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Formation and secretion of a unique quinone by hairy root cultures of *Lithospermum erythrorhizon*

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Abstract

A unique colorless quinone, named rhizonone, showing strong antifungal activity against spore germination of *Cladosporium fulvum* was isolated from the hairy root cultures of *Lithospermum erythrorhizon* in MS medium. The structure was elucidated as 5a-methyl-5a,6,10b,10c-tetrahydro-2H-anthra[9,1-bc]furan-7,10-dione mainly on the basis of spectroscopic data. This unique carbon skeleton, not previously known among the compounds isolated from this plant species, might be derived from geranylhydroquinone, an important intermediate of shikonin biosynthesis. Rhizonone was secreted into the medium while shikonin derivatives accumulated on the surface of the hairy roots and their debris.

Keywords: *Lithospermum erythrorhizon*; Boraginaceae; Quinone; Geranylhydroquinone; Rhizonone; Shikonin; Hairy root; Antifungal activity

1. Introduction

The hairy roots of *Lithospermum erythrorhizon* (Boraginaceae) produce large amounts of red-colored shikonin (**IV**) derivatives, the main secondary metabolites in this plant, in an ammonium-free liquid medium (Shimomura, Sudo, Saga, & Kamada, 1991). We (Fukui, Hasan, Ueoka, & Kyo, 1998) have reported that a new brown quinone, named hydroxyechinofuran B (**V**), is formed by hairy roots cultured in normal Murashige–Skoog (MS) liquid medium (Murashige, & Skoog, 1962) in which shikonin biosynthesis is repressed because of the presence of ammonium ion (Fujita, Hara, Ogino, & Suga, 1981). Recently, a bioautographical method of searching for biologically active components against spore germination of *Cladosporium fulvum* on TLC revealed the formation of a colorless antifungal metabolite (**I**, tentatively named rhizonone) by the hairy roots cultured in the same MS medium, which showed much stronger activity against spore germination than shikonin. This paper reports the characterization of the new antifungal compound as well as a possible biogenetic route to form a unique carbon skeleton from geranylhydroquinone, an important intermediate of shikonin biosynthesis.

2. Results and discussion

Rhizonone (**I**) was isolated as an oil (20 mg) from the culture medium (15 l) of *L. erythrorhizon* hairy roots. This compound (**I**) and a brown compound (hydroxyechinofuran B) reported previously (Fukui et al., 1998) were found to be present only in the filtrate through a No.2 filter paper, while almost all the shikonin derivatives in/on the hairy roots and their debris on the paper, indicating that the hairy roots entirely secreted these compounds into the medium.

The molecular formula of **I** was established to be $C_{16}H_{14}O_3$ by HREIMS; an IR band at 1660 cm^{-1} and UV max at 254 nm ($\log \varepsilon$: 4.29) showed the presence of a quinone moiety. These data suggest that **I** is de-

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Table 1
^{1}H– and ^{13}C NMR spectral data of rhizonone (**I**)

C	δ	DEPT	Significant HMBC/HMBC correlation with	H	δ	Multiplicity	J(Hz)
1	187.01	C=O	H-5, H-6, H-7, H-14				
2	145.15	=C=	H-8, H-14				
3	139.05	=C=	H-7, H-14				
4	186.31	C=O	H-5, H-6, H-14				
5	136.07	=CH		5 and 6	6.81	br.s	
6	136.22	=CH					
7	70.88	O–CH	H-15	7	4.68	dd	7.3, 2.4
8	49.41	CH	H-7, H-14a, H-15, H-16	8	2.88	m	7.3, 1.5
9	140.27	=C=	H-15				
10	114.25	=CH	H-9, H-12, H-15	10	5.86	m	
11	125.34	=CH	H-12 or -10, H-15	11	5.99	d	9.3
12	136.07	=H	H-16	12	5.95	dd	4.3, 9.3
13	31.44	=C=	H-8, H-14, H-16				
14	33.45	$-CH_2$	H-8, H-16	14a	2.24	dd	17.6, 2.4
				14b	2.87	d	17.6
15	70.96	$O-CH_2$	H-7	15	4.57	br.s	
16	16.1	CH_3	H-8, H-14	16	0.83	s	

rived from geranylhydroquinone, an important precursor of shikonin formation. However, although all known shikonin derivatives have two methyl groups in the side chain (Thompson, 1971), the NMR spectral data show the presence of a single methyl group suggesting that **I** has a unique carbon skeleton different from the compounds known in this plant.

Compound **I** was reductively acetylated with Zn–Ac_2O–pyridine to yield a leucodiacetate (**II**). The ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, DEPT and HMBC (COLOC) data of **I** and **II** are summarized in Tables 1 and 2, respectively. In the following sections, the numbering of the carbon skeleton of **I** and **II** is different from that of IUPAC, according to the biosynthetic information that rhizonone might be derived from geranylhydroquinone.

The ^{1}H NMR spectrum of **I** showed a 2H singlet at δ 6.81 which together with UV and IR data, suggested the presence of 2,3-disubstituted benzoquinone (partial structure A shown in graphical abstract). This was

Table 2
^{1}H and ^{13}C NMR spectral data of rhizonone leucodiacetate (**II**). (a), (b) and (c) indicate that the assigns are exchangeable between the same alphabet

C	δ	DEPT	Significant HMBC/COLOC correlation with	H	δ	Mult.	J(Hz)
1	148.61	=C–O-					
2	131.58(a)	=C=	H-14				
3	128.72(a)	=C=	H-7, H-14				
4	146.96	=C–O-	H-14				
5	121.48(b)	=CH-		5	6.98	d	8.8
6	122.41(b)	=CH-		6	7.03	d	8.8
7	72.36	O–CH	H-15	7	4.79	d	7.6
8	49.95	CH	H-14	8	2.93	d	7.6
9	141.27	=C=					
10	114.02(c)	=CH	H-11 or H-12	10	5.84(a)	m	
11	125.26(c)	=CH		11	5.99(a)	dd	9.2, 4.2
12	136.69(c)	=CH	H-16	12	5.89(a)	d	9.2
13	32.12	=C=	H-7, H-14, H-16				
14	34.91	$-CH_2$	H-16	14a	2.45	d	15.6
				14b	2.68	d	15.6
15	70.15	$O-CH_2$		15a	4.51	d	13.2
				15b	4.54	d	13.2
16	16.55	CH_3	H-14	16	0.72	s	
Ac	169.72						
	20.99	CH_3		Ac	2.31	s	
Ac	169.11						
	20.83	CH_3		Ac	2.35	s	

supported by a pair of 1H doublets ($J=8.8$ Hz) at δ 6.98 and δ 7.03 in the ^{1}H NMR spectrum of **II**. The presence of methylene protons (B) probably adjacent to the benzoquinone moiety was indicated by the signals at δ 2.24 (dd, $J=17.6$ and 2.4 Hz) and d 2.87 (d, $J=17.6$ Hz), the former probably showing a homoallylic coupling with another substituent on the quinone moiety. This partial structure was supported by a pair of 1H doublets at δ 2.45 and δ 2.68 ($J=15.6$ Hz each) in the ^{1}H NMR of **II**. A 3H singlet at δ 0.83 in **I** and a 3H singlet at δ 0.72 in **II** confirm the presence of a tertiary methyl (C). ABC-type signals (δ 5.86, m; δ 5.95, dd, $J=4.3$ and 9.3 Hz, and δ 5.99, d, $J=9.3$ Hz) in the ^{1}H NMR of **I** and the same pattern in that of **II** (δ 5.84 m, δ 5.89 d, $J=9.2$ Hz, and δ 5.99 dd, $J=4.2$ and 9.2 Hz) together with UV data and ^{13}C NMR data indicate the presence of C–C conjugated double bonds (D) having three straight protons. A 2H broad singlet (δ 4.57) assigned to two oxygenated methylene protons, a 1H double doublet at δ 2.88 ($J=1.5$ and 7.3 Hz) and a 1H double–doublet (δ 4.68, $J=7.3$ and 2.4 Hz) due to a methine proton on a carbon having the same oxygen as the above oxygenated methylene indicate the presence of a partial structure E, because two of the three oxygens in **I** are in the quinone moiety. This is supported by two AB-quartets in **II** (δ 2.93, $J=7.6$ Hz; δ 4.51, $J=13.2$ Hz; δ 4.54, $J=13.2$ Hz; δ 4.79, $J=7.6$ Hz). These five partial structures (A–E) were supported by the other NMR data of **I** and **II** as summarized in Tables 1 and 2.

The attachment of B to A which is estimated from the chemical shifts (δ 2,24 and 2,87) of the methylene protons in B was confirmed by the COLOC and HMBC correlation analyses between the methylene proton signals and the carbon signals (δ 139.05, 145.15, 186.31 and 187.01) of the quinone moiety, and also by the same correlation analyses between the carbon signals (δ 128.72, 131.58, and 146.96) assigned to the hydroquinone moiety and the methylene proton signals (δ 2.45 and 2.68) in **II** as shown in Tables 1 and 2.

The H–H COSY spectrum of **I** indicates that the signals at δ 2.24 of B showed a long-range coupling ($J=2.4$ Hz) with the oxygenated methine proton at δ 4.68 of E. The methine and the oxygenated methine protons (δ 2.88 and 4.68) in E showed the COLOC and HMBC correlation marks with the carbon signal (δ 145.15 and 139.05, respectively) of A in **I** and also the HMBC spectrum of **II** indicated the correlation peak between the oxygenated methine of E and the carbon signal (δ 128.72) of the hydroquinone moiety (A). These correlation peaks as well as the chemical shift (δ 4.68) of the oxygenated methine proton (E) confirm that the partial structure E is attached to the quinone moiety in an ortho-position of B.

The attachment of one end (the methine carbon) of E to C was confirmed by the correlation peaks, in COLOC and HMBC spectra of **I**, between the methine carbon at δ 49.41 of E and the methyl protons (δ 0.83) of C and between the quaternary carbon (δ 31.44) of C and the methine proton (δ 2.88) of E.

In the COLOC and HMBC spectra of **I**, a quaternary carbon resonance at δ 31.44 in C was correlated to the methylene protons (δ 2.24 and 2.87) in B, indicating the connectivity of the methylene (B) to the quaternary carbon in C. This was supported by the observation that, in 2-D spectra of **II**, the methylene protons (δ 2.45 and 2.68) of B were correlated with the methyl carbon (δ 16.55) and the quaternary carbon (δ 32.12) of C. The connectivity of the four partial structures (A, B, C and E) is reasonably explained by the formation of a six-membered ring as shown in Fig. 1.

The chemical shifts of the oxygenated methylene protons of E (δ 5.57 in **I** and a pair of doublets: δ 4.51 and 4.54 in **II**) suggest that the methylene is connected with the olefinic carbon (D) having no protons. Also, the signals (δ 114.25, 125.34, 140.24) assigned to the olefinic carbons (D) were found to be correlated with the oxygenated methylene protons (δ 4.57) of E in the HMBC and COLOC spectra of **I**. In the H–H COSY spectrum of **I**, the methine proton of E was correlated with the proton of D, confirming that the methine is also bonded to the partial structure D. In the HMBC and COLOC spectra of **I**, the correlation patterns were observed between one of the olefinic carbon signals (δ 136.07) of D and the methyl protons (δ 0.83) of C, indicating that the partial structure D is also connected with C. These assignments show that another six-membered ring is formed by the partial structures (C, D and E). Thus, the chemical structure of rhizonone was elucidated as **I** shown in the graphical abstract. The stereochemistry has not been elucidated yet although the positive correlations in NOESY spectra of rhizonone and its leucodiacetate were observed between 7-H, 8-H and 16-H.

Rhizonone (**I**) is a novel metabolite possibly derived from geranylhydroquinone, an important intermediate of shikonin biosynthesis. The carbon skeleton of rhizonone could be established, as shown in Fig. 2, by the same biochemical reactions as the formation of shikonin (Inouye, Ueda, Inoue, & Matsumura, 1979) and

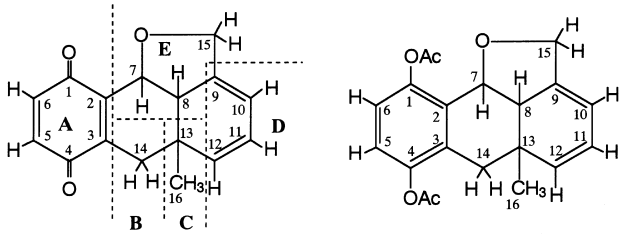

Fig. 1. Structures of rhizonone and its leucodiacetae.

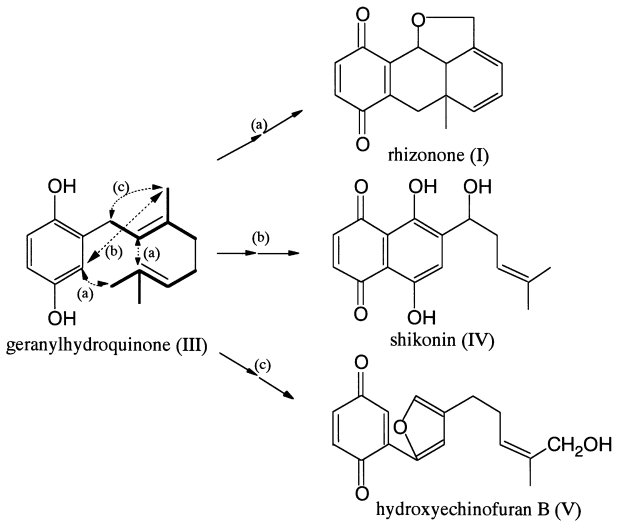

Fig. 2. Possible biosynthetic routes of rhizonone (a), shikonin (b) and hydroxyechinofuran B (c).

echinfuran (Fukui, Yoshikawa, & Tabata, 1984; Fukui, Tani, & Tabata, 1992) skeletons. Thus, the unique carbon skeleton of **I**, a novel quinone compound isolated from the hairy roots of *L. erythrorhizon*, is reasonably explained from the view point of biogenesis and is reported for the first time in this plant.

The inhibition zone against the spore germination of *C. fulvum* was produced with rhizonone at a concentration of less than 0.1 μg per spot, although with shikonin more than 0.5 μg was needed.

No rhizonone was produced in the undifferentiated cells cultured in MS liquid medium or in the ammonium ion-depleted medium. Much less rhizonone in the hairy root cultures was found in the ammonium ion-depleted MS medium, which stimulates the formation of shikonin. Rhizonone, which shows strong antifungal activity, might be formed and secreted against the invasion of pathogens under environmental conditions where antimicrobial shikonin (Tabata, Tsukada, & Fukui, 1982) is not produced. However, the production of rhizonone in the intact plant remains to be investigated.

3. Experimental

3.1. General

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

3.2. Plant material

Hairy roots of *L. erythrorhizon* were induced by direct infection of axenic shoot cultures with *Agrobacterium rhizogenes* strain 15834. The bacteria were eliminated on MS (Murashige, & Skoog, 1962) 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. (1983).

3.3. 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 a new antifungal compound, 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 rpm.

3.4. Bioautography

TLC on which the extracts and samples were spotted and developed were dried and sprayed with a suspension of the spores of *C. fulvum* in a medium containing 1 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 4 g KNO_3, 3 g K_2HPO_4, 7 g KH_2PO_4 and 50 g sucrose l^{-1}. The plate was kept at 25° under wet conditions in the dark for a week. The antifungal materials were detected as off-white spots on a gray–black background.

3.5. Extraction and isolation

The culture medium (10 l) of the hairy roots was combined and extracted with Et_2O (×3) and dried (Na_2SO_4). The extract was concd. in vacuo, and dissolved in $CHCl_3$, and subjected to column chromatography on silica gel (130–270 mesh) using a mixture of benzene and $CHCl_3$ (1:1) as an eluent. The fractions containing the colorless, UV-absorbing compound were concd. and purified by prep. TLC (silica gel, solvent: $CHCl_3$, R_f 0.3) to isolate the UV-absorbing compound (**I**) as an oil (20 mg). $[\alpha]_D$ ($c = 0.025$ MeOH) −240°, $[\alpha]_{500}$ −1140°, $[\alpha]_{437}$ +700°, $[\alpha]_{355}$ 0°, $[\alpha]_{286}$ −6560°; UV ($CHCl_3$) λ_{max} nm (log ε): 254 (4.29); IR ($CHCl_3$) ν_{max} cm^{-1}: 2850, 1660, 1590, 1300. 1120, 1070, 1030, 970, 920, 845. The data of various types of NMR measurements are summarized in Table 1. Found $[M]^+$ 254.0947, $C_{16}H_{14}O_3$ requires $[M]^+$ 254.0943. EI-MS (70 eV) m/z (rel. int.): 254 $[M]^+$ (40),

250 (35), 225 (100), 211 (50), 181 (63), 165 (50), 152 (47), 128 (50), 105 (60), 95 (40).

3.6. Reductive acetylation of compound *I*

A mixture of **I** (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 h. 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 HCI, 5% $NaHCO_3$ and H_2O, successively, dried (Na_2SO_4) and concd. in vacuo. The crude product was purified by prep. TLC (silica gel, $CHCl_3$, R_f 0.4) to yield a leucodiacetate (**II**) as an oil (10 mg). UV(CH_2Cl_2) λ_{max} nm ($\log \varepsilon$): 268 nm (3.81); IR ($CHCl_3$) ν_{max} cm^{-1}: 1750 br. and 1600. The data of the ^1H and ^{13}C NMR analysis are summarized in Table 2. EI-MS (70 eV) m/z (rel. int.): 340 (15), 300 (20), 283 (55), 258 (85), 240 (100), 236 (60), 211 (45). HR-EI-MS (Found: $[M]^+$ 340.1271; $C_{20}H_{20}O_5$ requires: $[M]^+$, 340,1311).

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