinone. In addition, the presence of 2,4-disubstituted furan ring was confirmed by two 1H singlets at δ 6.58 and 7.25 as in the leucodiacetates of echinofuran [4], and echinofurans B and C [3].

The ^1H NMR spectrum of **2** exhibited a quartet (δ 2.35, 2H, J = 7.3 Hz) due to allyl methylene protons, a triplet (δ 2.51, J = 7.3 Hz) due to methylene protons adjacent to the allyl methylene and a broad triplet (δ

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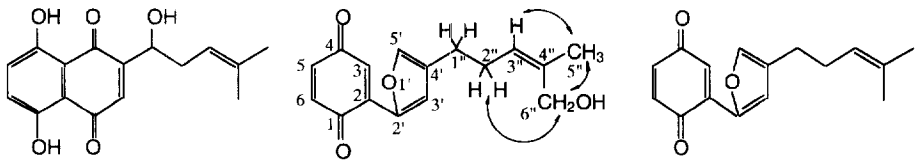

shikonin (**1**) hydroxyechinofuran B (**2**) echinofuran B (**3**)

Fig. 1. Structures of **1**, **2** and **3**, and NOE relationships in the side chain of **2**.

5.31, 1H, $J = 7.3$ Hz) for a vinyl proton also adjacent to the allyl methylene. These signals were similar to those of **3** [3] and of deoxyshikonin isolated from the roots of *L. erythrorhizon* and *Macrotomia euchroma* [5] except for a slightly lower-field shift of these signals, indicating the presence of a 4,4-disubstituted 3-butenyl group.

All the derivatives of shikonin and echinofuran isolated from boraginaceous plants have two geminal methyl groups at the 4-position of the 3-butenyl side chain [6], while the ^{1}H NMR spectrum of **2** showed the presence of an allyl methyl (δ 1.81, *s*) and a hydroxymethyl (δ 4.10, *s*), suggesting that one of the two geminal methyl groups is oxidized to a hydroxymethyl. This was supported by the observation from NOESY summarized in Figure 1. The observed correlations also showed that the *cis*-methyl of the geminal methyls on the side chain was oxidized to a hydroxymethyl. The ^{13}C NMR data (see Experimental) were in good agreement with the proposed structure and the assignments were supported by the C-H COSY spectral data as well as by comparison with those of **3** [3]. From the above evidence, the structure of hydroxyechinofuran B (**2**) was unambiguously confirmed.

Hairy roots [1] and undifferentiated cell cultures [2] of *L. erythrorhizon* were capable of producing large amounts of shikonin derivatives in an ammonium ion-free medium. When the hairy roots that were producing shikonin in an ammonium ion-depleted Murashige-Skoog (MS) medium were transferred to the normal MS medium, they ceased shikonin formation and started producing **2**. In MS medium containing half strength ammonium ion, the hairy roots were found to produce both **1** and **2**. When the culture medium was filtered through a filter paper (No. 2), which would retain shikonin derivatives, compound **2** passed through the paper, indicating that the shikonin derivatives are on/inside the roots and their debris due probably to their hydrophobic character, and that all of **2** is secreted into the medium.

Compound **2** as well as shikonins and echinofurans must be derived from *m*-geranyl-*p*-hydroxybenzoic acid through geranylhydroquinone, an important biosynthetic precursor of shikonin. Heide *et al.* [7] reported that undifferentiated cell cultures in Linsmaier-Skoog (LS) medium accumulate *p*-hydroxybenzoic acid glucoside inside the cells because LS medium, probably due to the presence of ammonium ion, inhibits the activity of *p*-hydroxybenzoic acid geranyltransferase. However, the present study suggests that a small amount of *m*-geranyl-*p*-hydroxybenzoic acid is formed and that this is then converted to an abnormal benzoquinone as observed in the undifferentiated cell cultures [3, 8] because the biosynthetic route leading from geranylhydroquinone to shikonin does not work well in the cells cultured in the medium containing ammonium ion [2, 7].

EXPERIMENTAL

General

UV-VIS: CH_2Cl_2 or $CHCl_3$; IR: $CHCl_3$; ^{1}H and ^{13}C NMR (JEOL Alpha-400, 400 and 100 MHz, respectively): $CDCl_3$ with TMS as an int. standard; EI–MS (JEOL JMS-SX102 Hybrid); TLC: silica gel, solvent $CHCl_3$.

Plant material

Hairy roots of *Lithospermum erythrorhizon* were induced by direct infection of axenic shoot cultures with *Agrobacterium rhizogenes* strain 15834. The bacteria were eliminated on MS [9] solid medium containing 1 g l^{-1} carbenicilline. The transformation was proven by the detection of the opines using paper electrophoresis according to the method of Petit *et al.* [10].

Culture method

The hairy roots were grown in the dark at 25° in a hormone-free and ammonium ion-depleted MS agar medium supplemented with sucrose (30 g l^{-1}) at pH 6. The stock cultures were subcultured on a monthly basis in 300 ml conical flasks containing hormone-free and ammonium ion-depleted MS medium (100 ml) solidified with 3 g l^{-1} gelrite. For the production of **2**, 1 g of fresh hairy roots were transferred to a 100 ml conical flask containing 30 ml MS liquid medium supplemented with 30 g l^{-1} sucrose and agitated on a rotary shaker at a speed of 70 strokes min^{-1}.

Extraction and isolation

The culture medium (30 ml × 50) of the hairy roots was combined and extracted with Et_2O (× 3) and dried

(Na_2SO_4). The extract was concd *in vacuo*, dissolved in $CHCl_3$, and subjected to CC on silica gel (130–270 mesh) using a mixture of C_6H_6 and $CHCl_3$ (1 : 1) as an eluent. The fractions that contained a brown compound were concd and purified by prep. TLC to give **2** as an oil (15 mg). UV–VIS ($CHCl_3$) λ max nm (log ε): 260 (4.13) and 465 (3.55); IR ν max cm^{-1}: 2925, 1655, 1600, 1565, 1290, 1090, 990, 920, 900. ^1H NMR: δ 1.81 (3H, *s*, 5″-H), 2.35 (2H, *q*, J = 7.3 Hz, 2″-H), 2.51 (2H, *t*, J = 7.3 Hz, 1″-H), 4.10 (2H, *s*, 6″-H), 5.31 (1H, *t*, J = 7.3 Hz, 3″-H), 6.74 and 6.75 (1H each, *s*, 5 & 6-H), 7.01 (1H, *s*, 3-H), 7.35 and 7.36 (1H each, *s*, 3′- and 5′-H). ^{13}C NMR: δ 21.22 (C-5″), 24.95 (C-1″), 27.79 (C-2″), 61.50 (C-6″), 119.98 (C-3′ or C-3), 125.21 (C-3 or C-3′), 126.91 (C-3″), 128.49 (C-4′), 133.49 (C-2), 135.59 (C-4″), 136.43 (C-5 or C-6), 136.53 (C-6 or C-5), 142.23 (C-5′), 146.09 (C-2′), 185.48 (C-1 or C-4), 187.28 (C-4 or C-1). Found $[M]^+$ 272.1058, $C_{16}H_{16}O_4$ requires $[M]^+$ 272.1049. EI-MS (70 eV) *m/z* (rel. int.): 272 $[M]^+$ (20), 243 (24), 223 (27), 187 (100), 160 (40), 137 (37), 131 (31), 103 (48), 77 (47), 43 (33).

Reductive acetylation of compound **2**

A mixture of **2** (10 mg), Zn powder (20 mg), Ac_2O (0.5 ml) and pyridine (0.5 ml) was allowed to stand at room temp. for 3 hr. Ice–H_2O was poured onto the mixture and the whole soln was extracted with Et_2O (15 ml × 3). The Et_2O layer was washed with 1 M HCl, 5% $NaHCO_3$ and H_2O, successively, dried (Na_2SO_4) and concd *in vacuo*. The crude product was purified by prep. TLC (Rf 0.4) to yield a leucotriacetate as an oil (10 mg). UV(CH_2Cl_2) λ max nm (log ε): 285 nm (3.96) and 228 (3.85); IR ν max cm^{-1}: 1750 br. and 1600; ^1H NMR: δ 1.76 (3H, br. *s*, 5″-H), 2.05 (3H, *s*, 6″-OAc), 2.31 (3H, *s*, 1- or 4-OAc), 2.35 (2H, *q*, J = 7.3 Hz, 2″-H), 2.38 (3H, *s*, 4- or 1-OAc), 2.49 (2H, *t*, J = 7.3 Hz, 1″-H), 4.56 (2H, br. *s*, 6″-H), 5.43 (1H, *t*, J = 7.3 Hz, 3″-H), 6.58 (1H, br. *s*, 3′-H), 6.99 (1H, *dd*, J = 2.9 and 8.8 Hz, 5-H), 7.11 (1H, *d*, J = 8.8 Hz, 6-H), 7.25 (1H, *s*, 5′-H), 7.51 (1H, *d*, J = 2.9 Hz, 3-H). (found: $[M]^+$ 400.1529; $C_{22}H_{24}O_7$ requires $[M]^+$, 400.1522).

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