Phytochemistry, Vol. 22, No. 3, pp. 721–722, 1983.
Printed in Great Britain.

0031–9422/83/030721–02$03.00/0

PRODUCTION OF CRYPTOTANSHINONE AND FERRUGINOL IN CULTURED CELLS OF *SALVIA MILTIORRHIZA*

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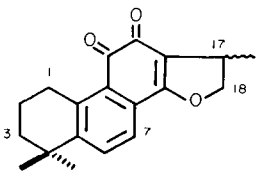
(*Received* 30 *June* 1982)

Key Word Index—*Salvia miltiorrhiza*; Labiatae; callus; cell line; diterpenes; cryptotanshinone; ferruginol.

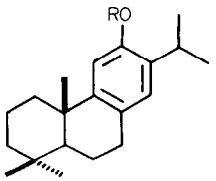
Abstract—Cell culture of *Salvia miltiorrhiza* on a solid medium followed by successive liquid culture produced a cell line, the cells of which contained predominantly two diterpenes, cryptotanshinone and ferruginol. Prior to this study, ferruginol had been isolated and first identified from the roots of the parent plant.

INTRODUCTION

The root (Tan-shen in Chinese and Tan-jin in Japanese) of *Salvia miltiorrhiza* Bunge is an ancient Chinese drug, ranked as a super-grade medicine in 'Shen-nung-pen-tsao-ching', the oldest classical Chinese herbal. It has been shown to contain a variety of quinonoid diterpenes (tanshinones), i.e. tanshinone II (ref. [1] and references cited therein), cryptotanshinone (**1**) [2] and related pigments [3–7] of an orange-red colour. For the last few decades, the root has been used in China as a remedy for angina pectoris [8] and recently Chinese pharmacologists suggested that cryptotanshinone (**1**) is an active principle in the root [8]. Therefore, we have undertaken to produce cryptotanshinone (**1**) from a cell culture of *S. miltiorrhiza*. This paper reports on the establishment of a cell line (cell line A), the cells of which contained abundant amounts of cryptotanshinone (**1**) together with the diterpene ferruginol (**2a**), a compound which we were also able to isolate from the roots of the parent plant.



1 Cryptotanshinone



2a R = H, Ferruginol
2b R = Ac, Ferruginol acetate

*Cf. *S. miltiorrhiza* roots from which salviol (2α-hydroxyferruginol) has been isolated [15].

RESULTS AND DISCUSSION

Identification of ferruginol (**2a**) *from* S. miltiorrhiza *roots*

The chloroform extract of dried roots of the plant was subjected to CC to give a diterpene (a pale yellow resin, $C_{20}H_{30}O$ $[M]^+$, *m/z* 286.2302), the mass spectrum of which was superimposable on that published for ferruginol (**2a**) [9]. The corresponding acetate, $C_{22}H_{32}O_2$ $[M]^+$, *m/z* 328.2399, prepared in a usual manner, had the same mp, optical rotation and mass fragmentation pattern [9], as those reported for ferruginol acetate (**2b**), and its ^1H NMR data (see the Experimental) were consistent with the structure **2b**. Final proof of the identity of the acetate was provided by mmp (80–81°) and by direct comparison of the IR, ^1H NMR and mass spectral data with those of an authentic sample of **2b** [10]. This is the first report of the isolation of ferruginol (**2a**) in the Labiatae and even in the herbal plant kingdom, it was previously found only in the woody plants *Podocarpus ferrugineus* (Podocarpaceae) [12], *Cupressus torulosa* D. (Cupressaceae) [11], *Juniperus communis* L. (Cupressaceae) [13] and *Cryptomeria japonica* D. Don. (Taxodiaceae) [14]. Co-occurrence of **2a*** and the tanshinones, such as **1**, in the plant kingdom was also found for the first time. On the assumption [16] that the tanshinones are biosynthesized via a diterpene having a ferruginol ring system, the biogenetic relationship between **1** and **2a** is worthy of study in the future.

Identification of cryptotanshinone (**1**) *and ferruginol* (**2a**) *in suspension cultured cells of cell line A.*

Of six cell lines derived from the seedlings of *S. miltiorrhiza* (see Experimental) only suspension cultures of cell line A produced abundant amounts of both **1** and **2a**. The harvested cells of this line afforded, after chloroform extraction and subsequent prep. TLC, **1** and **2a** in pure form. Confirmation of the identity of cryptotanshinone (**1**) was provided by direct comparison of its ^1H NMR, mass spectral, TLC and GC (column temperature 210°) data with those given by an authentic sample of **1** [2, 18]. The identity of the isolated ferruginol was confirmed by direct comparison [mass spectrum, TLC, GC (column temperature 190°)] with the standard isolated from the parent plant. The contents of **1** (0.8%) and **2a** (1.3%) in the dried cells were as high as those (0.7

and 1.1%, respectively) found in the dried roots of the plant (estimated by GC). In contrast to cell line A, all of the other cell lines produced only ferruginol (**2a**) in good amount.

EXPERIMENTAL

IR: $CHCl_3$; ^1H NMR: $CDCl_3$ with TMS as int. standard; MS and accurate MS: 75 eV; GC with FID: 2 m × 2 mm packed with 2% OV-1 (80–100 mesh); carrier, N_2 at 3 kg/cm^2. Si gel HF-254 and PF-254 were used for TLC and prep. TLC, respectively.

Plant material. Seeds of *S. miltiorrhiza* were provided by the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China in 1980. The plant was propagated from the seeds in the Medicinal Plant Garden of this University and was identified by one of us (K.Y.).

Isolation and identification of ferruginol (**2a**) *from plant roots.* The dried roots (196 g) were extracted in a Soxhlet with $CHCl_3$ for 6 hr. The extract (1.5 g) was chromatographed over Si gel (230–400 mesh; 110 g). Elution with C_6H_6 gave a pale yellow resin (**2a**) (28 mg). (Found: $[M]^+$ 286.2302. Calc. for $C_{20}H_{30}O$: 286.2297.) The phenolic compound (**2a**) was acetylated with Ac_2O (2 ml) and pyridine (1 ml) at 37° for 2 days. After usual work-up, the Et_2O extract was subjected to prep. TLC (0.5 mm; C_6H_6–*n*-hexane, 2:1; eluted with $CHCl_3$–MeOH, 20:1) and recrystallized from EtOH to afford the corresponding acetate (**2b**) (25 mg), colourless rods, mp 80–81° (uncorr.), $[\alpha]_D^{15}$ + 50° (EtOH; *c* 0.2). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1745, 1160 (OAc), 1365; MS: data identical with that cited in ref. [9]; ^1H NMR (100 MHz): δ 0.94 (3H, *s*), 0.97 (3H, *s*), 1.20 (3H, *s*, tert.Me × 3), 1.19 (3H, *d*, *J* = 7 Hz), 1.21 (3H, *d*, *J* = 6 Hz, sec.Me × 2), 2.32 (3H, *s*, OAc), 2.89 (3H, *m*, allylic H's), 6.82 (1H, *s*, aromatic H), 6.93 (1H, *s*, aromatic H). This acetate (**2b**) was identified with authentic ferruginol acetate [10] as described in the main text.

Culture method. Seeds of *S. miltiorrhiza* were sterilized in 70% EtOH for 30 sec, washed thoroughly with sterile H_2O, further sterilized in a 1% NaOCl soln for 1 hr, again washed × 3 with sterile H_2O and germinated in test tubes containing 0.6% agar in the dark at 25° for 1 week. The seedlings were transferred to a solid MS medium [17] containing 2,4-D (1 ppm), kinetin (0.1 ppm) and agar (0.6%). The pH of the medium was adjusted to 5.7 with 0.1 M NaOH and the soln was then sterilized by autoclaving for 15 min at 1.2 kg/cm^2 and 120°. Six cell lines (A–F) were established and the following procedures were typical for each cell line. The callus, derived from the seedlings, was maintained at 25° in the dark and was transferred every month to fresh medium. Suspension cultures were initiated from 3-month-old callus in the same medium as described above except that the agar was omitted. Cell suspension cultures were maintained at 25° in the dark with shaking on a gyratory shaker (100 rpm). The cultures were transferred every month to new medium. The 3-month-old suspension cultures were centrifuged and the cells collected were each inoculated into 50 ml liquid MS medium containing kinetin (0.1 ppm) in the absence of 2,4-D in 200-ml flasks and grown on a gyratory shaker (100 rpm) in the dark at 25°.

Identification of cryptotanshinone (**1**) *and ferruginol* (**2a**) *in the cultured cells.* Cells were harvested from the 1-month-old suspension cultures in the 2,4-D free medium by filtration with suction, dried at 80° for 2 hr and extracted in a Soxhlet with $CHCl_3$ for 3 hr. The extracts of cell line A were separated by prep. TLC to give cryptotanshinone (**1**) and ferruginol (**2a**) by development with C_6H_6–EtOAc (5:1) and C_6H_6–$CHCl_3$ (4:1), respectively. The ^1H NMR (200 MHz) data of the isolated cryptotanshinone (**1**): δ 1.31 (6H, *s*, Me-4), 1.36 (3H, *d*, *J* = 6.8 Hz, Me-17), 1.66 (2H, *br t*, H_2-3), 1.80 (2H, *m*, H_2-2), 3.22 (2H, *t*, *J* = 6.5 Hz, H_2-1), 3.61 (1H, *m*, H-17), 4.89 (1H, *t*, *J* = 9.5 Hz), 4.37 (1H, *dd*, *J* = 9.5 and 6 Hz, H_2-18), 7.59 (2H, AB*q*, *J* = 8 Hz, H-6 and H-7). The detailed characterization of both diterpenes (**1** and **2a**) is described in the main text. Each $CHCl_3$ extract of cell lines B–F was, without isolation procedure, subjected to GC analysis (column temp. 190°) and ferruginol (**2a**) was detected as a sole predominant product.

Acknowledgements—We are grateful to Professors R. C. Cambie, University of Auckland, New Zealand and C. R. Enzell, Swedish Tobacco Co., Sweden for kind gifts of authentic ferruginol acetate and to Emeritus Professor K. Takiura, Osaka University, Japan for that of authentic cryptotanshinone.

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