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TWO PHENOLIC ACIDS FROM *LITHOSPERMUM ERYTHRORHIZON* CELL SUSPENSION CULTURES

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Key Word Index—*Lithospermum erythrorhizon*, Boraginaceae, plant cell culture, biosynthesis, phenolic acid, rosmarinic acid, lithospermic acid

Abstract—Two phenolic acids isolated from colourless cell cultures of *Lithospermum erythrorhizon* were identified as rosmarinic acid and lithospermic acid However, these compounds were not detected in pigmented cell cultures producing shikonin derivatives

INTRODUCTION

Cultured cells (strain M-18) of *Lithosperum erythrorhizon* Sieb et Zucc [1–4] are capable of producing the same red naphthoquinone pigments (shikonin derivatives) as those found in the root bark of the original plant, when grown in a 'production medium' (M9) [6, 7] supplemented with IAA (10^{-6} M) and kinetin (10^{-5} M). When grown in Linsmaier–Skoog's liquid medium (LS) [8] containing the same growth regulators, however, the cells cease producing shikonin derivatives owing to excess ammonium ions present in this medium [6] Little is known as to what metabolites might accumulate in the unpigmented cells in place of shikonin This paper deals chiefly with the identification and quantitative estimation of two phenolic compounds isolated from these cells

RESULTS AND DISCUSSION

The methanol extract of fresh cells grown in LS medium for 2 weeks was concentrated and extracted successively with benzene, ethyl acetate and *n*-butanol TLC analysis showed that both the ethyl acetate and *n*-butanol extracts contained ferric chloride-positive phenolics, which were absent from shikonin-producing cultures grown in M9 medium The ethyl acetate extract was chromatographed on a Sephadex LH-20 column with 80% ethanol as solvent to give compound **1** as an off-white powder Its ^{1}H NMR (200 MHz, CD_3OD) spectrum showed two ABX systems [δ6 62 (*dd*, J = 2, 8 Hz, 1H), 6 69 (*d*, J = 8 Hz, 1H), 6 78 (*d*, J = 2 Hz, 1H), 6 78 (*d*, J = 8 Hz, 1H), 6 93 (*dd*, J = 2, 8 Hz, 1H), 7 02 (*d*, J = 2 Hz, 1H)] attributable to 1,3,4-trisubstituted benzene, an AB system [δ6 27 (*d*, J = 16 Hz, 1H), 7 51 (*d*, J = 16 Hz, 1H)] assigned to *trans*-olefinic protons, a 1H double-doublet [δ5 10 (*dd*, J = 5, 9 Hz, 1H)] assigned to a methine proton, and two 1H double-doublets [δ2 95 (*dd*, J = 9, 14 Hz, 1H), 3 10 (*dd*, J = 5, 14 Hz, 1H)] assigned to methylene protons on a carbon adjacent to the carbon bearing the above methine proton These data suggest that **1** is rosmarinic acid, which was first isolated from *Rosmarinus officinalis* (Labiatae) [9] The ^{1}H NMR and ^{13}C NMR data and $[\alpha]_D$ of **1** agreed with those previously reported [9–11], **1** was identified as rosmarinic acid by comparison (TLC, HPLC, $[\alpha]_D$ and IR) with an authentic sample

The *n*-butanol extract was fractionated by a similar method to that applied to the ethyl acetate extract to give compound **2** as an off-white powder Its ^{1}H NMR spectrum resembled that of **1** except that it showed two

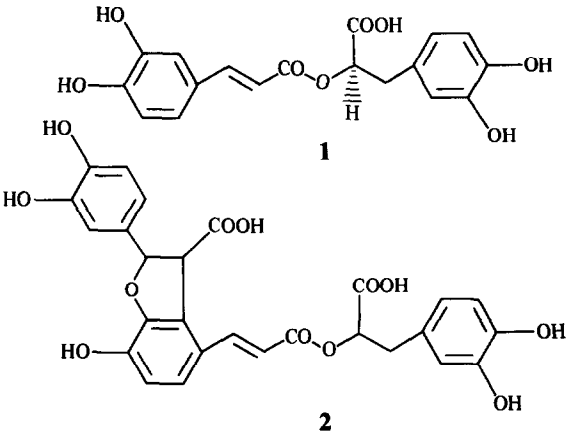

Table 1 Contents of **1** and **2** in the cultured cells and fresh root of *Lithospermum erythrorhizon*

	Content of phenolic acids (mg/l medium)		
Compound	Unpigmented cultured cells*	Pigmented cultured cells†	Fresh root (mg/g fr wt)
Rosmarinic acid (**1**)	61 2‡	—§	trace
Lithospermic acid (**2**)	12 9	—	2 49

*Culture strain M-18 grown in M9 medium
†Culture strain M-18 grown in LS medium
‡Equivalent to 5 5 mg/g dry wt
§Not detected

additional AB systems [δ4 35 (*d*, J = 5 Hz, 1H) 5 88 (*d*, J = 5 Hz, 1H), 6 81 (*d*, J = 8 Hz, 1H), 7 51 (*d*, J = 8 Hz, 1H)] which were assigned to two protons on a benzene ring and to two protons on two adjacent sp^3 carbons These data suggest that **2** is lithospermic acid, which was first isolated from *Lithospermum ruderale* (Boraginaceae) [12] The structure of **2** was confirmed by comparison of its spectral data (^{1}H NMR, ^{13}C NMR and IR) with those given in refs [10] and [11], furthermore, **2** behaved identically to an authentic sample on TLC (four solvent systems)

As the presence of **1** or **2** in *L erythrorhizon* was hitherto unknown, their contents in the fresh root and cultured cells were determined by HPLC The results showed that a comparatively large amount of **2** and a trace of **1** are present in the root which contains shikonin derivatives only in the cork layers By contrast to the unpigmented cells, neither of the two compounds was detected in pigmented cultured cells or in the culture medium

EXPERIMENTAL

Plant materials and culture method Callus cultures (strain M-18) of *Lithospermum erythrorhizon* were originally derived from the germinating seed on Linsmaier–Skoog's basal agar medium [8] containing 2,4-D (10^{-6} M) and kinetin (10^{-5} M), and subcultured on the same medium containing IAA (10^{-6} M) in place of 2,4-D for 10 years at 25° in the dark Cell suspension cultures initiated from the callus were grown in the same medium without agar on a reciprocal shaker (100 strokes/min) at 25° in the dark and subcultured at intervals of 2 weeks for *ca* 1 year Cells grown in LS medium were harvested for chemical analysis 2 weeks after inoculation To obtain shikonin-producing cells, unpigmented cells, which had been pre-cultured for 2 weeks in LS medium, were transferred to M9 medium [7] and cultured for 2 weeks before harvest Fresh roots of *L erythrorhizon* were obtained from a plant cultivated at Kyoto Herbal Garden of Takeda Chemical Industries Ltd

Extraction and isolation Cells (500 g fr wt) cultured in LS medium were extracted with MeOH (2 5 l) The extract was concd *in vacuo*, and extracted successively with C_6H_6, EtOAc and *n*-BuOH The EtOAc extract (470 mg) was chromatographed on a column of Sephadex LH-20 (45 × 2 cm) and eluted with 80% EtOH to collect 60 fractions (5 g each) Combined fraction Nos 24–28 (100 mg) gave chromatographycally pure **1** as an off-white powder The *n*-BuOH extract (1 42 g) was also fractionated according to the method described above Fraction Nos 23–27 (98 mg) afforded **1** as an off-white powder, fraction Nos 30–40 gave **2** (20 mg) as an off-white powder, fraction Nos 28 and 29 were found to be a mixture of **1** and **2** (23 mg) The physical data ($[\alpha]_D$, IR, ^{1}H NMR and ^{13}C NMR) of **1** and **2** were identical with those described in refs [9–11]

HPLC and TLC Pigmented cells (30 g fr wt) cultured in M9 medium, unpigmented cells (25 g fr wt) cultured in LS medium, and the fresh root of *L erythrorhizon* (9 g fr wt) were homogenized in MeOH (50 ml) The extracts were subjected to HPLC analysis, column, TSK-GEL LS410A (ODS, Toyo Soda, Japan), 150 × 4 6 mm, solvent, H_2O–MeOH–HOAc (20 30 1, v/v), flow rate 1 ml/min, pressure, 100 kg/cm^2, detection, absorption at 280 nm The quantity of **1** or **2** was estimated from the corresponding peak area recorded by Chromatopac C-RIA (Shimadzu, Japan) The purity of isolated **1** or **2** was checked by TLC using the following solvents EtOAc–MeOH–H_2O (100 16 5 13 5), toluene–EtOAc–HOAc (4 5 2), toluene–HCO_2Et–HCO_2H (14 10 3) and *n*-BuOH–HOAc–H_2O (10 2 1)

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