PERGAMON

Phytochemistry 53 (2000) 651–657

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Caffeic acid oligomers in *Lithospermum erythrorhizon* cell suspension cultures

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Received 15 June 1999; received in revised form 9 November 1999

Abstract

Lithospermum erythrorhizon cells cultured in pigment production (M-9) medium produced lithospermic acid B, a dimerized caffeic acid ester derivative, in quantities similar to the production of shikonin. The cells also produced a related dimer, (+)-rabdosiin. In Linsmaier–Skoog liquid medium, which suppresses shikonin production, both lithospermic acid B and (+)-rabdosiin were still formed. Lithospermic acid, a caffeic acid–rosmarinic acid conjugate, was isolated as a main constituent in *Lithospermum* hairy root cultures. In the aerial parts of *L. erythrorhizon*, the content of these phenylpropanoid oligomers was relatively low compared to that of rosmarinic acid.

Keywords: *Lithospermum erythrorhizon*; Boraginaceae; Cell suspension cultures; Caffeic acid oligomers; Lithospermic acid B; (+)-Rabdosiin; Shikonin; Secondary metabolite production

1. Introduction

Cultured cells of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) produce large amounts of shikonin derivatives, red naphthoquinone pigments, when cultured in pigment production (M-9) medium (Fujita, Hara, Suga & Morimoto, 1981). Shikonin is biosynthesized from two key precursors, *p*-hydroxybenzoic acid (PHB) formed through the phenylpropanoid pathway, and geranyl pyrophosphate formed through the mevalonate pathway (Schmid & Zenk, 1971; Inouye, Ueda, Inoue & Matsumura, 1979; Heide, Floss & Tabata, 1989. In Linsmaier–Skoog (LS) liquid medium (Linsmaier & Skoog, 1965) used for routine maintenance of *Lithospermum* cells, shikonin production is suppressed and the accumulation of a significant amount of PHB-*O*-glucoside (PHBOG) in the vacuoles is observed (Yazaki, Fukui & Tabata, 1986; Yazaki, Inushima, Kataoka & Tabata, 1995). These findings suggested that PHB synthesized in the cells could not be geranylated, but was glucosylated and accumulated as a storage form of shikonin precursor in the vacuoles. Thus, it follows that PHB geranyltransferase plays an important regulatory role in shikonin biosynthesis (Heide & Tabata, 1987; Heide, Nishioka, Fukui & Tabata, 1989).

In addition to PHBOG, shikonin non-producing cells also accumulate rosmarinic acid and lithospermic acid, which are biosynthesized through the phenylpropanoid pathway (Fukui, Yazaki & Tabata, 1984). The production of rosmarinic acid was reported to be stimulated by yeast extract (Mizukami, Ogawa, Ohashi & Ellis, 1992) and methyl jasmonate (Mizukami, Tabira & Ellis, 1993). Recently, Gaisser and Heide (1996) demonstrated that the content of rosmarinic acid was also increased in M-9 medium when shikonin production was inhibited by blue light irradiation. Fur-

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0031-9422/00/$ - see front matter
PII: S0031-9422(99)00623-8

thermore, they detected two compounds related to rosmarinic acid, but their exact structures were not determined. These results suggest that *Lithospermum* cells possess two pathways, one leading to polyphenol production and another to shikonin production, which share a common biosynthetic sequence in the early steps. To clarify the regulatory mechanisms operating in the secondary metabolism in *Lithospermum* cell cultures, it is necessary to determine the chemical structures of the end-products in the polyphenol pathway. In this paper, we report the production of two oligomeric caffeic acid conjugates in *L. erythrorhizon* cultured cells. We also report the quantitative analyses of these compounds in hairy root cultures and intact plants of *L. erythrorhizon*.

2. Results and discussion

The cold MeOH extract of fresh cells harvested from M-9 medium three weeks after inoculation was concentrated and partitioned between *n*-BuOH and water. HPLC analysis of the water-soluble part showed the presence of three compounds in addtion to a small amount of PHBOG. After acidifying (pH 2.0) with dilute HCl, the water phase was extracted with EtOAc and three compounds **1**–**3** were isolated from the EtOAc extract by use of Toyopearl HW-40 column chromatography and prep. HPLC. Compound **1** was identified as rosmarinic acid by comparison of its ^{1}H and ^{13}C-NMR spectra with those reported previously (Fukui et al., 1984). By comparison of its spectrometric data and $[\alpha]_D$ with those previously reported, compound **2** was identified as lithospermic acid B, a caffeic acid oligomer ester, which had been first isolated from *Salvia miltiorrhiza* radix (Tanaka et al., 1989). By comparison with literature data (Agata, Hatano, Nishibe & Okuda, 1988; Nishizawa, Tsuda & Hayashi, 1990), compound **3** was identified as (+)-rabdosiin, first isolated from *Macrotomia euchroma* (Nishizawa et al., 1990); identification was supported by analysis of various 2D-NMR spectra. This name was previously given for the (*1S,2R*) isomer of (−)-rabdosiin (Boqucki & Charlton, 1997).

This is the first report of the isolation of caffeic acid oligomers **2** and **3** from cell suspension cultures of *L. erythrorhizon*, and represents a novel co-occurrence of tetrameric phenylpropanoids with a dihydrobenzofuran skeleton and with a lignan skeleton in the same plant tissues.

In order to estimate the contents of caffeic acid oligomers and shikonin in cultured cells, hairy roots and intact plant of *L. erythrorhizon*, quantitative analysis of these compounds by HPLC was carried out. Prior to the analysis, the stability of caffeic acid oligomers was examined. Caffeic acid oligomers were partially decomposed by the lyophilization of cultured cells (ca. 30–40% of the extract from the fresh cells) and completely decomposed by drying in an oven at 50°C for three days (Yamamoto, Inoue & Yazaki, 2000). Shikonin (**5**) was also known to be unstable to heat. Therefore, fresh plant materials were used in this experiment.

Table 1 shows the content of caffeic acid oligomers and shikonin in the cells cultured in LS or M-9 medium. As previously reported (Fujita et al., 1981), shikonin production was induced when cells were inoculated into M-9 medium, reaching the level of 7.6 mg/g fr. wt. (ca. 6.3% of dry wt.) in three weeks. Production of caffeic acid oligomers was observed in cells cultured in LS and in M-9 medium as well, but the amount of **2** in cells cultured in M-9 medium was nearly eight times higher (12.4 mg/g fr. wt., ca. 10% of dry wt.) than that in the LS medium. In contrast to the remarkable difference in the content of **2** between LS and M-9 media, the contents of **1** and **3** were relatively unaffected by the change of culture media. In another experiment (Experiment 2), the content of **1** in the cells cultured in LS medium was nearly three times higher than that in the cells cultured in M-9 medium, but the content of **3** in both culture conditions was unchanged.

Table 1 also shows the content of these phenolic compounds in two different cell lines (CO and WM18) which lack the ability to produce shikonin even in the M-9 medium. Cell line CO produced an appreciable amount of caffeic acid oligomers, with rosmarinic acid (**1**), the main component in LS medium, and oligomer (**2**) predominant in M-9 medium. Cell line WM18 did not show as high productivity of **2** as did the other strains, but a slight stimulatory effect did accompany transfer into M-9 medium.

HPLC-photodiode array analysis of the MeOH

Table 1
Content of caffeic acid oligomers and shikonin in *L. erythrorhizon* cell suspension cultures

Cell strain	Medium	Contents (mg/g fr. wt.)				
		1	**2**	**3**	**4**	Shikonin[a]
M18TOM	Experiment 1					
	LS	1.76	1.6	1.21	–[b]	–
	M-9	1.74	12.45	1.70	–	7.58
	Experiment 2					
	LS	3.12	2.62	1.77	–	–
	M-9	0.81	7.74	1.32	–	15.58
CO	LS	2.51	0.42	0.10	–	–
	M-9	0.83	3.75	0.24	–	–
WM18	LS	0.58	0.02	0.05	–	–
	M-9	0.66	0.06	0.15	–	–

[a] Total amount of shikonin derivatives.
[b] (–): not detected.

extract of hairy root cultures of *L. erythrorhizon* revealed that caffeic acid oligomers **2** and **3** were produced much more abundantly than **1**. This finding was very different from that observed in de-differentiated cell cultures. Furthermore, hairy root cultures contained another caffeic acid derivative **4**, which was isolated by prep. HPLC. The positive ion FABMS spectrum of **4** gave a pseudomolecular ion at m/z539 ($[M + H]^+$), and the ^{1}H- and ^{13}C-NMR spectral data of compound **4** were found to be identical to those of lithospermic acid (Kelley, Mahajan, Brooks, Neubert, Breneman & Carmack, 1975; Kelley, Harruff & Carmack, 1976).

Table 2 shows the content of caffeic acid oligomers and shikonin in two independent hairy root clones cultured in hormone-free MS (Murashige & Skoog, 1962) or M-9 medium. The content of **4** and **2** increased after transfer of the tissues from MS medium to M-9 medium. On the other hand, the content of **3** was unaffected and only a trace amount of **1** was detected in both media.

Table 3 shows the distribution of caffeic acid oligomers and shikonin in the different tissues of 1- or 2-year-old intact plants of *L. erythrorhizon*. In the aerial part, **1** was the major caffeic acid ester, whereas in the underground part, the content of **1** was very low, and an appreciable amount of **4** was detected as a main component. This pattern is similar to that observed in hairy root cultures. In the root tissues, fibrous roots contained a relatively large amount of **4**. Compounds **2** and **3** were distributed throughout the plant, but the content of **2** in the root was higher than that in the aerial parts. It is noteworthy that the content of **2** and **4** in the cork layers of the main root were 3–10 times higher than those in the cortex and the central cylinder.

In contrast to the present results, Nishizawa et al. (1990) reported that **3** was undetectable in *L. erythrorhizon* plants, and Omoto et al. (1997) reported that both **1** and **4** were present in all parts of the plant. We cannot explain these discrepancies at present, but differences in the time of harvest, physiological status of the plants or the extraction procedures may be responsible.

Table 2
Content of caffeic acid oligomers and shikonin in *L. erythrorhizon* hairy root cultures

Cultures	Contents (mg/g fr. wt.)				
	1	**2**	**3**	**4**	Shikonin[a]
Clone Con F-1 (in MS medium)	tr.[b]	0.34	1.13	1.61	1.92
Clone Con F-1 (in M-9 medium)	tr.	0.87	0.97	3.03	9.96
Clone Con 5 (in MS medium)	tr.	0.35	1.21	1.76	1.78
Clone Con 5 (in M-9 medium)	tr.	0.87	1.01	4.91	9.42

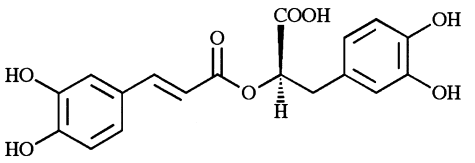
[a] Total amount of shikonin derivatives.
[b] tr: trace amount.

Caffeic acid oligomers such as rosmarinic acid (**1**), lithospermic acid (**4**) and lithospermic acid B (**2**) are widely distributed in the Labiatae and Boraginaceae. These oligomers, especially **2**, have many biological and pharmaceutical activities such as antioxidative, radical scavenging (Fung et al., 1993a), uremia-preventive (Tanaka et al., 1989; Yokozawa, Chung, Oura, Nonaka & Nishioka, 1988a; Yokozawa et al., 1988b; Lee et al., 1988), hypotensive (Kamata, Itzuka, Nagai & Kasuya, 1993; Kamata, Noguchi & Nagai, 1994; Yokozawa, Zhou, Oura, Tanaka, Nonaka & Nishioka, 1995), hepatoprotective effects (Hase, Kasimu, Basnet, Kadota & Namba, 1997), of the myocardial infarction improvement (Fung et al. 1993a, 1993b) and amplification of beating of myocardial muscles (Yokozawa et al., 1994). In order to identify a source of **2**, 30 plant species were surveyed (Omoto et al., 1997). However, its content in intact plants was found to be low in all except for *S. officinalis* and *L. erythrorhizon*. Furthermore, attempts to produce **2** by cell or hairy root cultures have been unsuccessful till date (Tada, Ikeda, Omoto, Shimomura & Ishimaru, 1996; Morimoto, Goto & Shoyama, 1994; Motoyama, Tada, Shimomura, Yoshihira & Ishimaru, 1996; Tada, Murakami, Omoto, Shimomura & Ishimaru, 1996).

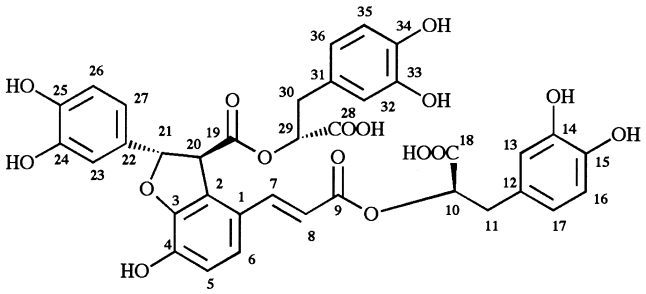
In the present study, we found that *Lithospermum* cell cultures are capable of producing a large amount of **2** (ca. 6–10% of dry wt.). This productivity is as high as that of shikonin (**5**), which has already been produced on an industrial scale (Fujita et al., 1981). This level of production of **2** was achieved using M-9 medium which was originally optimized for shikonin (**5**) production. However, our results demonstrate that this medium stimulates the biosynthetic pathways leading to both caffeic acid oligomers and shikonin (**5**) in *Lithospermum*.

It is noteworthy that the accumulation pattern of these caffeic acid oligomers in the aerial parts of the intact plant was different from that in underground parts, i.e. **1** was nearly undetectable in root tissues, but **4** was detected only in the underground tissues. However, it is not known whether light plays an important role in controlling the biosynthesis of these caffeic acid oligomers, as it does in shikonin (**5**) biosynthesis.

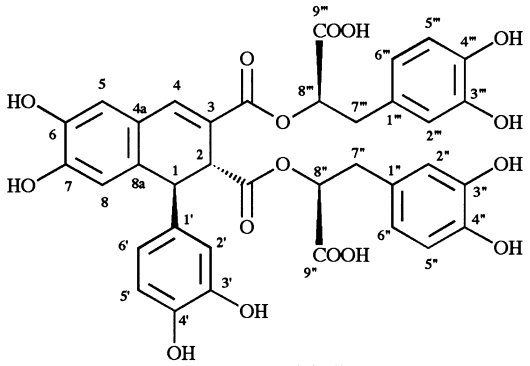
Contrary to the previous results in *Lithospermum* cell cultures (Fukui et al., 1984), we could not detect **4** in the culture strain used in this study, but compound **4** was the main caffeic acid oligomer both in hairy root cultures and the root tissues of the intact plant. This suggests that tissue differentiation may be correlated with the polymerization pattern of caffeic acid in *Lithospermum* plants. Alternatively, the difference between our results and those of Fukui et al. may reflect culture strain or handling differences.



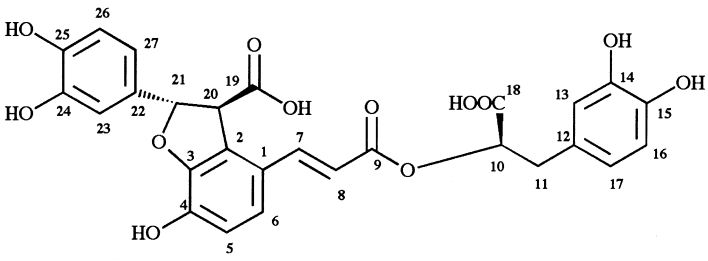
1: rosmarinic acid



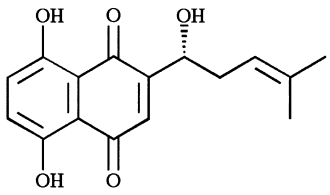
2: lithospermic acid B



3: (+)-rabdosiin



4: lithospermic acid



5: shikonin

3. Experimental

3.1. Materials and culture methods

The strain M18TOM of *L. erythrorhizon* cell suspension cultures derived from strain M18 (Mizukami, Konoshima & Tabata, 1978), was grown in 300 ml Erlenmeyer flasks containing 100 ml LS liquid medium (Linsmaier & Skoog, 1965) supplemented with 1 μM 3-indoleacetic acid and 10 μM kinetin, on a rotary shaker (100 rpm) at 23°C in the dark. The cells were subcultured in the same medium at intervals of two weeks. We also used two strains (CO and WM18) which were not capable of producing shikonin even in M-9 medium (Fujita et al., 1981). For the subculturing experiments, 1.5 g fr. cells were inoculated into LS medium containing the same hormones as above or 1 g fr. cells were inoculated into 100 ml-flasks containing 30 ml M-9 medium (Fujita et al., 1981) supplemented with 1 μM 3-indoleacetic acid and 10 μM kinetin, respectively, and agitated on a rotary shaker for three weeks.

The hairy roots (two clones; Con F-1 and Con 5) of *L. erythrorhizon* were generated from half-etiolated seedlings by direct infection with *Agrobacterium rhizogenes* (strain 15834), according to the method of Shimomura et al. (1991). The hairy roots were subcultured in MS liquid medium (Murashige & Skoog, 1962) on a rotary shaker (80 rpm) at 25°C in the dark at intervals of three weeks. Hairy roots used in the experiment were cultured in 100 ml Erlenmeyer flasks containing 30 ml MS medium under the same condition as above, and harvested by filtration through Miracloth (Calbiochem) three weeks after inoculation (inoculum size: 0.5 g). Intact plants of *L. erythrorhizon* were grown in the Kyoto Herbal Garden, Takeda Chemical, and were harvested in September 1997.

3.2. Isolation of constituents

Fresh *Lithospermum* cells (160 g) cultured in M-9 medium were extracted twice with 2 l MeOH in an ice–water bath for 90 min with ultrasonication. The MeOH extract was evaporated in vacuo to dryness, and the resultant residue was partitioned between H_2O (pH 7.0) and *n*-BuOH. The aqueous phase was acidified to pH 2.0 with 1 N HCl and extracted with EtOAc. The EtOAc extract was dried over Na_2SO_4, and then evaporated in vacuo to give the residue (1.6 g) which was purified by a combination of Toyopearl HW-40 (0.1% TFA containing 50% EtOH/H_2O) column chromatography and prep. HPLC. HPLC conditions were as follows: column, Hikarisil C18-2E (5 μM, 20 × 250 mm, Showa Denko Co., Japan); solvent

Table 3
Content of caffeic acid oligomers and shikonin in *L. erythrorhizon* intact plants

Material	Contents (mg/g fr. wt.)				
	1	**2**	**3**	**4**	Shikonin[a]
One-year old					
fibrous roots	tr.[b]	2.42	0.89	3.24	11.48
Lower part of main roots (2 mm i.d.)	tr.	0.58	–[c]	0.48	13.96
upper part of main roots (5 mm i.d.)	tr.	1.23	–	0.32	16.77
Middle leaf	3.02	0.26	1.97	–	–
Upper leaf	3.59	1.00	2.27	–	–
Middle stems	2.02	0.13	0.62	–	–
Upper stems	1.81	0.38	0.62	–	–
Average of underground parts	tr.	1.41	0.30	1.35	14.07
Average of aerial parts	2.61	0.44	1.39	–	–
Two-year old					
Fibrous roots	tr.	0.86	0.97	2.87	0.39
Lateral roots	tr.	2.93	0.09	2.58	3.69
Lower part of main roots (ca. 1 mm i.d.)	tr.	2.56	0.09	1.26	2.63
Middle part of main roots (ca. 5 mm i.d.)	tr.	1.52	0.05	0.81	2.51
Upper part of main roots (ca. 8 mm i.d.)	tr.	1.35	0.06	0.78	1.77
Lower leaf	1.26	0.08	1.02	–	–
Middle leaf	0.69	0.09	0.23	–	–
Upper leaf	1.38	0.05	0.79	–	–
Lower stem	1.97	0.94	0.38	–	–
Middle stem	0.52	0.09	0.16	–	–
Upper stem	0.50	0.01	0.19	–	–
Shoot apex (containing young leaf)	1.77	0.10	0.65	–	–
Average of underground parts	tr.	1.62	0.68	1.81	1.91
Average of aerial parts	1.16	0.20	0.35	–	–
Cork layer of main roots	tr.	2.88	0.04	1.31	8.08
Cortex and central cylinder of main roots	tr.	0.18	0.06	0.43	0.11

[a] Total amount of shikonin derivatives.
[b] tr: trace amount.
[c] (–): not detected.

system, 0.1% TFA-containing 15% MeCN/H_2O; flow rate, 10 ml/min; oven temperature, 40°C; detection, 254 nm. Compounds **1** (20 mg), **2** (320 mg) and **3** (59 mg) were isolated from 0.5 g of the EtOAc extract. Compound **4** (14 mg) was also isolated from *Lithospermum* hairy root cultures (10 g fr. wt.) in the same manner.

Identification of **2** was carried out by direct comparison (^{1}H-, ^{13}C-NMR, FABMS and $[\alpha]_D$) with authentic lithospermic acid B (**2**) $[[\alpha]_D^{20} + 115.5^\circ$ (H_2O)]. Compounds **1**, **3** and **4** were identified as rosmarinic acid (Fukui et al., 1984), (+)-rabdosiin (Agata et al., 1988; Nishizawa et al., 1990; Boqucki & Charlton, 1997) and lithospermic acid (Kelley et al. 1975, 1976), respectively, by comparison of their physical data (^{1}H-, ^{13}C-NMR, FABMS and $[\alpha]_D$) with those reported previously [(+)-rabdosiin (**3**): $[\alpha]_D^{20} + 137^\circ$ (H_2O)].

3.3. Quantitative analyses

The details of the extraction procedure and the quantitative analysis were as described (Yamamoto et al., 2000). In brief, fresh cultured cells, cultured hairy roots and several tissues of intact plants shown in Table 3 (ca. 0.5–1 g each) were extracted with 10 ml MeOH in an ice–water bath for 90 min with ultrasonication. β-Naphthol (1 mg) was added to each methanolic mixture as an internal standard for HPLC analysis. Cell debris was removed by centrifugation (21,000 × *g*, 5 min) and each resultant supernatant was subjected to reversed-phase HPLC to measure the quantities of caffeic acid derivatives and shikonin (**5**) on the basis of the peak area recorded by absorption at 254 nm using Chromatopac C-R4A (Shimadzu, Japan). The HPLC conditions were as follows: column, Hikarisil C18 (4.6 × 250 mm, Asahi Chemical, Japan); solvent system, 1% AcOH-containing MeCN/H_2O system with a gradient program (5% MeCN kept for 5 min, rising up to 20% MeCN in 1 min, then rising to 30% MeCN in 20 min, to 40% MeCN in 1 min, to 60% MeCN in 40 min, and finally increasing the MeCN concentration to 90% in 15 min: flow rate, 1 ml/min; oven temperature, 40°C.

Acknowledgements

We wish to thank Mr. Naohiro Fushimi and Mr. Hitoshi Watanabe, Kyoto Herbal Garden, Takeda Chemical for the generous gift of intact plants of *Lithospermum erythrorhizon*. We also are grateful to Dr. Koichiro Shimomura, Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences, Japan, and Prof. Isao Ageta, Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University for the gift of authentic samples of lithospermic acid B and rabdosiin, respectively, and for helpful suggestions. We thank Prof. Dr. Kanji Ishimaru for his advice on the quantitative analysis of caffeic acid oligomers. We also thank Prof. Tetsuro Shingu, Faculty of Pharmaceutical Sciences, Kobe Gakuin University and Dr. Kazuhiro Irie, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University for the NMR spectroscopic measurements.

References

Agata, I., Hatano, T., Nishibe, S., & Okuda, T. (1988). Rabdosiin, a new rosmarinic acid dimer with a lignan skeleton from *Rabdosia japonica*. *Chemical Pharmaceutical Bulletin*, *36*, 3223.

Boqucki, D. E., & Charlton, J. L. (1997). A non-enzymatic synthesis of (*S*)-(−)-rosmarinic acid and a study of a biomimetic route to (+)-rabdosiin. *Canadian Journal of Chemistry*, *75*, 1783.

Fujita, Y., Hara, Y., Suga, C., & Morimoto, Y. (1981). Production of shikonin derivatives by cell suspension cultures of *Lithospermum* erythorhizon. Part II: A new medium for the production of shikonin derivatives. *Plant Cell Reports*, *1*, 61.

Fukui, H., Yazaki, K., & Tabata, M. (1984). Two phenolic acids from *Lithospermum erythrorhizon* cell suspension cultures. *Phytochemistry*, *23*, 2398.

Fung, K. P., Wu, J., Zeng, L. H., Wong, H. N. C., Lee, C. M., Hon, P. M., Chang, H. M., & Wu, T. W. (1993a). Lithospermic acid B as an antioxidant-based protector of cultured ventricular myocytes and aortic endothelical cells of rabbits. *Life Sciences*, *53*, 189.

Fung, K. P., Zeng, L. H., Wu, J., Wong, H. N. C., Lee, C. M., Hon, P. M., Chang, H. M., & Wu, T. W. (1993b). Demonstration of the myocardial salvage effect of lithospermic acid B isolated from the aqueous extract of *Salvia miltiorrhiza*. *Life Sciences*, *52*, 239.

Gaisser, S., & Heide, L. (1996). Inhibition and regulation of shikonin biosynthesis in suspension cultures of *Lithospermum*. *Phytochemistry*, *41*, 1065.

Hase, K., Kasimu, R., Basnet, P., Kadota, S., & Namba, T. (1997). Preventive effect of lithospermate B from *Salvia miltiorhiza* on experimental hepatitis induced by carbon tetrachloride or D-galactosamine/lipopolysaccharide. *Planta Medica*, *63*, 22.

Heide, L., & Tabata, M. (1987). Geranylpyrophosphate:*p*-hydroxybenzoate geranyltransferase activity in extracts of *Lithospermum erythrorhizon* cell cultures. *Phytochemistry*, *26*, 1651.

Heide, L., Nishioka, N., Fukui, H., & Tabata, M. (1989). Enzymatic regulation of shikonin biosynthesis in *Lithospermum erythrorhizon* cell cultures. *Phytochemistry*, *28*, 1873.

Heide, L., Floss, H. G., & Tabata, M. (1989). Incorporation of shikimic acid into *p*-hydroxybenzoic acid in *Lithospermum erythrorhizon* cell cultures. *Phytochemistry*, *28*, 2643.

Inouye, H., Ueda, S., Inoue, K., & Matsumura, H. (1979). Biosynthesis of shikonin in callus cultures of *Lithospermum erythrorhizon*. *Phytochemistry*, *18*, 1301.

Kamata, K., Itzuka, T., Nagai, M., & Kasuya, Y. (1993). Endothelium-dependent vasodilator effects of identification of lithospermic acid B in the extracts. *General Pharmacology*, *24*, 977.

Kamata, K., Noguchi, M., & Nagai, M. (1994). Hypotensive effects of lithospermic acid B isolated from the extract of *Salvia miltiorrhiza* radix in the rat. *General Pharmacology*, *25*, 69.

Kelley, C. J., Mahajan, J. R., Brooks, L. C., Neubert, L. A., Breneman, W. R., & Carmack, M. (1975). Polyphenolic acids of *Lithospermum ruderale* Dougl.ex Lehm. (Boraginaceae). Part I: Isolation and structure determination of lithospermic acid. *Journal of Organic Chemistry*, *40*, 1804.

Kelley, C. J., Harruff, R. C., & Carmack, M. (1976). The polyphenolic acids of *Lithospermum ruderale*. Part II: Carbon-13 nuclear magnetic resonance of lithospermic and rosmaric acids. *Journal of Organic Chemistry*, *41*, 449.

Lee, T. U., Chung, H. Y., Yokozawa, T., Ohura, H., Tanaka, T., Nonaka, G., & Nishioka, I. (1988). Effects of lithospermic acid B magnesium salt on adenine-induced renal failure in rats. *Wakan Iyaku Gakkaishi*, *5*, 306.

Linsmaier, E. M., & Skoog, F. (1965). Organic growth factor requirements of tabacco tissue cultures. *Physiologia Plantarum*, *18*, 100.

Mizukami, H., Konoshima, M., & Tabata, M. (1978). Variation in pigment production in *Lithospermum erythrorhizon* callus cultures. *Phytochemistry*, *17*, 95.

Mizukami, H., Ogawa, T., Ohashi, H., & Ellis, B. E. (1992). Induction of rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures by yeast extract. *Plant Cell Reports*, *11*, 480.

Mizukami, H., Tabira, Y., & Ellis, B. E. (1993). Methyl jasmonate-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. *Plant Cell Reports*, *12*, 706.

Morimoto, S., Goto, Y., & Shoyama, Y. (1994). Production of lithospermic acid B and rosmarinic acid in callus tissues and regenerated plantlets of *Salvia miltiorrhiza*. *Journal of Natural Products*, *57*, 817.

Motoyama, E., Tada, H., Shimomura, K., Yoshihira, K., & Ishimaru, K. (1996). Caffeic acid esters in tissue cultures of *Heliotropium peruvianum*. *Plant Tissue Culture Letters*, *13*, 73.

Murashige, T., & Skoog, F. (1962). Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473.

Nishizawa, M., Tsuda, M., & Hayashi, K. (1990). Two caffeic acid tetramers having enantiomeric phenyldihydronaphthalene moieties form *Macrotomia euchroma*. *Phytochemistry*, *29*, 2645.

Omoto, T., Murakami, Y., Shimomura, K., Yoshihira, K., Mori, K., Nakashima, T., Tanaka, M., & Ishimaru, K. (1997). Caffeic acid deivatives in Laminaceae and Boraginaceae plants. *Japanese Journal of Food Chemistry*, *4*, 11.

Schmid, H. V., & Zenk, M. H. (1971). *p*-Hydroxybenzoic acid and mevalonic acid as precursors of the plant naphthoquinone alkannin. *Tetrahedron Letters*, *12*, 4151.

Shimomura, K., Sudo, H., Saga, H., & Komada, H. (1991). Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. *Plant Cell Reports*, *10*, 282.

Tada, H., Ikeda, Y., Omoto, T., Shimomura, K., & Ishimaru, K. (1996). Rosmarinic acid and related phenolics in adventitious root cultures of *Ocimum basilicum* L. *Plant Tissue Culture Letters*, *13*, 69.

Tada, H., Murakami, Y., Omoto, T., Shimomura, K., & Ishimaru,

K. (1996). Rosmarinic acid and related phenolics in hairy root cultures of *Ocimum basilicum*. *Phytochemistry*, *42*, 431.

Tanaka, T., Morimoto, S., Nonaka, G., Nishioka, I., Yokozawa, T., Chung, H. Y., & Oura, H. (1989). Magnesium and ammonium–potassium lithospermates B, the active principles having a uremia-preventive effect from *Salvia miltiorrhiza*. *Chemical Pharmaceutical Bulletin*, *37*, 340.

Yamamoto, H., Inoue, K., & Yazaki, K. (2000). *Journal of Chromatography B*, (in press).

Yazaki, K., Fukui, H., & Tabata, M. (1986). Accumulation of *p*-*O*-β-D-glucosylbenzoic acid and its relation to shikonin biosynthesis in *Lithospermum* cell cultures. *Phytochemistry*, *25*, 1629.

Yazaki, K., Inushima, K., Kataoka, M., & Tabata, M. (1995). Intracellular localization of UDPG:*p*-hydroxybenzoate glucosyltransferase and its reaction product in *Lithospermum* cell cultures. *Phytochemistry*, *38*, 1127.

Yokozawa, T., Chung, H. Y., Oura, H., Nonaka, G., & Nishioka, T. (1988a). Isolation of the active component having the uremia-preventive effect from *Salvia miltiorrhiza* radix extract. *Chemical Pharmaceutical Bulletin*, *36*, 316.

Yokozawa, T., Chung, H. Y., Oura, H., Nonaka, G., & Nishioka, T. (1988b). Effects of (dihydro)caffeic acid tetramer isolated from *Salvia miltiorrhiza* radix on renal function. *Wakan Iyaku Gakkaishi*, *5*, 163.

Yokozawa, T., Zhou, J. J., Sun, X. L., Hattori, M., Namba, T., Tanaka, T., Nishioka, I., & Oura, H. (1994). Effects of caffeic acid analogues on the rate and amplitude of beating of cultured myocardial sheets. *Natural Medicines*, *48*, 330.

Yokozawa, T., Zhou, J. J., Oura, H., Tanaka, T., Nonaka, G., & Nishioka, I. (1995). Effects on blood pressure of caffeic acid analogs isolated from *Salvia miltiorrhiza* radix in rats with adenine-induced renal hypertension. *Phytotherapy Research*, *9*, 105.